UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Laboratoire CAMB-UMR7199 CNRS/Université de Strasbourg

THÈSE présentée par :

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soutenue le : **27 septembre 2017**

pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : Aspects moléculaires et cellulaires de la biologie/ Immunologie

Development of innovative liposome-based constructs for non-invasive cancer immunotherapy in humans

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REMERCIEMENTS

Voilà ma thèse qui se termine; une page de ma vie qui tourne pour me mener à un nouveau chapitre. Cette longue aventure m'a introduit à tellement de gens qui ont tous contribué, chacun à sa manière, à rendre cette expérience plus enrichissante et plus précieuse, que ce soit sur le plan scientifique ou humain. Vous avez tous été des perles sur mon chemin.

Je tiens tout d'abord à exprimer mes sincères remerciements aux membres de mon jury de thèse pour l'honneur qu'ils m'ont accordé, Dr Anne Aubert, Pr Sylvain Fisson, Pr Pauline Soulas, Pr Pierre-Olivier Couraud, ainsi que Dr Vincent Flacher et Dr Béatrice Heurtault pour m'avoir fait l'honneur de juger mes travaux de thèse.

Une thèse est d'habitude dirigée par une ou deux personnes. De mon côté, j'ai eu la chance d'en avoir plus… Je vous raconte.

Comment puis-je trouver les mots pour remercier les personnes qui ont inspiré et dirigé ce travail, et qui, au-delà de cela, m'ont appris à comprendre les enjeux du monde de la recherche et à y grandir? A mes directrices immunologistes, Soulaïma et Sylvie, et à ma directrice galéniste, Béatrice (bien qu'il n'ait pas été possible d'avoir officiellement 3 co-directrices, et que tu sois un membre invité du jury), mille mercis du fond du cœur. Dès le début, vous avez été des accompagnatrices qui ont commencé par s'inquiéter de là où je vais dormir lors de mon premier voyage en France, pour rester à mes côtés tout au long de cette thèse. En étant toujours là pour me guider, mais tout en me laissant beaucoup de liberté, vous m'avez donné l'opportunité d'acquérir une vision large et mature de la science. Merci beaucoup pour votre encadrement, votre patience, votre compréhension, vos conseils précieux, votre disponibilité, et vos encouragements.

Je vous demande de m'excuser si je métends longuement sur cette partie, mais derrière ma thèse, il y a une histoire à raconter… L'histoire a commencé quand j'ai fini mon master et que je cherchais à m'inscrire en thèse. C'est là où, Soulaïma, tu m'as prise comme protégée, tu m'as soutenue sans faille et, grâce à toi, j'ai fait la connaissance de Sylvie et Béatrice. Depuis lors, tu as tout fait pour que je puisse avoir un financement au Liban et mener à bien ma thèse dans les meilleures conditions. Ça n'a pas toujours été facile ni évident. Au-delà de tout cela,

tu m'as été une grande sœur que je ne saurais pas assez remercier pour son accueil chaleureux (j'ai pratiquement passé la dernière semaine de la rédaction chez toi!). Sylvie, cette thèse n'aurait pas été possible sans ta confiance et ton engagement à m'assurer un financement en France. J'ai appris de ta rigueur et ton dévouement, et tu as si bien su me booster quand la situation devenait critique. Merci encore plus pour le plaisir que m'ont procuré toutes les discussions que nous avons partagées après les longues journées de travail, et merci pour avoir été si patiente et disponible. Que dire alors de toi Béatrice… Même si ton nom ne figure pas officiellement en tant que directrice de cette thèse, tu l'as été. Tu as été aussi précieuse pour moi que pour ce travail, et c'est grâce à toi que j'ai appris la galénique et que j'ai pu vivre une expérience inter-disciplinaire très enrichissante.

Je tiens à remercier du fond du cœur les directeurs de mes deux laboratoires et équipes d'accueil, Dr Jean Serge Remy (directeur de l'UMR7199), Dr Benoît Frisch (directeur de l'équipe de Biovectorologie à l'UMR7199) et Dr Hasnaa Bouharoun-Tayoun (co-directrice du laboratoire d'Immunologie de l'UL). Mille mercis pour m'avoir accueillie au sein de vos laboratoires, pour votre confiance et pour vos conseils. Hasnaa, déjà avant que je commence ma thèse, tu as été pour moi un mentor qui a vu une étincelle en moi et qui a su me guider afin que je puisse me faire une place dans le monde de la recherche.

Au cours de cette expérience j'ai eu la chance de travailler avec Dr Vincent Flacher. Merci Vincent pour m'avoir accueillie et formée, merci pour les expériences FACS, pour ta disponibilité et pour tes conseils précieux. Merci également d'avoir participé à mon jury et pris le temps de lire ce manuscrit.

Un grand merci aux stagiaires qui m'ont apporté de l'aide à la réalisation de certains travaux. Merci à Nisrine Ballout, maintenant docteur, et à Joëlle Richa (pour votre aide pendant les premières expériences sur les souris Hu-SPL-NSG), à Maroua Messous et à Yara El Murr (pour votre aide dans la vaccination TC), et à Yohan Gerber (mille mercis pour les travaux sur les liposomes fluorescents).

J'aurais difficilement pu terminer ma thèse dans de bonnes conditions sans le soutien et l'encouragement de mes deux équipes d'accueil. Merci pour avoir partagé mon quotidien durant cette longue expérience et de l'avoir enrichie. J'apprécie énormément la chance que

j’ai eue de nouer autant de relations humaines et de croiser autant de gens et de cultures. La liste est longue et je vais essayer de n’oublier personne.

A l’équipe Biovec en France, merci à Célia pour ta compagnie, ta gentillesse, ton soutien et pour ton aide pendant les jours fous des sacrifices. Merci à Zahra pour avoir formulé mes premiers liposomes et m’avoir formée à préparer tous les suivants, et à Neïla pour nos discussions sur les enjeux de la thèse et de la science. Merci à toutes les deux pour m’avoir «adoptée» pendant mon premier séjour en France et pour votre soutien continu. Cendrine, Anaïs et Evelyne, merci avant tout pour votre amitié, vos conseils et votre agréable compagnie. Anaïs et Cendrine, merci encore pour votre aide dans les expériences et pour votre soutien pendant les moments de forte déprime (Je n’ai pas oublié notre dernière longue discussion en live à minuit, vous maitrisez incroyablement l’art de booster les gens!). Merci à toutes de vos conseils judicieux. Je n’oublie pas de remercier Maria pour sa gentillesse, et Marcella, pour avoir été une « co-bureau » si sympa, mais aussi pour les pauses gourmandes avec vous et les cafés de 16h. Merci également à May (tu es un rayon de soleil), Alex (pour ton humour du second degré), à Eya, Amina et Salif.

A quelques milliers de kilomètres, à mon laboratoire Libanais, mille mercis à Nabil (mon guide lors de mes premiers pas dans la recherche), Stéphanie et Leila (mes «supporters» et les copines de 24/7), Renée (nous avons partagé le même sort et avons été synchrones pendant la thèse; qu’est-ce qu’il pourrait y avoir de plus encourageant?). Mille mercis à Catherine et Najibé pour le plaisir de nos discussions matinales et pour votre soutien dans les expériences. Merci à Sandy et Joëlle, à Elie, Silia, Elissa et Yara (encore), les jeunes motivés de la nouvelle génération qui ont contribué au plaisir que j’ai eu à vivre en ce lieu.

Je tiens également à remercier toutes les personnes que j’ai rencontrées chez les « voisins» (majoritairement des chimistes), pour toutes les pauses midi et les jeux de cartes. Un énorme merci chaleureux aux incontournables Malak et Marianne pour leur soutien sans faille, leurs conseils «logistiques» et leur compagnie. Merci à Fanny pour ses encouragements et sa bonne humeur (tue me tues!). Merci à Murielle et Nour et tous les amis que je n’ai pas cités, pour avoir vécu cette aventure avec moi.

Merci également à la faculté de médecine de l'Université Libanaise où j'ai eu mes premières expériences d'encadrement d'étudiants, et qui m'a donné l'opportunité de poursuivre cette thèse en parallèle. Je n'oublie pas de remercier la doyenne de la faculté de Santé Publique de l'UL, Dr Nina Zeidan, et le doyen de l'ED 414 à l'Unistra pour leur acte de foi qu'ils ont fait à l'égard de cette thèse.

Je ne serais certainement pas là à écrire ces lignes sans le soutien de mes parents, mon frère Tanios et ma sœur Hiba, à qui je dédie cette thèse. Vous m'avez été un véritable soutien sur tous les plans et avez cru en moi jusqu'au bout. L'ambition et la persévérance sont des qualités que j'ai apprises de vous. Mille mercis surtout pour cette phase de rédaction où je risquais d'être moins supportable que d'habitude (plutôt insupportable). Tanios et Hiba, n'oubliez pas, «aim high and think big». Je suis fière de vous autant que vous êtes fiers de moi.

Si mes parents m'ont formée dès mon plus jeune âge à avoir de l'ambition et des rêves, alors toi Fadi, «my other half», tu n'as hésité à aucun moment à renforcer ces traits de caractère en moi, à me soutenir jusqu'au bout quelqu'en soit le coût et à m'aider à rebondir après chaque chute. C'est grâce à toi que j'ai considéré la thèse avec un nouvel espoir après chaque déprime… Ce n'est pas pour me plaindre, mais j'en ai eu beaucoup, et c'est grâce à toi, Fadi que je me dis tous les matins que «tout ce qui ne te tue pas te rends plus fort ».

J'ai lu quelque part que « les remerciements sont la preuve que le scientifique n'est pas un robot»… Oui… J'espère avoir pu, en ces quelques lignes, exprimer ma sincère gratitude à tous les gens que j'ai croisés, et avec qui j'ai eu des relations aussi humaines que professionnelles pendant toutes ces années. Ayant franchi cette étape parsemée de difficultés qui nous pousse jusqu'à nos limites, je me retrouve prête à confronter d'autres défis…

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RESUME

Développement de constructions liposomiques innovantes pour l'immunothérapie humaine

Cette thèse est réalisée en co-direction entre Pr. Sylvie Fournel à l'UMR7199 CNRS à l'Université de Strasbourg et Pr. Soulaima Chamat au Laboratoire d'Immunologie à l'Université Libanaise.

Contexte

Les traitements antitumoraux classiques se basent pour la plupart sur la chimiothérapie et la radiothérapie. En raison de leur faible spécificité pour les cellules tumorales, ces traitements induisent de nombreux effets secondaires. La découverte que le système immunitaire du patient pouvait éliminer les tumeurs en utilisant par exemple des lymphocytes T cytotoxiques (CTL) a fait de l'immunothérapie anticancéreuse une stratégie attractive. Cette stratégie thérapeutique se base sur la capacité des cellules présentatrices d'antigènes (CPA) et en particulier des cellules dendritiques (DC), à capturer des antigènes associés aux tumeurs (TAA), puis à migrer suite à leur maturation induite par un signal de danger (adjuvant) jusqu'aux organes lymphoïdes secondaires pour y présenter des peptides issus des TAA aux lymphocytes $TCD4^+$ et $TCD8^+$. Les premiers, lorsqu'ils sont différenciés en T helper 1 (Th1) procurent des signaux de maturation sous forme de cytokines et de molécules de costimulation à la DC, qui va alors être capable d'induire la différenciation des $TCD8^+$ en lymphocytes T cytotoxiques (CTL), principales cellules effectrices de la réponse antitumorale. La mise en place d'une réponse immunitaire efficace contre les tumeurs nécessite donc 1) une activation de la DC par des signaux de danger fournis par une molécule immunostimulatrice, comme, par exemple, un agoniste de TLR, 2) l'activation de $TCD4^+$ helper suite à la reconnaissance d'un **épitope $TCD4^+$** présenté par la DC et 3) l'activation de $TCD8^+$ cytotoxiques suite à la présentation par une DC **d'un épitope $TCD8^+$.**

Classiquement, l'administration d'un vaccin se fait à travers la peau, celle-ci étant un site immunologique particulièrement riche en DC. En effet, l'épiderme comprend les cellules de

Langerhans (LCs) et le derme comprend plusieurs sous-populations de DCs dermiques (dDCs) qui expriment ou pas la langerine. La voie d'administration cutanée la plus conventionnelle est la voie sous-cutanée (SC). Toutefois, celle-ci implique un drainage du vaccin de l'hypoderme qui est dépourvu de DC, vers les ganglions drainant la zone d'administration. Alternativement, la voie intradermique est difficile à cibler. C'est pourquoi l'immunisation transcutanée (TC) est envisagée comme voie intéressante qui cible préférentiellement les LC et les dDCs. Les antigènes peptidiques sont adaptés, du fait de leur petite taille, au passage à travers la peau. L'encapsulation de ces peptides dans des nanoparticules, tel que les liposomes, augmente leur immunogenicité et leur absorption par la peau.

L'objectif général de mon projet de thèse est donc de developper des constructions liposomiques anti-tumorales pour une administration TC chez l'homme.

Mon laboratoire d'accueil à l'Université de Strasbourg a développé des constructions liposomiques peptidiques contenant tous les éléments indispensables à la réponse immunitaire antitumorale (épitopes $TCD4^+$, $TCD8^+$, agoniste de TLR) qui induisent une réponse immunitaire antitumorale après administration par voies SC et intranasale chez la souris. **Pour atteindre ce but, mon 1er objectif spécifique est donc d'optimiser ces constructions vaccinales liposomiques pour induire une réponse immunitaire après administration par voie TC.**

Par ailleurs, les réponses immunitaires induites chez l'animal lors des essais précliniques des vaccins divergent souvent de celles qui sont ensuite observées lors des essais cliniques, ce qui rend nécessaire le développement de modèles animaux plus prédictifs de la réponse immunitaire humaine aux vaccins. Un tel modèle a été développé dans mon laboratoire d'accueil libanais. Il repose sur la reconstitution de souris immunodéficientes avec des cellules immunitaires humaines provenant de la rate ou du sang périphérique humain, et est deisgné par Hu-SPL-NSG. **Le deuxième objectif de ce projet est donc de tester les constructions vaccinales liposomiques, dans un modèle de souris humanisée afin de les optimiser pour une application humaine ultérieure.**

Objectifs et stratégie de l'étude:

Objectif 1 : Développement de constructions liposomiques adaptées pour la vaccination TC contre le cancer et évaluation de leur immunogenicité.

Les résultats de cette partie sont présentés dans l'article #1, en préparation.

1- **Optimisation de la composition de la construction liposomique pour la vaccination TC** à partir de la construction précédemment validée au laboratoire, en optimisant 3 éléments : la molécule immunostrimulatrice, la présence ou non d'une molécule de ciblage des DC, et la nature de la vesicule lipidique. Nous avons aussi formulé des liposomes fluorescents incorporant un fluorochrome lipophile dans leur bicouche lipidique. Cette stratégie nous offre la possibilité de suivre les cellules qui internalisent les liposomes dans la peau, et leur migration jusqu'aux ganglions lymphatiques.

2- **Evaluation de la réponse immunitaire locale et systémique induite par les constructions**. Dans ce but nous avons d'abord évalué la réponse immunitaire induite par la construction d'origine par la voie d'administration TC en comparaison à la voie SC. Nous avons ensuite évalué l'influence de deux molécules immunostrimulatrices sur cette réponse, des agonistes de TLR2/6 et de TLR4, ainsi que l'effet de l'addition du mannose. Nous avons finalement évalué l'influence de la fluidité de la vésicule phospholipidique sur la réponse immunitaire induite.

3- **Evaluation de la migration des DC de la peau** induite par une immunisation TC par les formulations liposomiques, vers les ganglions lymphatiques drainant la zone d'application.

Objectif 2 : Evaluation de l'immunogénicité des liposomes dans le modèle Hu-SPL-NSG

Les résultats de cette partie sont présentés dans l'article #2, en préparation.

La capacité des souris humanisées à répondre à des formulations liposomiques n'est pas bien établie dans la littérature. Pour cela nous avons choisi une formulation-modèle comprenant un épitope B au lieu de l'épitope $TCD8^+$, en addition a l'épitope $TCD4^+$ et a un agoniste de TLR. Ce choix nous a permis d'évaluer la capacité de suivre l'induction d'une réponse humorale ainsi qu'une réponse cellulaire.

1- Evaluation de la toxicité des agonistes de TLR vis-à-vis des splénocytes humains : Dans une première étape, nous avons évalué les liposomes incorporant différents agonistes de TLR vis-à-vis des splénocytes humains en culture pour leur capacité d'induire une toxicité ou une prolifération. En plus, nous avons évalué leur effet sur la reconstitution des souris Hu-SPL-NSG.
2- Evaluation de la capacité des liposomes à induire une réponse immunitaire chez la souris Hu-SPL-NSG, contre l'épitope B et l'épitope T $CD4^+$

Résultats :

1. Formulation et caractérisation des vaccins liposomiques

Pour répondre au 1^{er} objectif, nous avons modifié des constructions vaccinales validées pour des immunisations par voie SC pour formuler de nouvelles constructions potentiellement plus adaptées pour la voie TC. Dans ces constructions, nous avons associé un peptide $TCD4^+$ issu de l'hémagglutinine du virus de la grippe (HA) et un peptide $TCD8^+$ issu de la protéine ErbB2 humaine. Nous avons fait varier la nature de l'adjuvant en utilisant soit le Pam_2CAG (dipalmitoyl-cystéine-alanyl-glycine), agoniste de TLR2/6, soit le MPLA (monophosphoryl lipid A), agoniste de TLR4. De plus, nous avons modifié la composition et les propriétés physicochimiques de la vésicule lipidique, en utilisant soit des liposomes conventionnels soit des liposomes ultradéformables, appelés transfersomes. Finalement, nous avons testé l'avantage potentiel de l'addition du mannose (dioleyl glycérol-dimannose ou DOG-$Man_{2)}$, molécule de ciblage connue pour cibler les DC et ainsi favoriser la capture de la construction liposomique.

En addition, nous avons formulé des liposomes fluorescents (Lp DiI) en incorporant dans leur bicouche lipidique un fluorochrome lipophile.

Pour répondre au $2^{ème}$ objectif, nous avons préparé des liposomes incorporant uniquement un agoniste de TLR. Nous avons varié la nature de cet agoniste en utilisant soit le MPLA (agoniste de TLR4), soit Pam_3CAG (tripalmitoyl-cystéine-alanyl-glycine), ligand de TLR2/1, soit le Pam_2CAG, ligand de TLR2/6. En se basant sur l'évaluation de l'effet de ces agonistes sur les

splénocytes humains en culture, nous avons choisi le Pam_2CAG pour l'associer aux peptides B, issu de la pilline *de Pseudomonas euruginosa* (PAK), et TCD4+ (HA).

Les liposomes et les transfersomes ont été préparés par la technique d'hydratation d'un film lipidique à partir de phospholipides additionnés de l'adjuvant Pam_2CAG ou MPLA et d'une ancre amphiphile fonctionnalisée qui permet l'ancrage des épitopes peptidiques à la surface de la construction. Lorsque nécessaire, les résidus mannose sont ajoutés au mélange de départ. La suspension aqueuse obtenue, contenant des vésicules multi-lamellaires, a été soniquée ou extrudée pour obtenir une population homogène de liposomes unilamellaires de petite taille (SUV). Sur les SUV ainsi obtenus ont été ensuite greffés les peptides épitopiques issus des protéines ErbB2 ou PAK, en addition à HA. La caractérisation physicochimique de ces formulations a montré qu'elles présentent des diamètres moyens de l'ordre de 70 à 90 nm avec une distribution étroite. Les indices de polydispersité étaient tous inférieurs à 0.3 indiquant une homogénéité des échantillons. Le rendement de couplage des épitopes était entre 90 et 100% pour les liposomes et de l'ordre de 75% pour les transfersomes.

Ainsi, grâce à une technique de formulation robuste et maitrisée, nous avons préparé des constructions liposomiques homogènes tout au long de ce travail, ce qui représente un atout incontestable pour leur évaluation *in vivo*.

2. Evaluation des constructions vaccinales administrées par voie TC dans la souris BALB/c

Pour évaluer la capacité des différentes constructions vaccinales à induire une réponse immunitaire à médiation cellulaire après administration TC, les liposomes et les transfersomes portant les épitopes peptidiques associés à un adjuvant (Pam_2CAG ou MPLA), et portant ou non une molécule de ciblage (DOG-Man_2) ont été administrés par massage précédé d'une application d'éthanol à des souris BALB/c (J0, J2, J8). Après 30 jours, le nombre de lymphocytes T spléniques ou ganglionnaires spécifiques des peptides portés par les liposomes a été évalué à l'aide d'un test ELISPOT mesurant la production d'IFN-γ.

En comparant la réponse immunitaire induite par la formulation d'origine par les 2 voies SC et TC, nous avons démontré une sécrétion d'IFN-γ par les cellules de la rate et des ganglions. Ceci montre que la voie TC est capable d'induire une réponse immunitaire aussi puissante que celle

induite par la voie SC. Cette preuve de concept constitue un rational qui nous permet d'adapter nos formulations pour la voie TC.

Pour choisir le ligand TLR qui est le plus efficace par la voie TC, nous avons ensuite comparé l'immunogénicité de constructions liposomiques incorporant différents agonistes de TLR, un agoniste de TLR2/6 (Pam_2CAG) et un agoniste de TLR4 (MPLA), pour leur effet immunostimulateur par application TC chez la souris. Nos résultats ont montré que les liposomes porteurs de Pam_2CAG ont induit, en réponse aux peptides HA et ErbB2, une sécrétion d'IFN-γ par les cellules ganglionnaires (réponse locale) aussi bien que par les cellules de rate (réponse systémique). Par contre, les liposomes porteurs de MPLA ont induit une sécrétion d'IFN-γ uniquement par les cellules de la rate. Ces résultats montrent que les deux agonistes de TLR sont convenables pour une vaccination transcutanée, cependant, Pam_2CAG semble être meilleur que MPLA comme il induit à la fois une réponse locale et systémique.

Pour poursuivre l'optimisation de nos constructions liposomiques pour la voie TC, nous y avons incorporé une molécule de ciblage des DC, le di-mannose, et nous évalué la réponse induite par ces formulations. L'addition du di-mannose aux liposomes n'a pas significativement amélioré la réponse immunitaire observée.

Pour vérifier si une augmentation de la déformabilité de la vésicule lipidique ne pouvait pas améliorer la réponse induite par immunisation TC, nous avons remplacé, dans nos constructions, les liposomes conventionnels par des transfersomes, et nous avons évalué leur effet sur la réponse locale et systémique. De manière générale, les transfersomes n'ont pas amélioré la réponse immunitaire observée. Chez les souris immunisées par les transfersomes, nous avons noté dans les splénocytes et dans les ganglions une production d'IFN-γ en réponse au peptide issu d'ErbB2 mais pas ou peu de réponse contre le peptide issu de HA. Ces résultats suggèrent qu'en dépit de leur ultradéformabilité les formulations vaccinales à base de transfersomes ont induit en TC une réponse immunitaire moins bonne que les liposomes conventionnels.

Nos résultats montrent pour la première fois que ces constructions liposomiques sont immunogènes par voie TC et qu'elles sont capable induire aussi bien une réponse $CD8^+$ qu'une réponse T $CD4^+$. Ils montrent aussi que le Pam_2CAG est supérieur au MPLA pour cette voie

d'immunisation, puisqu'il induit à la fois une réponse immunitaire locale et une réponse systémique. Toutefois, les transfersomes et le di-mannose ne semblent pas améliorer la réponse.

3. Etude de l'activation immunitaire locale induite par les constructions liposomiques

Dans le but d'étudier la migration des DC de la peau vers les ganglions drainant la zone d'application après immunisation TC, nous avons préparé des liposomes fluorescents incorporant un fluorochrome dans leur bicouche lipidique. Des souris BALB/c ont reçu, par massage précédé d'une application d'éthanol, ces liposomes incorporant ou non un ligand de TLR. Cette partie du travail a été menée en parallèle à la première, donc comme nous n'avions pas encore d'indications sur l'identité du meilleur agoniste de TLR, nous avons aléatoirement choisi le MPLA. Les souris ont été sacrifiées après 48 heures pour l'analyse de la migration des DC de la peau vers les ganglions brachiaux drainant la zone d'application. L'analyse en cytométrie en flux des suspensions de cellules ganglionnaires a montré une absence de fluorescence dans les ganglions. Toutefois, le nombre de DC provenant de la peau était augmenté, indiquant ainsi que les constructions liposomiques sont capables d'induire la migration des DC de la peau vers les ganglions après application TC. Les cellules qui migrent préférentiellement sont les LCs et les dDCS lang^{-}. Toutefois, nous avons observé que les liposomes blancs sont également capables d'induire cette migration. Ceci pourrait être expliqué par une contamination des liposomes blancs par des molécules pouvant induire la migration de DC. Une autre hypothèse serait que même si les liposomes blancs sont capables d'induire la migration des DC de la peau, seuls les liposomes incorporant un ligand de TLR sont capables d'induire leur maturation.

4. Evaluation des constructions liposomiques dans les souris Hu-SPL-NSG

En parallèle, j'ai analysé au Liban l'immunogénicité des liposomes dans les souris humanisées Hu-SPL-NSG, un autre modèle préclinique plus prédictif de la réponse immunitaire humaine que le modèle murin classique. Dans ce modèle, des souris immunodéficientes sont reconstituées par des splenocytes humains normaux, provenant de donneurs d'organes décédés ou d'accidentés de route splenectomisés.

Dans un premier temps nous avons testé des liposomes incoprporant plusieurs agonistes de TLR, MPLA, Pam_2CAG et un agoniste de TLR2/1, Pam_3AG, pour leur effet sur les cellules spléniques humaines *in vitro*, en recherchant l'induction d'un effet toxique et/ou d'une prolifération de ces cellules. Nous avons noté l'index de prolifération le plus élevé avec le Pam_2CAG, d'où il a été choisi pour incorporation dans les liposomes peptides à évaluer *in vivo.*

Pour avoir une preuve de concept, nous avons d'abord choisi une formulation modèle à évaluer dans la souris Hu-SPL-NSG. Alors qu'une réponse cellulaire ne peut être analysée que dans les organes lymphoïdes secondaires après euthanasie, une réponse humorale offre l'opportunité d'être suivie en cours de l'expérience par ELISA dans les sérums des souris immunisées. Pour cette raison, nous avons remplacé le peptide ErbB2 dans la formulation d'origine par un peptide B, issu de la pilline de *P.aerigunosa* souche K (PAK), et nous avons gardé le peptide HA, en addition au Pam_2CAG.

Pour évaluer les liposomes chez la souris Hu-SPL-NSG, des splénocytes humains ont été cultivés pendant 3 jours avec des constructions liposomiques puis injectés au J3 à des souris NSG. Les souris ont reçu des injections de rappel par ces mêmes constructions par voie intrapéritonéale aux J7 et J21. Aux jours 28 ou 35, les souris ont été sacrifiées et nous avons déterminé la concentration des IgG humaines dans leur sérum (pour vérifier « l'humanisation » des souris) ainsi que la réponse humaine contre HA de leurs cellules spléniques et la reponse specifique anti-PAK dans leur serum.

Nous avons pu mettre en évidence que le sérum de toutes les souris Hu-SPL-NSG contenait des IgG humaines, ce qui reflète une bonne reconstitution et indique que les cellules humaines restent viables et fonctionnelles. Pour évaluer la circulation ciblée des splenocytes humains vers les rates de ces souris, un test d'immunofluorescence indirecte (IFI) a été réalisé et a démontré un «homing» des leucocytes humain vers cet organe. Ces résultats indiquent que l'immunisation des souris Hu-SPL-NSG par les liposomes n'influence pas la viabilité et la fonctionnalité des cellules humaines.

Nous avons ensuite évalué la response immunitaire spécifique induite par la construction modèle chez la souris Hu-PSL-NSG. Un ELISpot réalisé à partir de cellules de rate a indiqué une sécrétion d'IFN-γ humain en réponse au peptide HA, signalant ainsi l'induction d'une réponse $CD4^+$ spécifique aux constructions liposomiques. A notre connaissance, notre travail

figure parmi les premiers qui ont démontré l'immunogénicité de liposomes porteurs de peptides épitopiques dans un modèle de souris humanisée.

Toutefois, nous n'avons pas pu détecter des anticorps spécifiques anti-PAK dans les sérums de ces souris.

Ces résultats constituent une preuve de concept sur l'immunogénicité de la plateforme liposomique sélectionnée dans la souris Hu-PSL-NSG, et reflètent l'utilité de ce modèle dans leur l'évaluation. En plus, ils suggèrent un potentiel prometteur des liposomes comme véhicule vaccinal anti-tumoral pour l'homme.

Conclusion

L'ensemble des résultats de ce projet a permis de démontrer, dans la souris BALB/c, la faisabilité et l'immunogénicité de la vaccination anti-tumorale par la voie TC avec des liposomes portant à leur surface des peptides T $CD8^+$ de TAA et complétés par les éléments nécessaires à l'activation des DC et de cellules Th1. Nos travaux nous ont également permis de démontrer dans un modèle de souris humanisée que la plateforme vaccinale sélectionnée dans les tests réalisés dans le modèle murin reste immunogène vis-à-vis des cellules humaines. Ainsi, la vaccination TC de l'homme avec ce type de formulations pourrait représenter une stratégie non invasive efficace et prometteuse pour l'immunothérapie active antitumorale.

Ces résultats seront complétés par l'évaluation de la capacité de ces constructions à inhiber la croissance de tumeurs exprimant la protéine ErbB2 humaine chez la souris BALB/c, ainsi qu'à leur capacité d'induire une réponse $CD8^+$ chez la souris Hu-SPL-NSG. Le but ultime de ce travail étant le développement, à long terme, d'un modèle de vaccination TC chez la souris humanisée.

ABSTRACT

This thesis project is carried out in co-direction between Prof. Sylvie Fournel at the UMR7199 CNRS at the University of Strasbourg and Pr. Soulaima Chamat at the Laboratory of Immunology at the Lebanese University.

Cancer immunotherapy is gaining more attention thanks to a better understanding of the immune system's role in fighting tumors. Tumor vaccines are intended to induce tumor specific cytotoxic T lymphocytes (CTL) *via* 1- maturation of dendritic cells (DCs) by danger signals provided by the immunostimulatory molecule, 2- activation of $CD4^+$ T cells following recognition of a CD4 epitope presented by the DC, iii) activation of $CD8^+$ T cells following recognition of a CD8 epitope presented by this DC.

The skin is an attractive route of tumor-specific vaccination because of its richness in dendritic cells (DCs) and its capacity to induce robust CTL responses. Skin DCs internalize vaccines and migrate to draining lymph nodes where they induce a systemic immune response. They are especially endowed with the capacity to cross-present antigens to both naive $CD4^+$ and $CD8^+$ T cells, thus, resulting in the induction of a CTL response. Convenient targeting of skin DCs is ensured by transcutaneous (TC) vaccination. However, the skin is impermeable for conventional vaccine preparations. Therefore, peptide-based vaccines are desirable for TC vaccination because their small size facilitates their diffusion through the skin. Additionally, the use of various nanoparticles, such as liposomes and transfersomes, as peptide delivery vectors increases their skin crossing and capture by DCs and subsequently, their immunogenicity in presence of an immunostimulatory molecule.

Therefore, the general objective of my thesis was to develop these liposome-based constructs adapted for cancer immunotherapy by the TC route in humans.

My host laboratory at the University Strasbourg developed highly versatile liposomal constructs to co-deliver all the three crucial elements for an efficient tumor-specific immune response (a CD4 epitope, a CD8 epitope and an adjuvant). These constructs were shown to induce specific anti-tumor immune responses after subcutaneous injection in normal mice.

The first specific objective of my work is to optimize these constructs to induce a potent immune response after transcutaneous (TC) application.

In addition, responses induced in animal models may deviate partially or totally from those observed later in clinical trials. In order to optimize these vaccine formulations for human application, we proposed to evaluate them in an animal model which is more predictive of the human immune response. This model is a humanized mouse developed by my host laboratory at the Lebanese University, in which immunodeficient mice are engrafted with human splenocytes in order to mimic human immune responses. These humanized mice are called Hu-SPL-NSG mice. **The second specific objective of my thesis was therefore to determine whether liposome constructs that were previously validated in the conventional murine can induce detectable human immune responses in the Hu-SPL-SCID model.**

To meet the 1st objective, the previously developed vaccine constructs were optimized in order to be more suitable for the TC route. These constructs express a universal $CD4^+$ T cell epitope-containing peptide from the influenza virus hemagglutinin (HA) and a $CD8^+$ T cell epitope-containing peptide from the human ErbB2 tumor antigen, in addition to an immunostimulatory molecule (TLR2/6 agonist). The optimized vaccine constructs differ by the TLR agonist and the physicochemical properties of the lipid vesicle, resulting in either conventional liposomes or more flexible ones called transfersomes. A DC-targeting molecule, di-mannose, could also be added.

Vaccine constructs were evaluated for their immunogenicity after TC application on previously shaved dorsum of a normal mouse model. Liposomes bearing the peptides in combination with a TLR2/6 (Pam_2CAG) or a TLR4 agonist (MPLA) resulted in the induction of peptide-specific cellular immune response. However, Pam_2CAG seemed to be superior to MPLA, since it induced an immune response both in the spleen (systemic response) and the lymph nodes (local response) of the immunized mice. In contrast, MPLA-bearing liposomal constructs induced only systemic responses. Di-mannose addition to the constructs did not improve the immune response. Similarly, the replacement of the conventional liposomal vesicle with an ultradeformable one, called transfersome, did not improve the immune response. Transfersomes rather seemed to impair HA-specific responses. Our results show that the

liposomal constructs are immunogenic by the TC route. Liposomes incorporating Pam_2CAG as an immunostimulatory molecule seem the most adapted for the TC route.

After confirming the constructs immunogenicity, we investigated their capability to induce skin DC migration to the draining lymph nodes after TC immunization. Lymph node DCs were analyzed by flow cytometry, and revealed that the liposomal constructs incorporating MPLA as a danger molecule induced the migration of skin DCs. However, the same effect was observed with the plain constructs, suggesting either a contamination of these constructs, or a migration that is not accompanied by a maturation of the DCs.

We show herein for the first time that liposomal constructs are immunogenic by the TC route and induce both a $CD8^+$ response and a $CD4^+$ T cell response.

To meet our 2nd objective, we first formulated liposomal constructs incorporating different TLR agonists, namely MPLA, Pam_2CAG, and a TLR2/1 agonist, Pam_3CAG. The evaluation of their safety profile *in vitro* towards human splenocytes indicated Pam_2CAG to be the most appropriate TLR agonist for *in vivo* evaluation.

The immunogenicity of a model liposomal constructs was then tested in the Hu-SPL-NSG mouse model. Liposomes carrying a B cell epitope peptide instead of the ErbB2 peptide, the HA peptide and Pam_2CAG were injected intraperitoneally in NSG mice previously reconstituted with human splenocytes. These liposomal constructs were shown to induce a specific human immune response against HA, inducating that the liposomal constructs are able to induce a specific $CD4^+$ response. However, we were unable to detect specific anti-PAK antibodies in the sera of these mice.

These results are a proof of concept on the immunogenicity of our liposomal platform in the Hu-PSL-NSG mouse, and reflect the utility of the Hu-SPL-NSG model in their evaluation. In addition, they indicate a potential of liposomes as an anti-tumor vaccine vehicle for humans.

In conclusion, all the results of this project demonstrated the feasibility and efficacy of tumor vaccination by the TC route in BALB/c mice with liposomes carrying $CD8^+$ TAA peptides on their surface and incorporating the necessary elements for activation of DCs and Th1 cells. Our work also allowed us to demonstrate in a humanized mouse model that the vaccine platform

selected in the tests carried out in the murine model remains immunogenic to human cells. Thus, human TC vaccination with this type of formulations could represent an effective and promising noninvasive strategy for anti-tumor active immunotherapy.

These results will be completed by evaluating the constructs ability to inhibit the growth of tumors expressing the human ErbB2 protein in the BALB/c mosue as well as their ability to induce a $CD8^+$ response in the Hu-SPL-NSG mouse. On the long run, the ultimate goal of this work is to develop a TC vaccination model in the Hu-SPL-NSG mouse.

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LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cell Cytotoxicity
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
Bcl	B-cell lymphoma
BCR	B cell receptor
BLT	Bone marrow, Liver, Thymus
CDC	Complement-Dependant Cytotoxicity
CEA	Carcinoembryonic Antigen
Chol	Cholesterol
ConA	Concanavalin A
CT	Core of the Tumor
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte-Associated Antigen 4
CV	Coefficient of variation
DAMP	Damage-Associated Molecular Pattern
DC	Dentritic Cells
dDC	Dermal Dendritic cell
DiI	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DLS	Dynamic Light Scattering
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic
DOG-Man_2:	Dioleyglycerol-di-Mannose
DPG-mal	Dipalmitoyl Glycerol-Maleimide
dSEARCH	Dendrite Surveillance Extensions and Retraction Cycling Habitude
EDTA	Ethylenediaminetetraacetic acid
EGFR	Endothelial Growth Factor Receptor
ELISA	Enzyme-Linked lmmunosorbent Assay
ELISpot	Enzyme-Linked lmmunospot
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
gp	glycoprotein
GVHD	Graft Versus Host Disease
HA	Hemaglutinin
HCl	Hydrochloric acid
Her2/ErbB2/Neu	Human epidermal growth factor Receptor-2
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte antigen

HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
HSC	Hematopoietic Stem Cells
Hu	Humanized
ID	Intradermal
IDO	Indolamine 2’3’-Dioxygenase
IFI	Indirect Immunofluorescence
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IFN-γ	Interferon Gamma
ISCOM	Immune Stimulating Complex
Jak	Janus kinase
KAR	Killer Activating Receptor
KIR	Killer-Cell Immunoglobuline-Like Receptors
Lang	Langerin
LC	Langerhans cell
LHRH	Luteinizing-Hormone-Releasing Hormone
ln	Natural log
Lp	Liposome
LPS	Lipopolysaccharides
mAb	monoclonal antibody
MAGE-A3	Melanoma-Associated Antigen 3
MAMP	Microbe-Associated Molecular Pattern
Man	Mannose
MCA	Methylcholanthreme
MDDC	Monocyte-Derived Dendritic Cell
MDSC	Myeloid-Derived Supressor Cells
MHC	Major Histocompatibility Complex
MLV	Multilamellar Vesicle
MPER	Membrane Proximal External Region
MPLA	Monophosphoryl Lipid A
MUC	Mucin
MVV	Multivesicular Vesicles
NK	Natural Killer
NLR	NOD-Like Receptor
NOD	Non-Obese-Diabetic
NSG	NOD-SCID-Gamma null
o/w	oil-in-water
Ova	Ovalbumin
PAK	Pseudomona aeruginosa strain K
Pam_2CAG	Dipalmitoyil Cysteine-Alanyl-Glycine
Pam_3CAG	Tripalmitoyil Cysteine-Alanyl-Glycine
PAP	Prostatic Acidic Phosphatase
PBL	Peripheral Blood Lymphocytes

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PC	Phosphatydilcholine
PDI	Polydisrpersity Index
PD-L1	Programmed Death Protein Ligand 1
PG	Phosphatydilglycerol
PGE2	Prostaglandine E2
PLGA	Poly(Lactic-co-Glycolic Acid)
Poly-ICLC	Polyinosinic-Polycytidylic acid with Polylysine and Carboxymethylcellulose
Prkdc	Protein Kinase, DNA activated, Catalytic polypeptide
PRR	Pattern-Recognition Receptor
PSA	Prostate Specific Antigen
RAG	Recombination-Activating Gene
RNA	Ribonucleic
ROS	Reactive Oxygen Species
SALT	Skin-Associated Lymphoid Tissue
SC	Subcutaneous
SCID	Severe Combined ImmunoDeficiency
SDC	Sodium Deoxycholate
SIS	Skin Immune System
SPC	Soy Phosphatydilcholine
SPL	Splenocytes
SRC	SCID Repopulating Cells
STAT	Signal Transducer and Activator of Transcription
TAA	Tumor Associated Antigen
TAM	Tumor-Associated Macrophages
TBHSP70	Tuberculosis Heat Shock Protein 70
TBS	Tris Buffer Saline
Tc	Transition temperature
TC	Transcutaneous
TCI	Transcutaneous immunization
TCR	T Cell Receptor
Tf	Transfersome
TGF	Transforming Growth Factor
Th1	T helper 1
Th2	T helper 2
TIL	Tumor-Infiltrating Lymphocyte
TLR	Toll-like receptor
TMB	Trimethylbenzidine
TNF-α	Tumor Necrosis Factor Alpha
TRAIL	TNF-Related Apoptosis Inducing Ligand
Treg	Regulatory T Cell
UV	Unilamellar vesicle
VEGF	Vascular Endothelial Growth Factor

FOREWORD

The role of the immune system in fighting tumors

1. Overview of the immune system

The role of the immune system is not only to fight potential intruders from the external environment, known as “non-self”, mainly pathogenic microbes, but also to control harmful modifications within our own cells, that may arise following infection or cancerous transformation, known as “modified self”. It comprises a multitude of cells and molecules that cooperate in an integrated network. The immune system is divided broadly in two arms, respectively the innate immune system and the adaptive immune system.

1.1. Components of the innate and adaptive immune systems

The innate immune system is present in all taxa from cnidarians to mammals but with various modalities. It includes mainly the epithelial barriers, phagocytes (macrophages and neutrophils), dendritic cells (DCs) and different subsets of innate lymphoid cells (ILC) among which the most important are the natural killer (NK) cells, as well as free molecules such as the complement system (figure 1). The defense mechanisms of the innate immunity are designed to respond rapidly to infections and cell transformations. To recognize danger, cells of innate immunity rely only on a limited number of receptors that can bind to molecules which are common to groups of related microbes (these are called microbial-associated molecular patterns or MAMPs) or that are expressed or released by stressed or dying cells (these are called danger-associated molecular patterns or DAMPs) but not by healthy cells. These receptors, called pattern-recognition receptors or PRRs, are identical for all members of the same animal species. Some members of the PRR family are called Toll-like receptors or TLRs; their engagement with MAMPs or DAMPs leads to the activation of the immune cell.

The adaptive immune system is present in all taxa of the jawed Vertebrates. It comprises T and B lymphocytes and antibodies secreted by plasma cells, which derive from activated B cells (figure 1). Components of adaptive immunity rely on a huge number of receptors that are extremely diversified and that recognize a virtually unlimited number of molecules of microbial or non-microbial origin. Molecules recognized by T and B lymphocytes are called antigens. T and B cells are clonally distributed, meaning that each cell acquires during its differentiation a specific antigen receptor that can only recognize a single antigenic determinant called epitope. The B cell receptor (BCR) recognizes “native” epitopes of unprocessed antigens, while the T cell receptor (TCR) can only bind to small “degraded” linear

peptide epitopes generated by the “processing” of antigens within our own cells and loaded on molecules of the major histocompatibility complex (MHC). B and T cells may be activated only after encounter with their specific antigen. Following activation, a B or T cell proliferates, giving rise to a clone of identical cells. Some differentiate into effector cells that participate in the ongoing immune response while others are kept as “memory cells” to better respond to a potential future reencounter with the same antigen. Therefore, while adaptive immunity is delayed in comparison to innate immunity upon a first encounter with intruders, its memory allows it to be more rapid and amplified upon re-challenge. This property is the cornerstone of the principle of vaccination. A vaccine is an antigenic preparation that activates specific lymphocytes and generates memory capable of mounting a more potent secondary antigen-specific immune response.

B cells are in charge of the adaptive humoral immunity, as their main role is to secrete antibodies after differentiation into plasma cells. T cells are in charge of the adaptive cellular immunity. They are divided in three major effector populations, respectively “helper” T cells (Th) which are responsible of secreting cytokines to initiate and regulate the adaptive immune response and amplify innate immunity, cytotoxic T cells (Tc or CTL) that can kill target cells expressing non-self antigens (mostly virally infected cells) or modified-self antigens (transformed cells), and regulatory T cells (Treg) that downregulate other effectors of immunity to avoid hypersensitivity and auto-immune diseases.

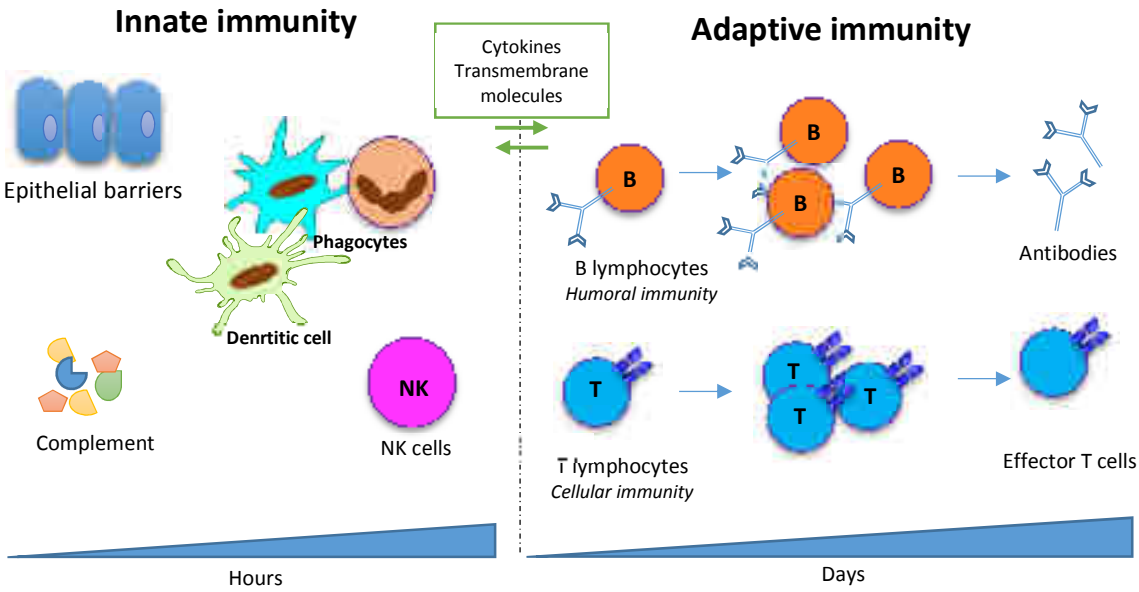


Figure 1 : Cells and molecules of the innate and adaptive immune system.

1.2. Lymphocyte differentiation

All cells of the immune system arise in the bone marrow. While B cells also differentiate before being released in the circulation, T cells arise in the bone marrow but they differentiate in the thymus. To prevent auto-immunity, lymphocyte differentiation involves acquisition of a functional antigen receptor followed by elimination of auto-reactive lymphocytes whose receptor can recognize self antigens. Since BCRs recognize native epitopes, B cell differentiation implies only a negative selection of cells expressing an auto-reactive BCR (figures 2). On the other hand, since TCRs recognize processed antigenic epitopes loaded on MHC molecules, T cell differentiation includes 2 steps: the first is a positive selection of those expressing a TCR capable of binding to self MHC molecules, the second is a negative selection of those that have a high affinity for the complex made by a self-epitope loaded in the MHC molecule (figure 3). There are 2 pathways of antigen processing (figure 4). Proteins present in the cytosol, such as the proteins synthetized by the cell, are called endogenous antigens. They are processed and their peptides are loaded on MHC class I molecule. T cells that are selected on class I molecules differentiate to become $CD8^+$ T cells, which turn mainly into cytotoxic T cells. Proteins that derive from endocytosed or phagocytosed material are called exogenous antigens. They are processed in the endosomal vesicles and their peptides are loaded on MHC class II molecules. T cells that are selected on class II molecules differentiate to become $CD4^+$ T cells, which turn mainly into helper T cells or regulatory T cells.

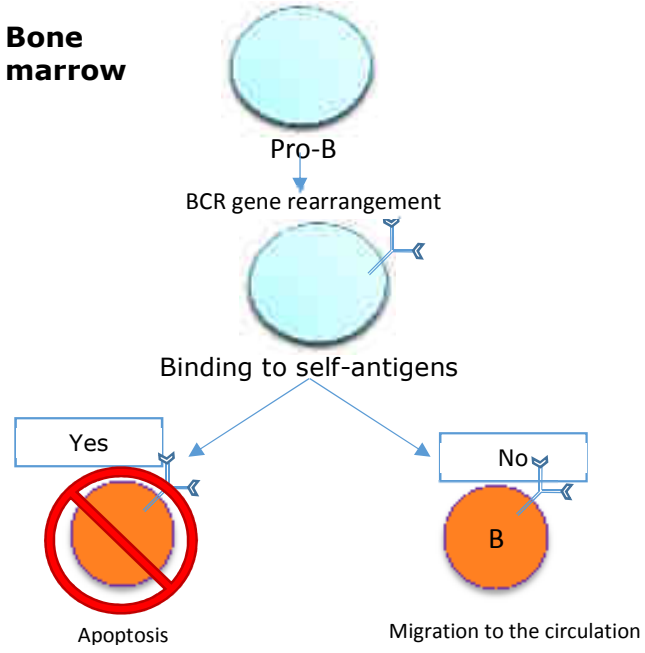


Figure 2 : Negative selection of B cells. *B cell differentiation implies only a negative selection of cells expressing an auto-reactive BCR*

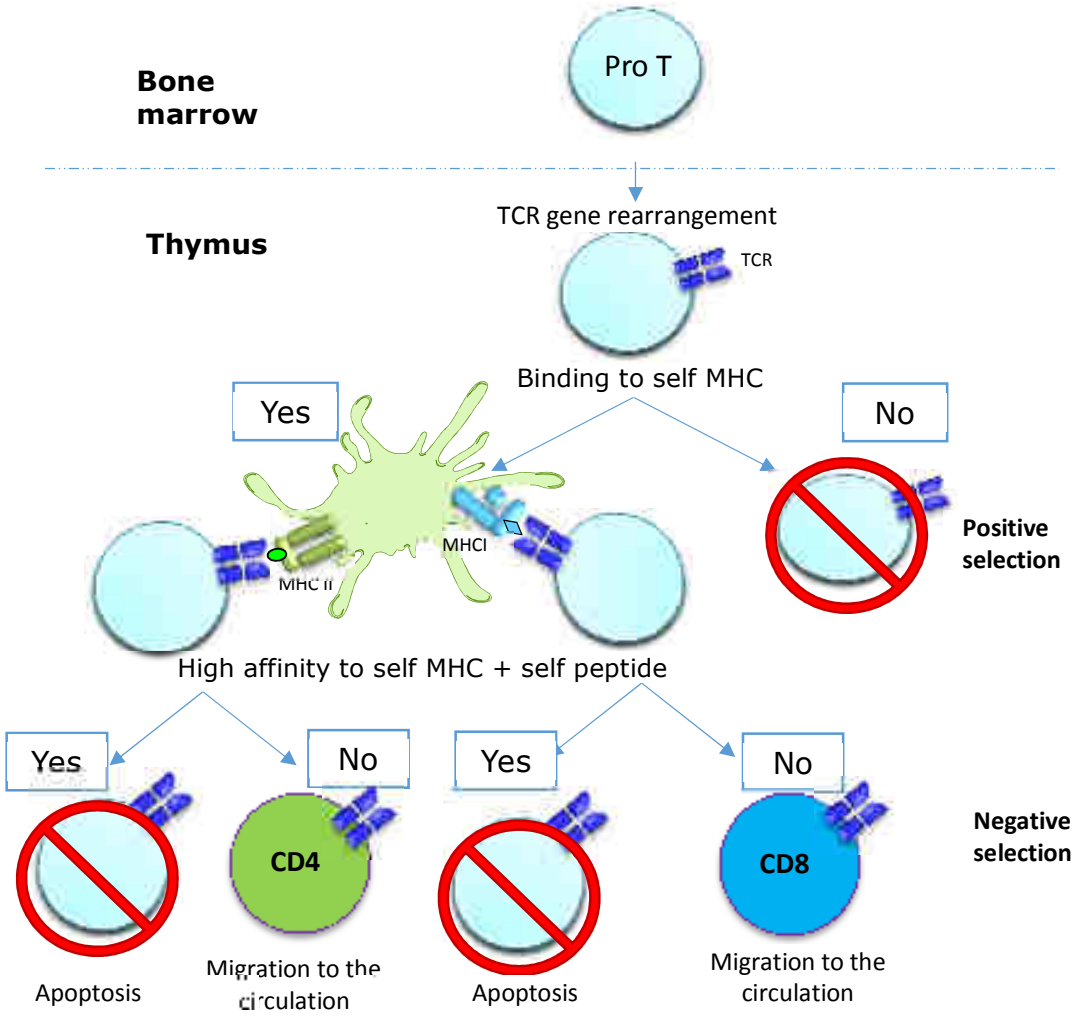


Figure 3: Positive and negative selection of T cells. *Since TCRs recognize processed antigenic epitopes loaded on MHC molecules, T cell differentiation includes 2 steps: the first is a positive selection of those expressing a TCR capable of binding to self MHC molecules, the second is a negative selection of those that have a high affinity for the complex made by a self-epitope loaded in the MHC molecule*

1.3. Innate immune response

The immune system is a powerful defense system that has to be very well controlled in order to avoid inappropriate activation that may lead to harmful inflammation. Therefore, activation of any component requires usually more than one signal, and "cross-talk" between the different components is maintained at all times, mainly by a complex network of cytokines to ensure targeted, effective yet balanced responses.

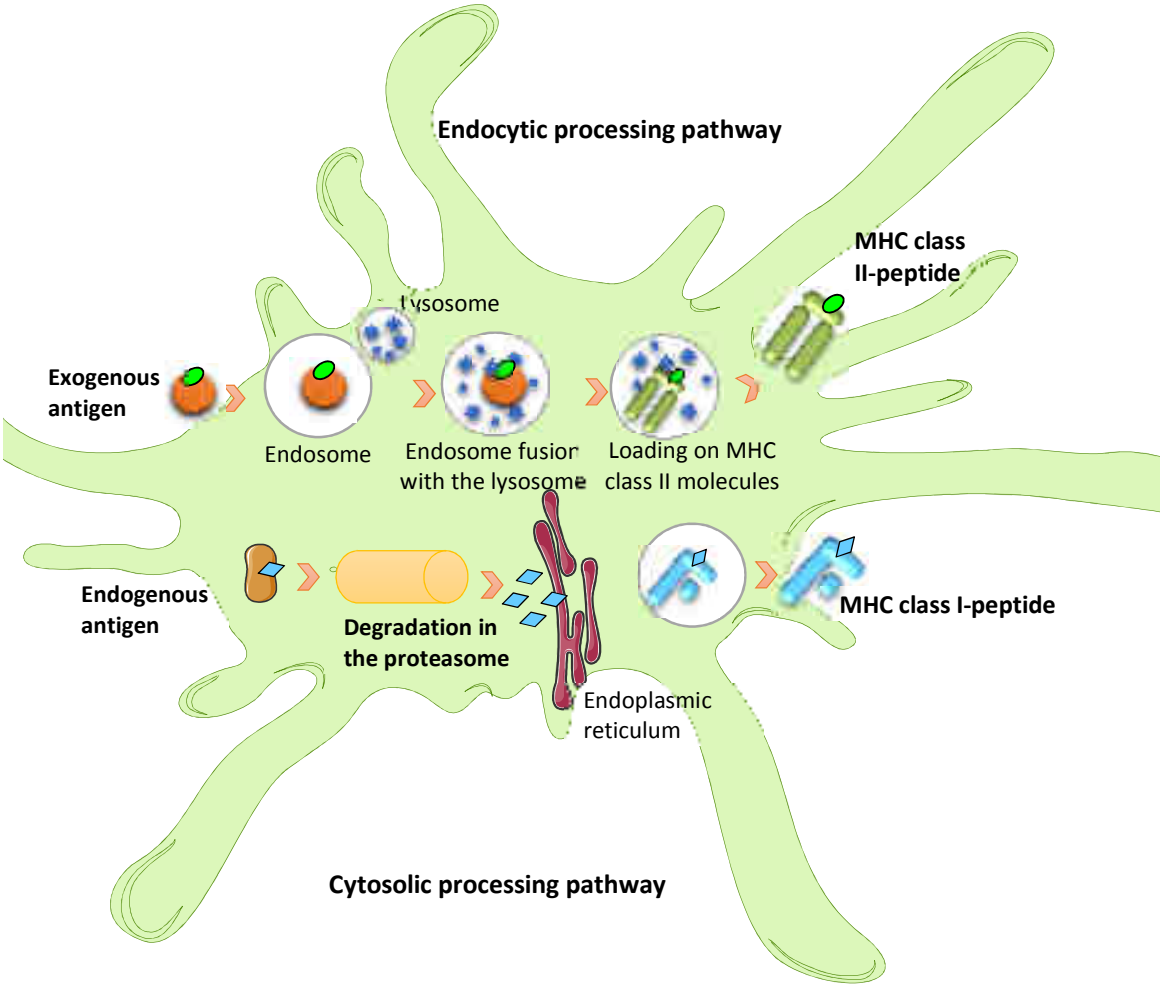


Figure 4 : The two pathways of antigen processing and presentation. *In the exogenous or endocytic pathway, proteins internalized by the cells are degraded in the endosome, and the derived peptides are presented on MHC class II molecules. In the cytosolic or endogenous pathway, proteins which are present in the cytosol, are degraded in the proteasome and the resulting peptides are loaded on MHC class I molecules.*

In case of infection or abnormal cell transformation, an immune response is triggered first when the effectors of the innate immune system recognize danger by binding to MAMPS or DAMPS. Innate immunity has 3 roles: 1) it provides an immediate defense line that eliminates the source of danger or at least keeps it in check 2) it alerts and activates the effectors of the adaptive immune system and 3) even when the adaptive immunity becomes effective, it keeps contributing, in cooperation with lymphocytes and antibodies, to an optimal defense against the intruder.

The type of elicited immune response depends on the nature of the triggering event. For example, in case of viral infection or cell transformation, a prompt response may be provided by NK cells. Indeed, in stressed cells, the amount of expressed MHC class I molecules is

typically reduced. This reduction disturbs the balance between activating and inhibiting signals detected by NK cells and subsequently triggers them to exert a cytotoxic effect on the target. Conversely, in case of infection with extracellular microbes, the most important effectors are neutrophils, which can either ingest pathogens and kill them intracellularly or secrete digestive enzymes and oxidative molecules that are cytotoxic to these pathogens.

Macrophages are present in most tissues. They do not only play a phagocytic role towards microbes and cell debris, but they can also express antigenic peptides derived from phagocytosed material on their MHC class II molecules, to be recognized and receive help from Th1 cells, in order to increase their lytic activity. Finally, macrophages and other inflammatory cells (mast cells, eosinophils, basophils) and molecules (cytokines, complement components…) recruit more effectors to the "battle field" and induce the vascular changes that are needed for this recruitment.

1.4. Activation of adaptive immune responses

The main immune cell players at the interface between the innate and the adaptive arms of the immune system are the dendritic cells (DCs). Their main role is to be antigen presenting cells (APCs). These cells are members of the innate immune system present in most tissues, mainly the skin and the mucosa. They are endowed with long extensions called dendrites, hence their name. Their main role is to constantly "sample" cellular and soluble components from their environment, to endocytose them and to process them in order to present derived peptides to T cells.

Processing of exogenous proteins in endocytic vesicles and loading of resulting peptides on MHC class II molecules leads to antigen presentation to specific $CD4^+$ T cells (figure 4). In the DCs, some exogenous proteins can also leak from the endocytic vesicles and be degraded in the cytosol, generating peptides that are loaded on MHC class I molecules. This unique property of DCs is called cross-presentation of peptides on class I and class II molecules and is crucial for the induction of $CD8^+$ T cell responses. When a DC uptakes foreign material or cell debris containing MAMPs or DAMPs, its PRRs, mainly TLRs, are engaged and mediate its maturation, which is characterized by the expression of co-stimulatory molecules that can be recognized by T cells. Mature DCs migrate to secondary lymphoid organs or tissues where they

present antigens to naïve $CD4^+$ and $CD8^+$ T cells in order to prime antigen-specific T cells (figure 5 A).

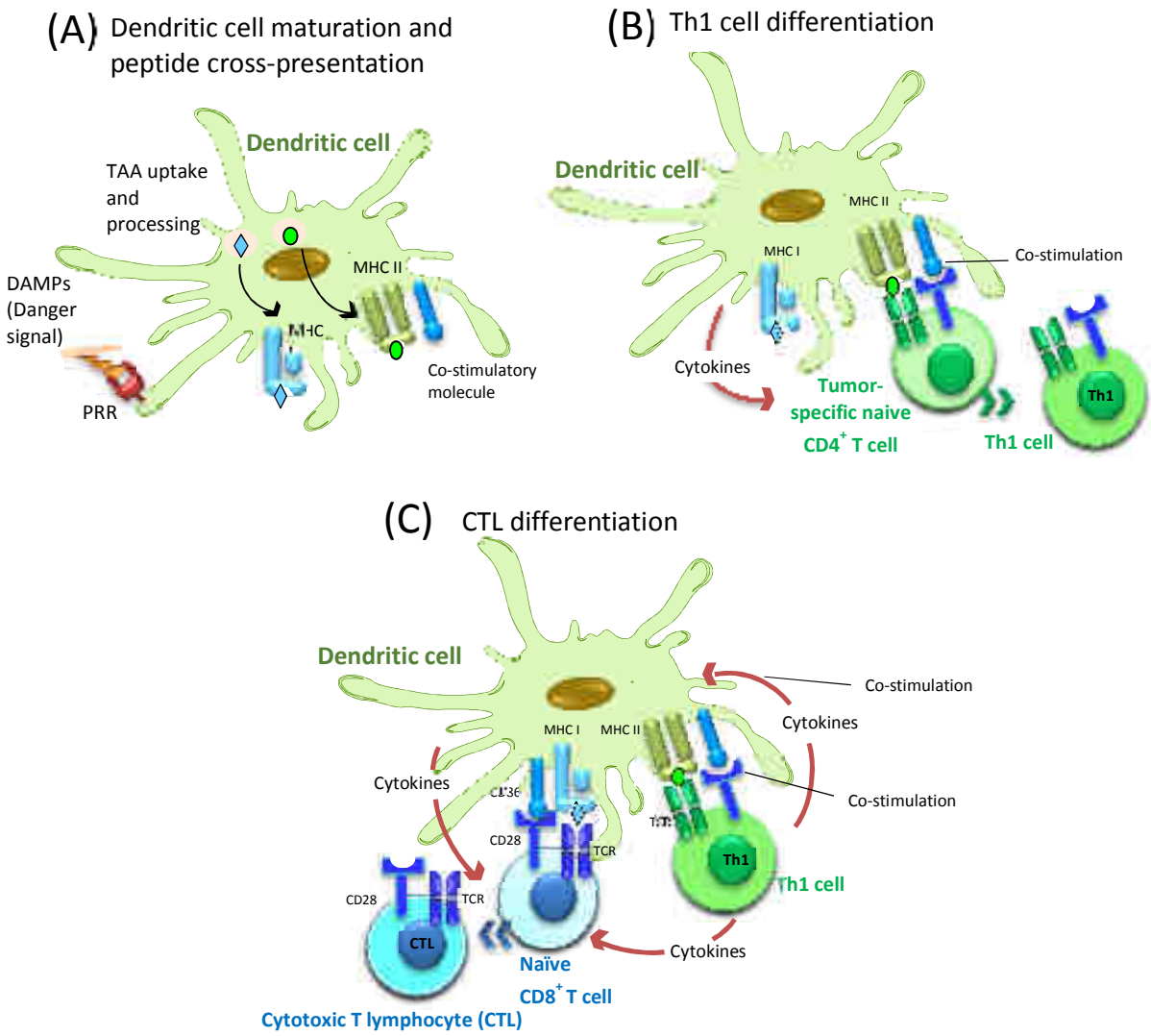


Figure 5 : The induction of cellular adaptive immune response. *(A) DCs internalize cell debris, proteins and microorganisms and cross-present them on MHC class I and II. Signaling through DAMP induces their maturation: they express co-stimulatory molecules. (B) Binding of $CD4^+$ T cells to MHCII-peptide complexes and co-stimulatory molecules, and cytokines received by DCs induce their maturation into TH1. (C) Th1 cytokines induce DCs to upregulate co-stimulatory molecules and secrete cytokines. Binding of $CD8^+$ T cells to DCs and signals received from Th1 and DC induce CTL differentiation*

Differentiation of Th cells: $CD4^+$ T cells are activated by receiving 2 signals from the DC, namely by TCR binding to the class II MHC-peptide complex and by engagement of complementary

co-stimulatory molecules (figure 5 B). They secrete IL-2 and express IL-2 receptors, thus inducing their own proliferation. Following their activation, $CD4^+$ T cell differentiation into Th will depend on the context of cytokines that are secreted by the DC, which is itself dictated by the type of response needed. For instance, IL-12 secretion by the DC promotes mainly differentiation of Th1 that secrete IFN-γ and activate an adaptive cellular immune response to face intracellular infections and cell transformations. Conversely, in the presence of IL-4, Th cells differentiate mainly into Th2 cells that secrete IL-4, IL-5 and IL-13 and activate an adaptive humoral immune response to combat extracellular infections. Other differentiated Th cell populations include, but are not restricted to, Th17 that secrete IL-17 and play an important role in anti-infectious immune responses by activating neutrophils, and induced T regulatory cells (iTreg) that secrete IL-10 and play an immunosuppressive role.

Differentiation of CTL: Differentiation of CTL requires two signals resulting from a triangular engagement of $CD4^+$ T cells, DCs, and $CD8^+$ T cells.

1- Indeed, exogenous peptides are first cross-presented by the DC on MHC class I and MHC class II molecules to $CD8^+$ and $CD4^+$ T cells respectively. The interaction of the T cell receptor (TCR) of the $CD8^+$ T cell with the peptide-MHC class I complex induces the first signal.
2- The second signal is provided by co-stimulatory molecules and cytokines expressed by the DC. These co-stimulatory molecules and cytokines are upregulated following the interaction of this DC with a Th1 cell which previously differentiated from a $CD4^+$ T cell upon its interaction with a peptide-MHC class II complex.

Together, these two signals induce the differentiation of $CD8^+$ T cells specifically bound, along with the Th1 cell, to the same DC into CTL (figure 5 C). Then, CTL upregulate the production of their apoptose-inducing molecules, including perforin and granzymes.

Activation of B cells and plasma cell differentiation: B cells that are selected through BCR binding to native antigens that reach the secondary lymphoid organs or tissues usually need Th cell help to be fully activated; these antigens are called T-dependent antigens. Only complex carbohydrate antigens can activate B cells without TH help; they are referred to as T-independent antigens. Activated B cells give rise to plasma cells that secrete antibodies.

1.5. Effector phase of specific immunity

CTL can kill their target cells, usually virally infected or cancer cells, by inducing apoptosis, a type of programmed cell death. Antibodies binding to soluble antigens help in their elimination by forming immune complexes. Antibodies binding to cellular antigens bridge between the target cell and phagocytes by binding to Fc receptors on the latter; this process is called opsonization.

2. Cancer immunity and immunotherapy

Cancers arise from malignant cell transformations that lead to uncontrolled cell growth and invasion of tissues. These are usually, but not always, accompanied by mutations in cellular antigens, leading to expression of so-called tumor-associated antigens or TAA. Cancer cells are poorly antigenic as the large majority of their proteins are unchanged as compared to the normal cells from which they derive. Moreover, they usually grow in an immunosuppressive microenvironment. As a result, they tend to be tolerated rather than induce an immune response. Nonetheless, recent findings have proven that it is possible to enroll the immune system in cancer therapy. Passive immunotherapy has been used for more than 20 years. It relies on administration of tumor-specific monoclonal antibodies that control cell growth or mediate killing of cancer cells by different effectors of the immune system. More recently, attempts of cancer active immunotherapy are intended to boost the patient's own immune system to better fight the tumor. One strategy is to design cancer vaccines, which would have all the minimum components needed to elicit a protective immune response. These would include i) MAMPs or DAMPs that target and activate dendritic cells, ii) CD8 epitopes derived from TAA to bind specific $CD8^+$ T cells, and iii) CD4 epitopes to activate $CD4^+$ T cells, in order to ultimately initiate a tumor-specific CTL response.

INTRODUCTION

Chapter 1: Eliminating cancer cells using cancer vaccines

1. Overview on cancer development and treatments

1.1. Oncogenesis: a historical perspective

Throughout history, explanations of cancer and carcinogenesis have extensively changed. From the old beliefs of Hippocrates in the "humoral" origin of tumors that stem from an imbalance in the fluids of the body, to the discovery by Muller in 1838 that cancer originates from cells, 19 centuries have elapsed. In the years that followed, multiple theories were stated. Some believed that cancer was induced by trauma, others thought it was caused by parasitic infections or even by chronic irritation. It is only after DNA was discovered by Watson and Crick that the achievements of molecular biology lead to concrete discoveries about cancer origin and development. The modern theories of carcinogenesis started with the identification of oncogenes (figure 6). These are defined as genes involved in normal cells growth, which, when mutated, cause an uncontrolled cell division, thus leading to cancer development. Mutations may occur either spontaneously or because of carcinogens or viruses, thus, mediating the initiation phase. The rapidly proliferating cancer cells grow into tumors depending on various conditions that impose a certain rhythm, particular to each cancer type and to each individual (progression phase). Growing tumors can subsequently acquire an invasive potential and establish metastases in other tissues (invasion phase) (Lonardo *et al.*, 2015).

1.2. Different approaches to cancer therapy

The oldest treatments of cancer relied on total or partial surgical ablation of tumors. As of today, ablation remains the first line treatment for solid tumors. Surgery is usually combined with chemotherapy or radiotherapy, resulting in improved survival of cancer patients. Surgery and radiation are used to locally treat cancer, whereas chemotherapy is systemically administered, and therefore reaches cells that have spread throughout the body. Chemotherapy involves cytotoxic drugs that drive cells into apoptosis through various mechanisms, such as causing DNA damage and strand breakage in dividing cells, blocking folate receptors and inhibiting key enzymes in folate metabolism or even by targeting death receptors.

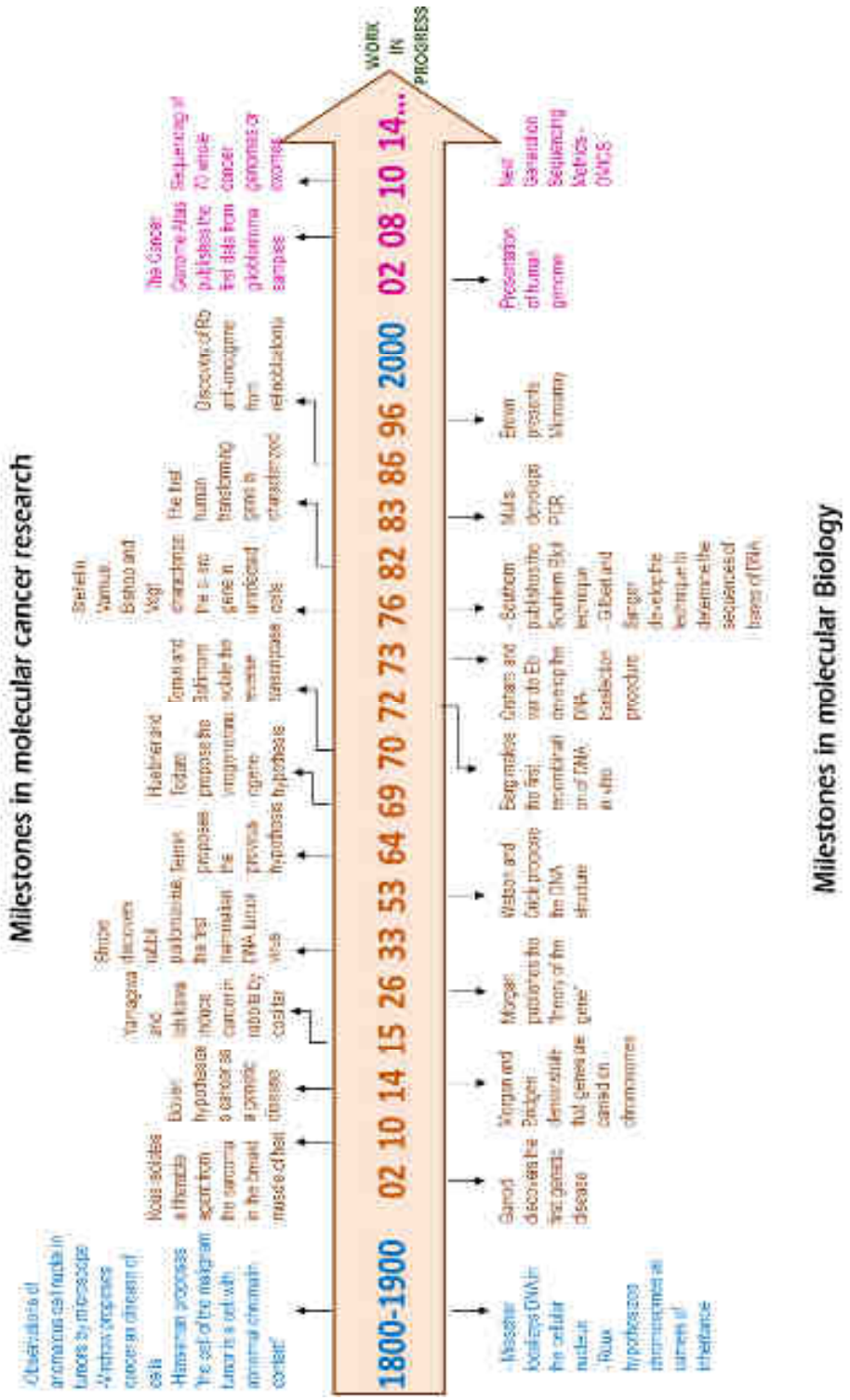


Figure 6: Milestones in molecular cancer research (upper part) and molecular biology (lower part) *(Lonardo et al., 2015). If the lower and the upper timelines are compared, it becomes quite evident that the advancement of our knowledge of molecular processes was decisive in the elaboration of the current understanding of tumor genetics. In particular, DNA structure discovery by Watson and Crick was followed by the modern theories of carcinogenesis and the oncogenes identification. More recently, genome wide sequencing projects and the –omics fields are allowing a broader molecular view of cancer which is accessible to a wider population of the research community.*

Since these mechanisms are poorly specific of tumor cells, they affect normal cells as well and, therefore, they have multiple side effects. Additionally, drug-resistant tumor cells emerge frequently. Combined therapies involving co-administration of two or more cytotoxic drugs that achieve synergistic effects were recently found to improve the clinical outcome. Drugs are chosen to have different mechanisms of action in order to minimize drug resistance and overlapping toxicities while increasing tumor cell killing (Al-Lazikani *et al.*, 2012). To cope with emerging resistance and side effects, the search for alternative cancer therapies has been ongoing for several decades with the aim of finding optimal drugs that would have high efficacy and low intrinsic toxicity to normal tissues, improving the patients' quality of life.

One of the considered alternative approaches is immunotherapy, a strategy that stems from our growing understanding of the immunogenicity of cancer. Passive immunotherapy relies on the administration of pre-formed antibodies targeting either proteins expressed by malignant cells (such as Her2/ErbB2/neu, EGFR, CD20) or soluble growth factors required for tumor growth (such as vascular endothelial growth factor), or on adoptive transfer of autologous *in-vitro* modified tumor-specific lymphocytes, as well as on administrating immunostimulatory molecules (such as TLR agonists) that activate the intrinsic immune response against tumor. Active immunotherapy aims to induce the patient's own immune system to fight tumors by administrating cancer vaccines.

2. Evidence of cancer immunogenicity

2.1. The first evidence of cancer immunogenicity

The first reports on the immune system's ability to fight established tumors were based on the observation that febrile episodes were sometimes followed by complete spontaneous remission in a number of cancer patients (Challis and Stam, 1990; Køstner *et al.*, 2013). These cases caught the attention of William Coley who made, in 1893, the first systematic attempt of tumor immunotherapy. Coley administered to cancer patients a mixture of killed *Streptococcus pyogenes* and *Serratia marcescens* known as "Coley's toxin", and reported that it was able to induce complete remission in a number of soft and bone tissue cancer patients. This "immunostimulant" was intended to induce a non-specific immune activation that favors the induction of a tumor specific immune response. However, poor patient follow-up and variable effectiveness of different Coley's toxin preparations led to severe criticism of his work.

Yet, Coley's theory for cancer immunotherapy was not abandoned. Throughout the following Century, attempts to treat a wide range of cancers, like hepatocellular carcinoma and nodular lymphoma, were carried out using the same strategy, without, however, reaching statistical significance (McCarthy, 2006). Nowadays, bladder cancer is treated with an intra-cystic injection of a variant of Coley's toxin, the Bacillus Calmette-Guerin (BCG) vaccine, which has proven to induce a beneficial clinical outcome by eliciting a local protective immune response against tumor cells. Activation of the antitumor response appeared to be independent of the BCG-specific response, but to rather rely on danger signals provided by the vaccine to the immune system, thus inducing and amplifying the tumor-specific response (Redelman-Sidi *et al.*, 2014; Talat Iqbal and Hussain, 2014; Wu *et al.*, 2015).

In 1909, Ehrlich was the first to propose the hypothesis of cancer immunosurveillance when he suggested that in immunocompetent individuals host defense eliminates aberrant cells and thus prevents them from turning into tumors. However, the lack of biotechnological tools at that time prevented him from proving his hypothesis. It wasn't until 50 years later that this notion was brought back based on growing evidence in clinical practice and in experimental settings, when Gross and Foley attempted to vaccinate mice against a sarcoma or chemically induced tumors (Ribatti, 2016). It is only in 2001 that an elegant study was published by Shankaran *et al.*, demonstrating the role of the immune system, and more specifically, that of T cells and IFN-γ , in protecting mice against chemically-induced and spontaneous tumors (Shankaran *et al.*, 2001).

2.2. Immune response against tumors

2.2.1. The immune system can fight tumors

2.2.1.1 Immunodeficient patients display an increased frequency of malignancies

Individuals with congenital or acquired immunodeficiencies, or transplant recipients undergoing chronic pharmacological immunosuppression, were found to exhibit an increased incidence of various neoplasms (Boshoff and Weiss, 2002; Gatti and Good, 1971; Penn *et al.*, 1971). Congenital deficiency disorders in cellular and/or humoral immunity led to a cancer incidence that could reach 15.4%, a percentage which is 128-fold higher than in age-matched general population (Penn, 1981). Studies conducted on large cohorts of immunocompromised transplant recipients over many decades and in several countries, such as Finland, Denmark,

Norway, Sweden, Australia and New Zealand revealed a constantly increased risk ratio of non-virally-induced tumors including, bladder, colon, pancreas, kidney and ureter cancers, melanoma, lymphomas and endocrine tumors (Dunn *et al.*, 2004; Penn, 1996; Sheil, 1984, 1986). Altogether, these data suggested that cancer formation in these patients is tightly related to the underlying immunodeficiency.

2.2.1.2 Cancer immune infiltration is associated with improved prognosis

Solid tumors are sometimes found to be infiltrated with functionally distinct populations of innate and adaptive immune cells. Among cells of the innate immune system, we can find macrophages, NK cells and DCs, the only APCs capable of initiating adaptive responses. We can also find cells of the adaptive immune response such as naïve and memory B lymphocytes and effector T lymphocytes including Th1, Th2 and CTLs, as well as Treg lymphocytes (figure 7).

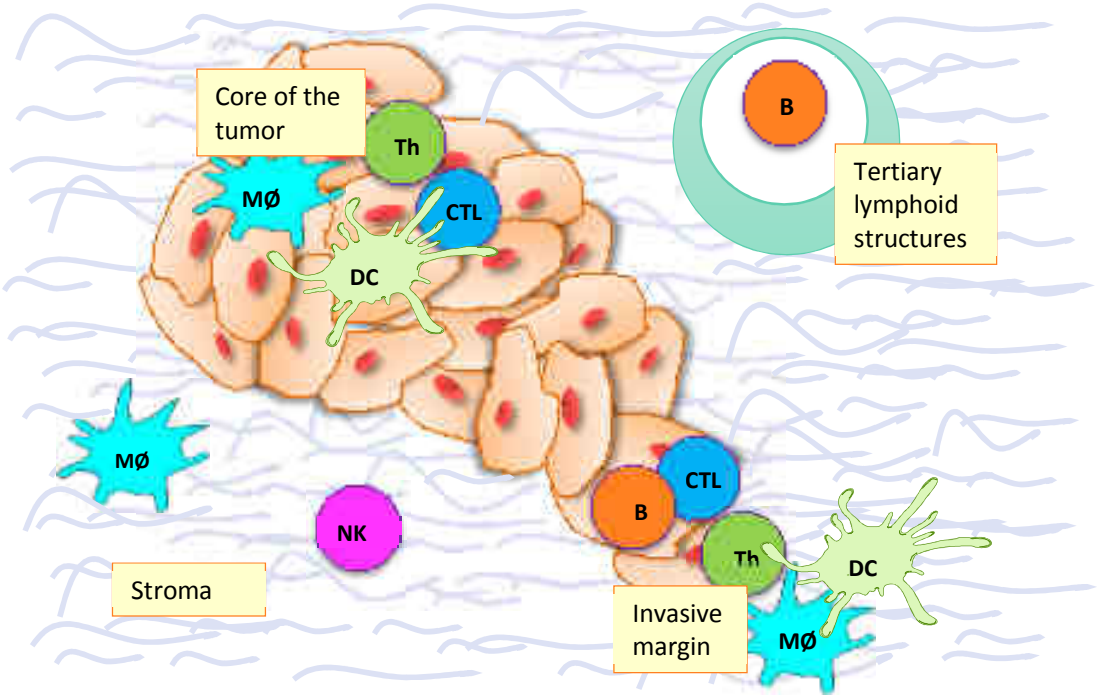


Figure 7: Tumor infiltration with different immune cells. *The distribution of the different immune cells is shown in the tumor core, invasive margin, tumor stroma, and the tertiary lymphoid structures adjacent to the tumor zone. MØ: macrophage; DC: dendritic cell, NK: natural killer; Th: T helper lymphocyte, CTL: cytotoxic T lymphocyte, B: B lymphocyte*

Such immune infiltrates were found to be excessively heterogeneous and to distribute in different locations in the tumor according to various tumor types and to different individuals within the same tumor type. However, in the majority of cases, macrophages and DCs were

found to infiltrate the tumor core or to surround its invasive front. Lymphocytes appeared to be less randomly distributed. NK cells were found to be located in the stroma, the supporting matrix in which tumors grow and which is basically composed of connective tissue and infiltrated by immune cells. T cells were found to colonize either the core of the tumor (CT) or the invasive margin or both. Finally, B cells were found at the invasive margin and in the tertiary lymphoid structures, which are lymphoid aggregates arising in the adjacent zone to the tumor due to chronic immune stimulation (Fridman *et al.*, 2012) (figure 7).

The phenotype, density and location of tumor infiltrating cells were found to largely influence patient outcome. In particular, NK cells and CTLs are of a major importance since they both are cytotoxic effectors against cancer cells. The activity of CTLs depends on a well-established Th1 immunity, whereas it is downregulated by Treg cells. Table 1 summarizes a number of studies that demonstrated associations between the different signatures of the immune infiltrating T cells and prognosis in cancer.

Table 1: Association of immune T cell infiltrates with prognosis in cancer (edited) *(Fridman et al., 2012).*

Cancer type	Memory $CD8^+$ T cells	Th1 cells	Th2 cells	Treg cells
Melanoma	Good			None/ Poor
Head and neck cancers	Good			Good
Breast cancer	Good	Good/ None	Good/ None	None/ Poor
Bladder cancer	Good	Good		Good
Ovarian cancer	Good	Good	Poor	Good /Poor
Oesophageal cancer	Good	Good		
Colorectal cancer	Good	Good	None	Good/None
Renal cell carcinoma	Good /Poor	Good		Poor
Prostatic adenocarcinoma	Good			
Lung carcinoma	Good/ None	Good		Poor
Pancreatic cancer	Good		Poor	Poor
Cervical cancer		Good		
Anal squamous cell carcinoma				None
Brain cancer				None
Hepatocellular carcinoma	Good /Poor	Good		Poor
Gastric cancer		Good	Poor	
Medulloblastoma		Good		
Merkel cell carcinoma	Good			
Urothelial cell carcinoma	Good			
Follicular lymphoma and hodgkin's lymphoma			Good	Good/ None/ Poor

The majority of studies report a favorable influence of memory CD8+ and Th1 cell infiltration. Whereas Th2 cells are less investigated, they may correlate with a good prognosis in certain histological types of cancers, such as breast cancer.

2.2.2. Innate immunity to cancer: NK cells

2.2.2.1. Cancer immune infiltration with NK cells

Natural Killer (NK) cells were first described as non-B and non-T lymphocytes that were capable of killing certain cancer cells in vitro (Herberman *et al.*, 1975). Early reports found that tumor infiltration with NK cells was correlated with improved prognosis in different malignancies. However, these studies used the phenotypic marker CD57, whose expression is shared by NK and activated CTLs. Later reports using the NK-restricted phenotypic marker NKp46 refuted these findings. Tumor-infiltrating NK cells were found to exhibit an anergic state, suggesting that their role in immunosurveillance may be limited to the early stages of cancer development (Fridman *et al.*, 2012).

2.2.2.2. Do NK cells play a role in cancer immunosurveillance?

Suggestions that NK cells contribute to cancer immunosurveillance relied on the observation that NK cell-deficient mice injected with NK-sensitive tumors were found to exhibit an accelerated tumor growth rate, a faster induction time and an increased metastatic tumor spreading, as compared to normal mice (Kärre *et al.*, 1983; Talmadge *et al.*, 1980a, 1980b). By contrast, NK cell depletion or blocking in mice bearing developed tumors did not seem to influence tumor evolution, which strongly suggested that their role is limited to the early stages of cancer development (Vesely *et al.*, 2011). Interestingly, most of the therapeutic antibodies clinically used in cancer treatment (anti-ErbB2, Anti-CD20, anti-CD30 etc…) (table 2) activate NK cell cytotoxicity. These antibodies bind to their cognate tumor-associated antigen (TAA, see below paragraph 2.2.3.3), and subsequently engage their constant part (Fc) with specific receptors on NK cells, thereby activating them to kill the target tumor cells (Wang *et al.*, 2015)(figure 8). This mechanism is called antibody-dependent cell cytotoxicity (ADCC).

Table 2: Antigenic targets, cancer indication and mechanism of action of the therapeutic monoclonal antibodies currently approved by the Food and Drug Administration (FDA) for cancer therapy that involve ADCC *(Coulson et al., 2014)*

Therapeutic monoclonal antibody	Antigenic target	Mode of action	Main cancer indication(S)
RITUXIMAB	CD20	ADCC, CDC, induces apoptosis	Non-Hodgkin's lymphoma
ALEMTUZUMAB	CD52	Induces apoptosis, CDC, ADCC	Chronic lymphocytic leukemia
TOSITUMOMAB	CD20	ADCC, induces apoptosis	Non-Hodgkin's lymphoma
CETUXIMAB	EGFR	ADCC, inhibition of EGFR signaling	Colorectal cancer, head and neck cancer
CATUMAXOMAB*	EpCAM	ADCC, T-cell mediated lysis, phagocytosis *via* FcγR accessory cells	Malignant ascites in patients with EpCAM $^{+}$positive cancers
OFATUMUMAB	CD20	ADCC, CDC	Chronic lymphocytic leukemia
TRASTUZUMAB EMTANSINE	HER2	Inhibition of HER2 signalling, ADCC	Breast cancer

**Approved by European Medicines Agency and undergoing trials in the USA.*

ADCC: antibody-dependent cell cytotoxicity; CDC: complement dependent cytotoxicity; EGFR: Endothelial Growth Factor Receptor; VEGF: Vascular Endothelial Growth Factor.

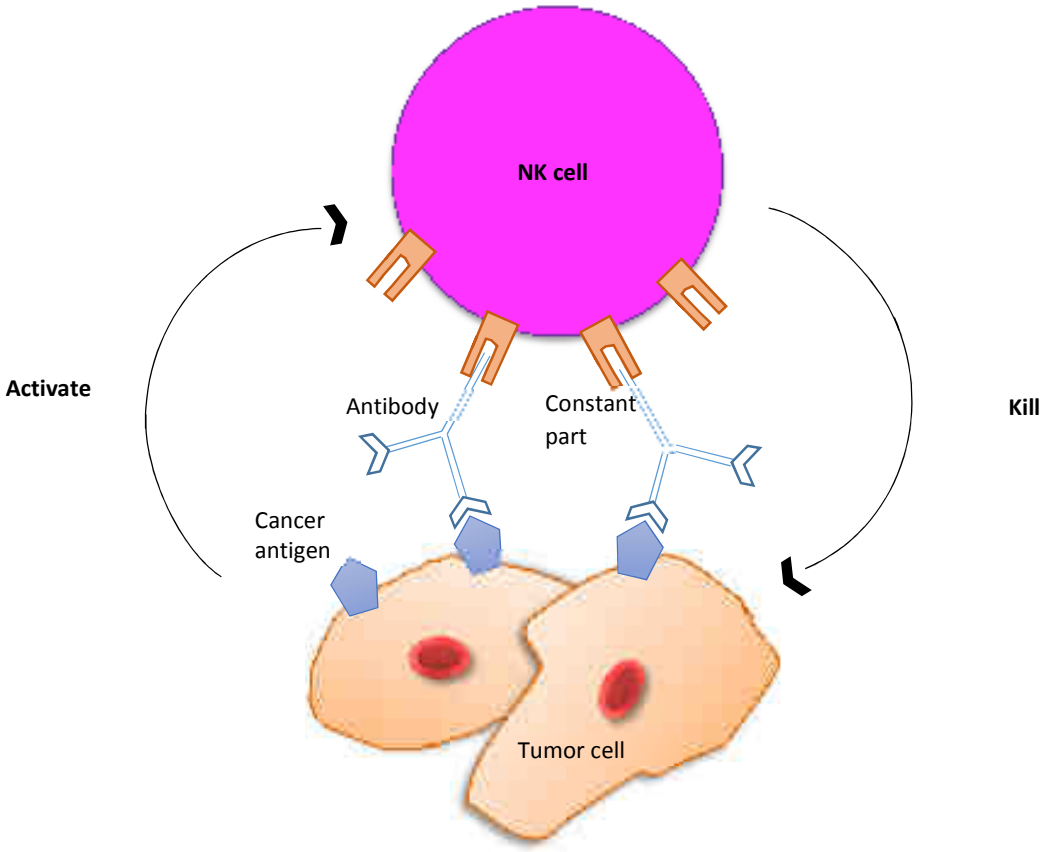


Figure 8: The role of NK cell in monoclonal antibody therapies (ADCC). *Antibodies recognizing their targets on cancer cells engage their constant part with their specific receptors on NK cells, and activate them to kill their target.*

2.2.2.3. How do NK cells attack cancer cells?

NK cells can differentiate between normal cells and transformed cells due to their expression of a panel of activating and inhibitory receptors. The first identified NK inhibitory receptors, termed Killer-cell Immunoglobulin-like Receptors (KIR), were described to recognize MHC class I molecules expressed by normal cells, which are interestingly decreased in cancer cells (Deng and Mariuzza, 2006). More recently, studies revealed that NK cell cytotoxicity is also inhibited by other self-ligands such as E-cadherin (Gründemann *et al.*, 2006), a transmembrane protein that mediates cell-cell adhesion, and collagen, a component of the extracellular matrix (Lebbink *et al.*, 2006). NK cells were additionally found to possess activating receptors that can recognize ubiquitous intracellular self-structures indicating abnormal cellular physiology upon externalization. Other Killer Activating Receptors (KAR) recognize self-ligands that have low

expression level in most tissues, but are preferentially expressed or upregulated in case of cellular distress (Marcus *et al.*, 2014).

Therefore, the summation of activating and inhibitory signals determines whether NK cytotoxic activity is stimulated or dampened upon interaction with a target cell (Vivier *et al.*, 2011). Normal cells expressing high levels of MHC I molecules and other self-ligands, together with low levels of stress molecules inhibit NK cell activity and are spared (figure 9 A). In contrast, cancer cells are killed because they usually express decreased levels of MHC class I molecules (figure 9B). This is the principle of the "missing self-theory" (Kärre, 2008). They may also be killed because of high expression of stress molecules (figure 9C).

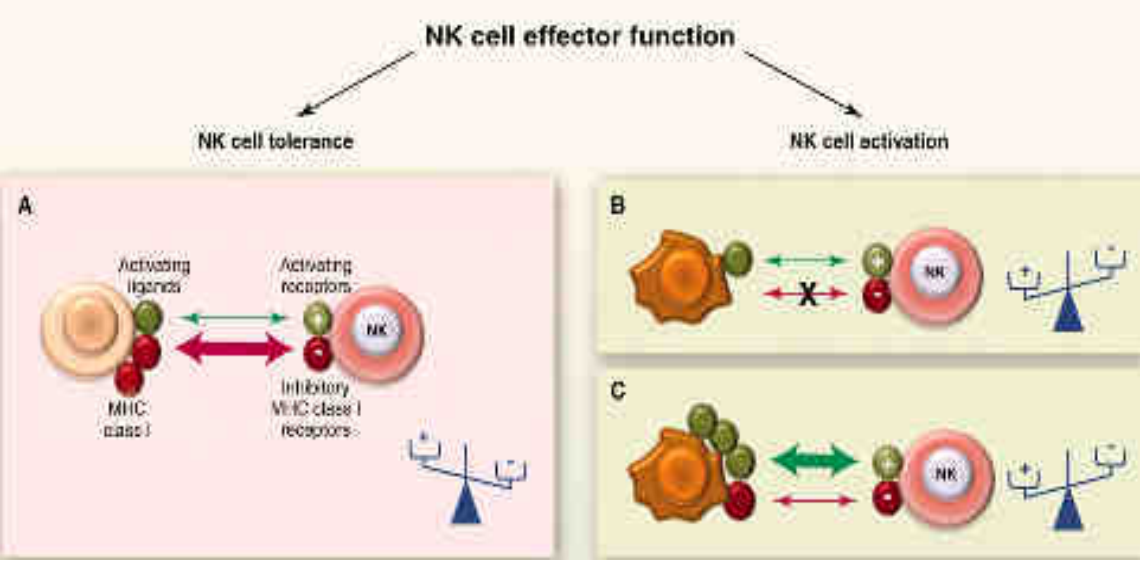


Figure 9: The dynamic regulation of NK cell effector function. *NK cells sense the density of various cell surface molecules expressed at the surface of interacting cells. The integration of these distinct signals dictates the quality and the intensity of the NK cell response. NK cells spare healthy cells that express self-MHC class I molecules and low amounts of stress-induced self-molecules (**A**), whereas they selectively kill target cells "in distress" that down-regulate MHC class I molecules (**B**) or up-regulate stress-induced self-molecules (**C**). +, activating receptors; –, inhibitory receptors. (Vivier et al., 2011).*

NK cells mediate cytotoxicity by inducing apoptosis of target cells, either by death receptor-mediated pathways such as TNF-Related Apoptosis Inducing Ligand (TRAIL) and Fas ligand (FasL) or through the perforin-granzyme pathway. When released perforin forms transmembrane pores in the target cell, granzymes diffuse in to its cytosol and initiate

apoptosis. NK cells can additionally produce cytokines to activate other immune effectors. In particular, they activate macrophages by secreting IFN-γ and induce their polarization towards the pro-inflammatory M1 profile, which coordinates with the Th1 cell type and secretes IL-1, IL-6, TNF-α and IL-12.

2.2.3. Cancer adaptive immune response: the importance of T lymphocytes

2.2.3.1. Cancer immune infiltration with T lymphocytes: from the immune contexture to the immunoscore

Tumor-infiltrating lymphocytes (TILs) correlate with protection. In various independent studies, the number of TILs was found to be significantly and consistently correlated with improved prognosis, such as in melanoma (Clemente *et al.*, 1996) and in colorectal (Naito *et al.*, 1998) and ovarian (Sato *et al.*, 2005; Zhang *et al.*, 2003) cancer patients. In particular, $CD8^+$ T lymphocytes, which differentiate into CTLs, are specialized in killing transformed, infected, or damaged cells. Indeed, studies on colorectal cancer patients revealed that among TILs, the $CD8^+$ T cells were those that conferred protection (Naito *et al.*, 1998). However, in epithelial ovarian cancer, the beneficial effect of $CD8^+$ T cell infiltration was found to be influenced by the $CD8^+/CD4^+$ ratio (Sato *et al.*, 2005; Zhang *et al.*, 2003). Since $CD4^+$ T cells can be either Th1 cells, associated with a CTL response, or Treg cells, which inhibit CTL function, the profile of accompanying $CD4^+$ T cells is decisive in defining the efficacy of $CD8^+$ T cell infiltration.

A broad view of cancer immune infiltration: the immune contexture. The close interactions between various immune cell types makes the conventional "reductionist" research approach, which studies a limited number of elements at a time, insufficient to reveal the complex interrelations arising between the various immune cells and the tumor. Therefore, the type, density, location and functional orientation of immune cells within distinct tumor regions, were altogether defined as the "immune contexture", and their correlation with the clinical outcome was investigated using robust studies of systems immunology (figure 10).To this end, genomic profiling, and immunostaining were performed on resected tumors of large cohorts of colon cancer patients by Gerome Galon and his collaborators. To assess the type of the tumor-specific immune response in these patients, gene and marker clusters, such as a Th1 markers, inflammatory response markers or regulatory response markers, were chosen to indicate the polarization of the immune response. To reveal the location of the immune cells,

samples from two distinct regions of the tumor were taken, namely the core of the tumor (CT) or its invasive margin. Then, robust statistical analysis was conducted to visualize the correlation between the expression of the different gene clusters and cell localization on one hand, and patient outcome on the other hand (Galon *et al.*, 2006, 2007, Pagès *et al.*, 2005, 2009).

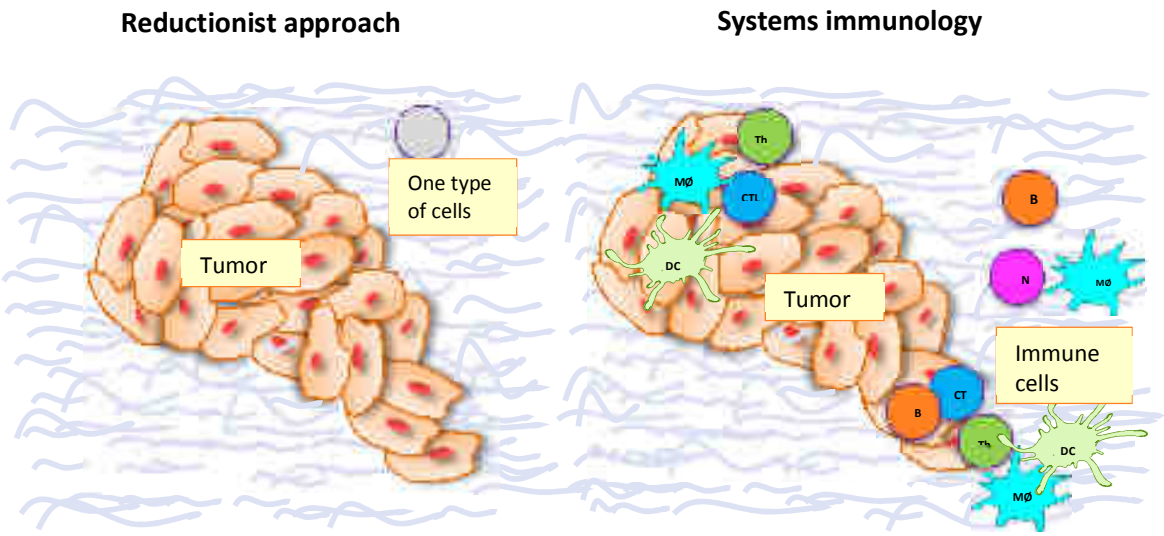


Figure 10: The reductionist view of cancer versus the integrative view of systems immunology. *Tumor cells do not exist alone in their microenvironment. They rather interact with a multitude of immune cells that infiltrate the tumor and its stroma. Fibroblasts also have a major impact on tumor development (not shown). Therefore, the study of the influence of any cell type cannot be performed alone, but has to take into consideration all, or a large number, of accompanying cells, to elucidate the interrelations that continuously arise between them. MØ: macrophage; DC: dendritic cell, NK: natural killer; Th: T helper lymphocyte, CTL: cytotoxic T lymphocyte, B: B lymphocyte*

From the immune contexture to the immunoscore. The results of this study have extensively marked the course of our understanding of cancer immunosurveillance. Indeed, the authors found a strong inverse correlation between the expression level of Th1 effector T-cell markers in distinct tumor region on one hand, and tumor recurrence and early metastatic signs on the other hand (Galon *et al.*, 2006, 2007, Pagès *et al.*, 2005, 2009). These findings indicate that a well-established Th1 response mediates an efficient cytotoxic response, which, in turn, controls cancer cells. Therefore, a new scoring system known as the "immunoscore" was suggested to predict the clinical outcome of patients (figure 11). It is defined by the density of two of the three important cell types, T cells ($CD3^+$), $CD8^+$ cells and effector memory T cells ($CD45RO^+$), together with their localization at the tumor center and invasive margin (Galon *et*

al., 2013). The immunoscore ranges from 0 (I0) when low densities are present in both tumor regions, to 4 (I4) when high densities are found in both regions (Galon *et al.*, 2014) (figure 12).

The potency of the immunoscore in predicting tumor recurrence and survival, along with the failure of standard histopathological staging of tumors to do so, argued for its implementation as a new component of tumor classification. To prove its feasibility and validate its major prognostic power for routine use in colon cancer patients, a retrospective study involving several thousands of tumors is currently ongoing in 23 different centers in 17 countries (Galon *et al.*, 2014).

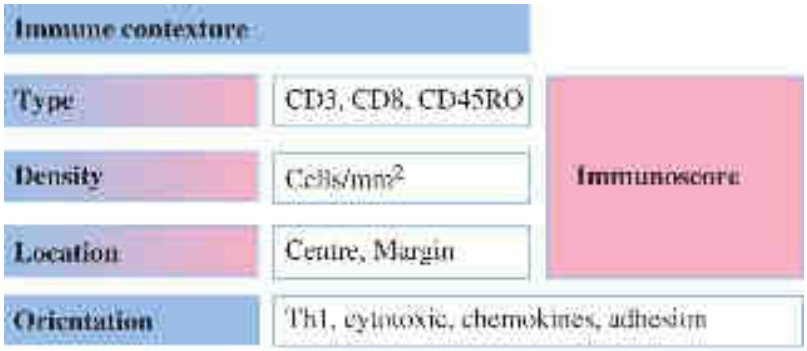


Figure 11: Correspondence between the immune contexture and the Immunoscore *(Galon et al., 2014).*

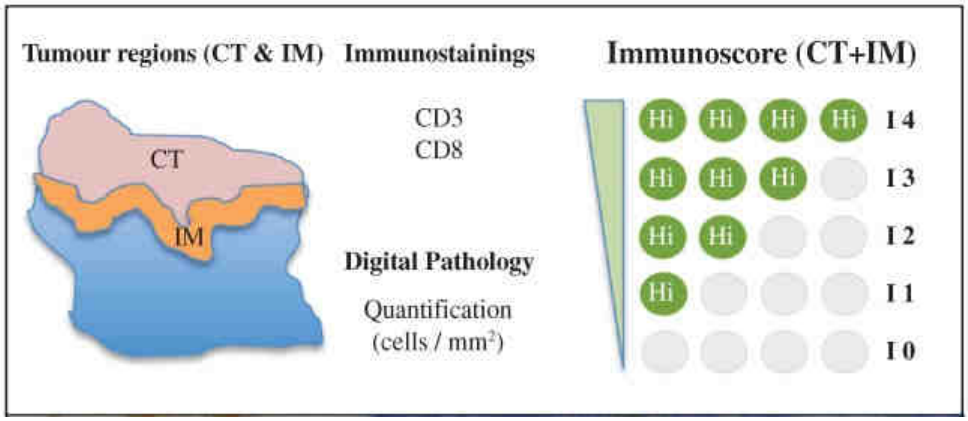


Figure 12: Immunoscore definition and methodology *(edited). (Galon et al., 2014). The immunoscore ranges from 0 (I0) when low densities are present in both the core of the tumor (CT) and the invasive margin (IM), to 4 (I4) when high densities are found in both regions.*

2.2.3.2. Experimental evidence of the role of T cells in cancer immunosurveillance

Lymphocytes control cancer development in mice. Since the early phases of cancer research, lymphocytes were experimentally shown to play a central role in controlling the distinct steps of tumor development.

- *Lymphocytes control early cancer development phases.* Both normal mice and nude mice (mice that lack T lymphocytes) develop tumors following subcutaneous or intramuscular injections of carcinogens such as methylcholanthrene (MCA). Interestingly, monitoring of the delay to sarcoma induction and tumor size showed that nude mice consistently developed tumors more frequently and more rapidly than normal mice (Engel *et al.*, 1997). Despite that nude mice lack only T lymphocytes, they cannot mount B cell responses due to the lack of the "help" of $CD4^+$ T lymphocytes. Therefore, these results can be attributed to a deficiency in lymphocyte functions. Later, studies were conducted on Recombination-Activating Gene (RAG)-deficient mice, which are deficient for B, T and NKT cells. In a hallmark study published in 2001, it was shown that RAG-2 deficient 129/SvEv mice were more susceptible to develop both chemically-induced and spontaneous primary malignancies, as compared to wild type 129/SvEv mice (Shankaran *et al.,* 2001). These findings are in agreement with the clinical data found in immunocompromised humans, and clearly indicate that tumors can trigger an immune response capable of controlling their development. They further show that this surveillance is lymphocyte-mediated and particularly controls the first steps of tumor initiation.
- *Properly-activated lymphocytes control the development of pre-established tumors.* Additionally, *in vitro*-activated lymphocytes were shown to control tumors established in C57BL/6 mice by injection of the sarcoma cell line MCA 38. Adoptive transfer of TILs activated *in vitro* in the presence of IL-2 was shown to confer immunity against pre-developed tumor cells as it resulted in the cure of 50-100% of metastases-bearing mice (Rosenberg *et al.*, 1986). This protocol was adapted for clinical trials as early as 1988 in melanoma patients and was found to induce tumor regression (Rosenberg *et al.*, 1988). These data conferred a comprehensive basis for the ability of lymphocytes to prevent tumor outgrowth during progression and metastasis. As of today, extensive efforts are being deployed in this branch of active cancer immunotherapy.

CTLs mediate tumor cell killing. Whereas transfer of serum could not confer immunity to cancer, administration of *in vitro* generated CTLs specific for adenovirus-transformed cells into tumor-bearing nude mice resulted in the destruction of established subcutaneous tumors (Kast *et al.*, 1989). Similarly, *in vitro*- generated CTLs from melanoma-bearing patients were shown to exhibit a cytotoxic activity against autologous melanoma cells (Knuth *et al.*, 1984).

Additional evidence of the role of CTL in tumor rejection was done in a mouse model of Simian Virus 40-induced tumors in which their presence was correlated to protection against osteosarcomas (Schell *et al.*, 2000).

The role of Th1 cells and interferon (IFN)-γ. Strong evidence emerged from multiple studies supporting a role for Th1 responses and IFN-γ in tumor immunosurveillance. Certain observations indicated that the phenotype, but not the number, of $CD4^+$ T cells in the lymph node of tumor-bearing mice was correlated to tumor rejection. Indeed, syngeneic cells of the P 815 tumor cell line injected in hind footpads of DBA/2 mice were found to regress only in the presence of IFN-γ-producing $CD4^+$ T cells in the tumor-draining lymph nodes (Fallarino *et al.*, 1996). Similarly, transfection of tumor cells with IL-2, which has a major role in the establishment of Th1 responses, and immunization of mice with irradiated transfected cells, proved to be efficient since it resulted in the regression of pre-established tumors (Fallarino *et al.*, 1997). Additional evidence supporting a major role for Th1 responses in tumor rejection was obtained in mice that have Th1-biased immune responses due to the lack of IL-4 and IL-13 (Th2 cytokines) signaling. These mice rejected immunogenic cancer cells that were accepted by wild-type mice (Kacha *et al.*, 2000).

A direct role for IFN-γ in tumor immunosurveillance was later evidenced. In contrast to normal mice, those depleted in IFN-γ by monoclonal antibody administration prior to tumor implantation failed to reject implanted tumors (Dighe *et al.*, 1994). Similarly, in IFN-γ insensitive mice, the frequency of tumor development was increased and the delay to tumor onset was shortened as compared to normal mice (Kaplan *et al.*, 1998).

Interestingly, beside its established role in inducing CTLs, IFN-γ was found to act on cancer cells by enhancing their immunogenicity. When tumor cells were engineered to be IFN-γ insensitive, they exhibited an enhanced *in vivo* tumorigenicity, as compared to IFN-γ sensitive ones. Mice rejecting normal tumor cells were incapable of subsequently rejecting IFN-γ insensitive tumors (Dighe *et al.*, 1994; Kaplan *et al.*, 1998).

Altogether, these data provide rational evidence for the role of IFN-γ producing Th1 cells and CTLs in fighting cancer initiation and development. Furthermore, they underline a

high level of cooperation that exists among these cell types and which is primordial for their function.

2.2.3.3. How do lymphocytes kill tumor cells?

How are tumors recognized by lymphocytes? The role of Tumor Associated Antigens (TAAs). Normal cells presenting endogenous peptides associated to MHC class I molecules on their cell surface (figure 4) are not antigenic. Lymphocytes recognizing these self-peptides are either eliminated during T lymphocytes differentiation in the thymus (foreword, figure 3) or rendered tolerant, thus, preventing the induction of autoimmunity against these peptides. To be able to induce efficient CTL responses in patients and immune rejection in genetically compatible hosts, tumors were speculated to possess specific antigens that were recognized by the immune system, thus triggering their immune-mediated destruction. Thus, to induce a tumor-specific immune response, the first requirement is the expression of modified peptide sequences that are recognized by the immune system. An additional requirement is the presentation of these peptides in an immunostimulatory context (will be detailed in the next paragraph).

Tumor-associated Antigens (TAA) were first identified in tumor transplantation experiments, which prompted a continuous search for TAAs that can serve as targets for immunotherapy (Baldwin, 1971; Van der Bruggen *et al.*, 1991; Van den Eynde and Van der Bruggen, 1997). Up to date, a plethora of TAAs has been identified and comprehensive overview of their classification and their utility is provided by the “database for T-cell defined tumor antigens” (Vigneron *et al.*, 2013).

TAAs can classified according to several ways. Depending on the pattern of expression of the parental gene, tumor specific antigens are divided into viral antigens, unique mutated antigens and cancer germline antigens. Non-tumor specific antigens are either overexpressed or tissue-specific antigens linked to a differentiation process.

1. **Viral antigens** derive from viral proteins synthesized inside virus-induced tumor cells, such as in cervical carcinoma, nasopharyngeal carcinoma, hepatocarcinoma, and some leukemias (Vigneron, 2015).

2. **Unique mutated antigens** derive from point mutations that usually change one amino acid in the peptide sequence. Less frequently, point mutations produce totally new peptides due to a frameshift (Vigneron, 2015).

3. **Cancer germline antigens or cancer-testis antigens** are expressed in several tumors but not in most normal tissues due to the methylation of their genes. In some advanced tumors, cancer germline gene promoters are demethylated due to the genome-wide demethylation that takes place and therefore, they have their products expressed. The exclusive expression of cancer germline antigens by tumors makes them attractive targets for immunotherapy and especially for cancer vaccines (Vigneron, 2015).

4. **Differentiation antigens** are encoded by genes with a tissue-specific expression. They are found on the normal tissue as well as on the derived tumor. They were identified mostly on lymphoid and myeloid leukemia but also on melanoma cells (tyrosinase, gp100, Melan-A/MART), on prostate cancer cells (prostate specific antigen PSA, prostatic acidic phosphatase PAP) and on colorectal cancer cells (carcinoembryonic antigen CEA) (Vigneron, 2015).

5. **Overexpressed antigens** are TAAs that have a low expression in normal cells, yet, as a result of gene amplification or increased transcription, they are overexpressed in malignant cells. Therefore, they are interesting cancer vaccine candidates. Examples include the oncogene and growth factor receptor ErbB2 (HER2/NEU), which is overexpressed in a number of epithelial tumors, such as ovarian and breast carcinoma (Vigneron, 2015).

Table 3: Classification and examples of TAAs based on molecular criteria (Zarour et al., 2003).

CATEGORY	EXAMPLE ANTIGEN	CANCER HISTOLOGY
ONCOFETAL	CEA	Colorectal carcinoma
	TAG-72	Prostate carcinoma
ONCOVIRAL	HPV E6, E7	Cervical carcinoma
OVEREXPRESSED/ACCUMULATED	BING-4	Melanoma
	9D7	RCC
	Ep-CAM	Breast carcinoma
	EphA3	Multi
	Her2/neu	Multi
	Telomerase	Multi
	Survivin	Multi
CANCER-TESTIS	BAGE family	Multi
	CAGE family	Multi
	GAGE family	Multi
	MAGE family	Multi
CT9, CT10	NY-ESO-1/LAGE-1	Multi
	PRAME	Multi
	SSX-2	Melanoma, Multi
LINEAGE RESTRICTED	Melan-A/MART-1	Melanoma
	Gp100/pmel17	Melanoma
	Tyrosinase	Melanoma
	TRP-1/-2	Melanoma
	P.polypeptide	Melanoma
	Prostate-specific antigen	Prostate
MUTATED	β-catenin	Melanoma, Prostate, HCC
	BRCA1/2	Breast, ovarian carcinoma
	Fibronectin	Multi
	MART-2	Melanoma
	p53	Multi
	Ras	Multi
POSTTRANSLATIONALLY ALTERED	MUC1	Ductal carcinoma, RCC
IDIOTYPIC	Ig, TCR	B, T leukemia, lymphoma, myeloma

Depicted are the various classes of TAAs. Beside the 1) oncoviral antigens, we find 2) unique mutated antigens, 3) cancer germline antigens, which are cancer testis antigens, CT9 and CT10. We also find 4) differentiation antigens, which may be oncofetal, idiotypic (found in hematological malignancies), lineage-restricted antigens, or post-translationally altered antigens. Finally, 5) overexpressed antigens such as Survivin are also shown. BRCA = breast cancer antigen; CDK4 = cyclin-dependent kinase-4; CEA = carcino-embryonic antigen; CML66 = chronic myelogenous leukemia (antigen) 66; CT= cancer testis; HPV = human papilloma virus; Ep-CAM = epithelial cell adhesion molecule; Ig = immunoglobulin; MART-1/-2 = melanoma antigen recognized by T cells-1/-2; MC1R = melanocortin-1-receptor; SAP-1 = stomach cancer- associated protein tyrosine phosphatase-1; TAG-72 = tumor antigen-72; TCR = T cell receptor; TGF-βRII = transforming growth factor-β receptor II; TRP = tyrosinase-related protein.

How is a T-cell response mounted against the TAA? Tumor cell destruction depends on the differentiation of $CD8^+$ T cells into CTLs capable of specifically recognizing the TAAs. Upon binding to their peptides expressed on the tumor cell surface, CTLs induce tumor cell apoptosis through the perforin-granzyme pathway or the FasL pathway.

The initiation of the tumor specific cytotoxic response relies on the cooperation between 3 cell types: the DCs, the $CD4^+$ T cells, and the $CD8^+$ T cells. First, DCs internalize tumor cell debris and constituents (such as lysates, apoptotic bodies, or exosomes), generated either by spontaneous cancer cell lysis or NK cell-mediated destruction, or by nibbling from live cancer cells. The internalized material is processed and the resulting TAAs are cross-presented as peptide-MHC class II and MHC class I complexes to $CD4^+$ and $CD8^+$ T cells respectively.

In parallel, the DC receives from the tumor cell a danger signal that induces its maturation. Danger signals are provided by endogenous molecules that have a physiological role in normal cells, but serve as immunostimulatory molecules that indicate cell damage when released from stressed cells, such as ATP and DNA, or when exposed on their surface, such as α-integrin and phosphatidylserine. They are therefore termed DAMPs. Similar to MAMPs, usually sensed by the organism to identify the presence of pathogens, DAMPs bind to pattern recognition receptors (PRRs), like toll-like receptors (TLRs) and NOD-like receptors (NLRs). Mature DCs upregulate the expression of MHC II molecules that present tumor cell derived-peptides and express co-stimulatory molecules, such as B7.1 and B7.2 (also known as CD80 and CD86) (Kurts *et al.*, 2010) that subsequently engage with the CD28 co-receptor on T cells.

Binding of $CD4^+$ and $CD8^+$ T cells to their specific peptides presented by the mature DC induces a cascade of events. First, engagement of $CD4^+$ T cells with their specific peptide-MHC class II complexes, together with the engagement of co-stimulatory molecules induce their differentiation into Th1 cells. Subsequently, while Th1 cells remain bound to the DC, they ligate their CD40 ligand (CD40L) with the CD40 receptor expressed on the DC. Additionally, they secrete IFN-γ that acts on the DC by increasing its expression of co-stimulatory molecules and, thus, license it to induce the differentiation of the $CD8^+$ T cells into CTLs. For this differentiation to happen, $CD8^+$T that recognize their cognate peptide loaded on MHC class I molecules on the DC also need IL-2 and IFN-γ secreted by the Th1 (figure 13). Finally, effector CTLs migrate, infiltrate the tumor and recognize and lyse malignant cells expressing the TAA.

Therefore, the induction of an effective tumor-specific CTL response depends first on the capacity of the DC to cross-present TAAs on MHC class I and class II molecules, a major characteristic of DCs, and on its maturation state. In other words, it depends on the simultaneous presence of $CD4^+$ and $CD8^+$ T cell epitopes along with a danger signal.

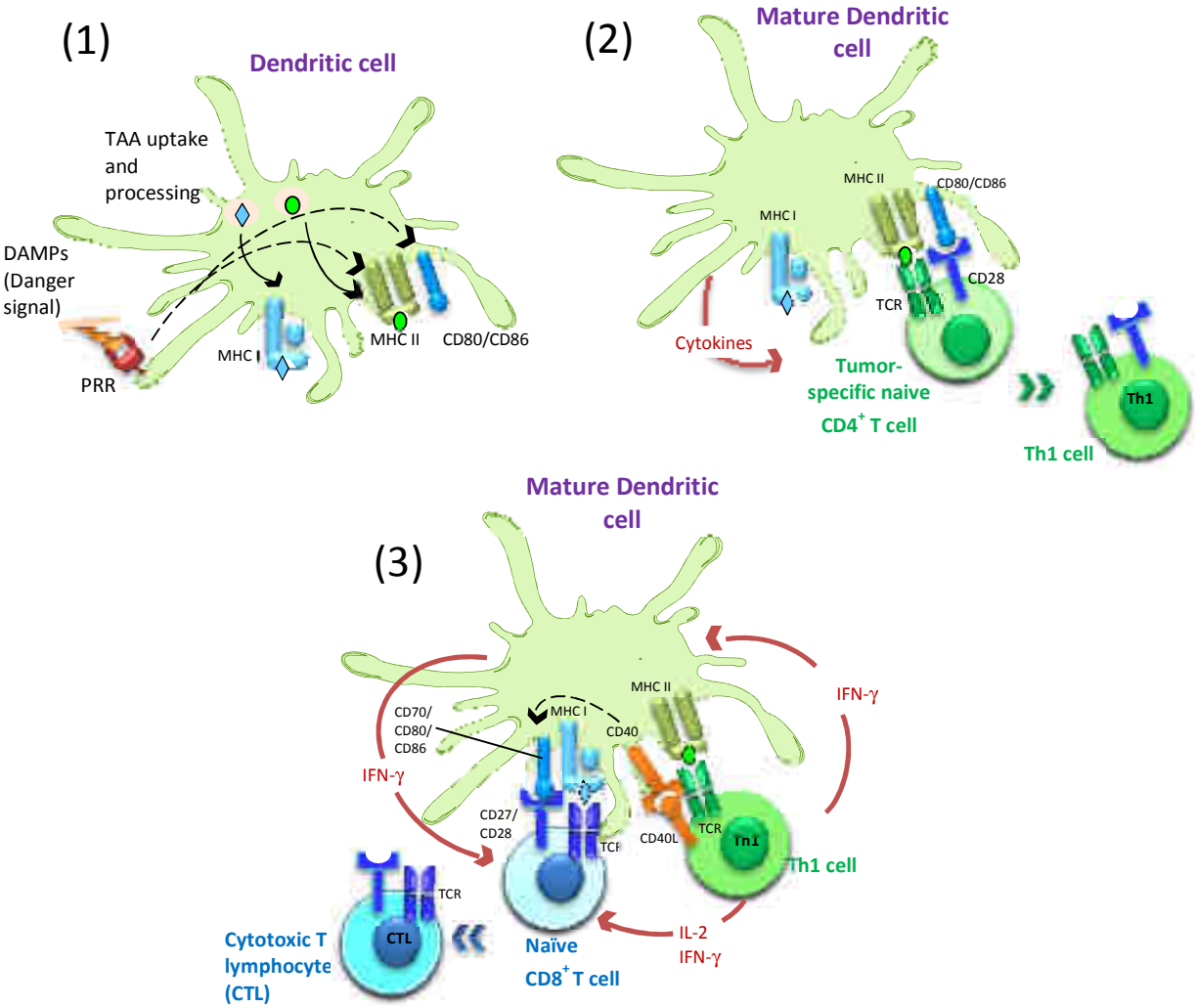


Figure 13: The molecular mechanisms involved in CTL induction. *(1) Dendritic cells (DCs) uptake TAAs in exosomes and apoptotic bodies released from cancer cells (not shown), and cross-present their derived peptides to $CD4^+$ T cells and $CD8^+$ T cells on MHC class II and MHC class I molecules respectively. A danger signal such as a TLR agonist induces the DC maturation and upregulates the expression of MHC class II molecules and of co-stimulatory molecules.(2) Activated $CD4^+$ T cells differentiate into Th1 cells. (3) that produce IL-2 and licence the DC for cross-priming through CD40 ligand (CD40L)–CD40 interactions. Licensed DCs upregulate their expression of co-stimulatory molecules, such as CD70, CD80 and CD86. Under the effect of its interaction with the DC, and of the IFN-γ and IL-2 cytokines, $CD8^+$ T cells differentiate into CTLs.*

2.2.4. Cancer adaptive immune response: the role of B lymphocytes

The potential contribution of natural B cell responses to cancer-specific immunosurveillance is far less understood than that of the T cell immunity. Some reports documenting the infiltration of tumors with TILs of the B-cell lineage (B-TILs) correlated it to better survival in certain tumors (Iglesia *et al.*, 2014; Schmidt *et al.*, 2008). Additionally, B-cell depletion was found to impair tumor-specific effector and memory IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells in a melanoma mouse model (DiLillo *et al.*, 2010). By contrast, the occurrence of tumor-specific antibodies was reported in a few cancer patients and was not frequently correlated with protection (Toubi and Shoenfeld, 2007). Therefore, the favorable role of tumor infiltration with B cells remains limited to certain histological types of tumors (table 1), and the contribution of a natural tumor-specific humoral response to protection against tumor development and spreading is not yet well understood.

3. Cancer immunoediting and other escape mechanisms

Immunosurveillance is an efficient process that constantly controls a great number of transformed cells, preventing tumors from becoming apparent. Sometimes however, several factors then lead to uncontrolled tumor growth. They include modulation of tumor immunogenicity, or immunoediting, rapid tumor cell division and survival strategies, modulation of the immune response and other escape mechanisms. Altogether, these mechanisms result in a progressive shift of the cancer-immune system interplay from successful elimination of cancer cells to evasion of the tumor.

3.1. The theory of immunoediting: tumors become less immunogenic

The theory of tumor immunoediting first derived from the observation that tumors that develop in immunodeficient mice were consistently more immunogenic than those in immunocompetent hosts. Therefore, these tumors were more frequently rejected when transplanted into normal mice than those initially grown into immunocompetent hosts (Shankaran *et al.*, 2001). These findings suggested that immunosurveillance leads to an immunoselection phenomenon, during which the immune system destroys highly recognizable or antigenic malignant cells, thus selecting non-antigenic ones and leaving them to grow (Engel *et al.*, 1997). The theory of immuno-editing postulates that tumors are edited

with time, thus becoming increasingly heterogeneous and allowing cancer cells to escape immunosurveillance. During the multiplication process, a myriad of mutations and epigenetic alterations accumulate and affect many levels of the tumor cell biology (figure 14). Above all, they change the profile of TAA expression. Progressively, they exhibit lower levels of strong antigens and escape detection by the immune system. This is usually accompanied by downregulation of the antigen presentation machinery, and MHC and costimulatory molecules expression, which all favor tumor cell escape from effector $CD8^+$ T cells.

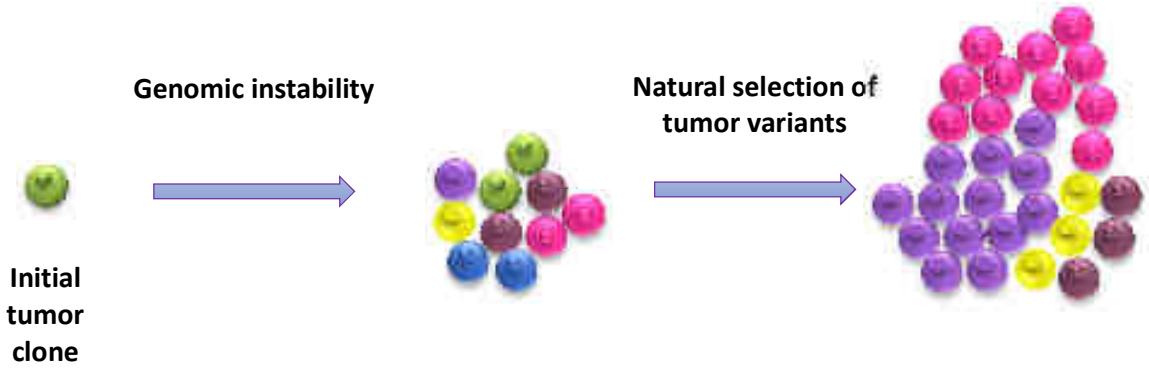


Figure 14: Natural selection of tumor variants in the generation of "tumor escape" phenotypes*. Genomic instability gives rise to genetic diversity in tumors. Natural selection of tumor variants occurs by differential propagation of tumor subclones in their microenvironment. Reproduced from (Khong and Restifo, 2002).*

3.2. Tumors increase their own survival and evade destruction

Tumors increase their survival and resistance to apoptosis by upregulating pro-survival and growth factors, such as B-Cell Lymphoma (Bcl)-2 and Human Epidermal growth factor Receptor (Her)-2. They also enhance their resistance mechanisms against cytotoxic effectors of immunity, such as NK cells and CTLs. These mechanisms include the upregulation of the Signal Transducer and Activator of Transcription (STAT)-3. Constitutive or inflammation-induced activation of STAT-3 in tumor cells was reported to induce the secretion of Vascular Endothelial Growth Factor (VEGF), a potent angiogenic factor responsible of tumor neovascularization and the expression of immune inhibitory receptor ligands.

3.3. Tumors modulate tumor-specific immune response

Tumors establish an immunosuppressive microenvironment. To do this, they secrete immunosuppressive cytokines, such as IL-10, which inhibits differentiation of DCs and downregulates costimulatory molecules, and Transforming Growth Factor (TGF)-β, which

inhibits activation and proliferation of T cells and NK cells. They can also produce metabolic factors such as Prostanglandin E2 (PGE2) and adenosine, as well as growth factors such as VEGF, which inhibits differentiation and maturation of DCs (Teng *et al.*, 2015) (figure 15).

This immunosuppressive microenvironment favors the induction and recruitment of immunosuppressive cells, such as regulatory T cells (Treg), Myeloid-Derived Suppressor Cells (MDSC) and macrophages (Teng *et al.*, 2015) (figure 15). MDSCs are a heterogeneous class of immature myeloid cells, defined, as their name suggests, by their ability to suppress immune responses (Elliott *et al.*, 2017). They impair T cell activation and trafficking between tumor site and lymph nodes and reduce their viability. In addition, they induce Treg cells and negatively influence macrophages (Safarzadeh *et al.*). Similarly, tumor-associated macrophages (TAM) in the tumor microenvironment gradually shift from an M1 phenotype, which coordinates with a cancer-protective Th1 immune response, to an M2 phenotype, which functions in coordination with a Th2 immune response. They inhibit T cells and recruit Treg cells (Galdiero *et al.*, 2013; Guo *et al.*, 2016).

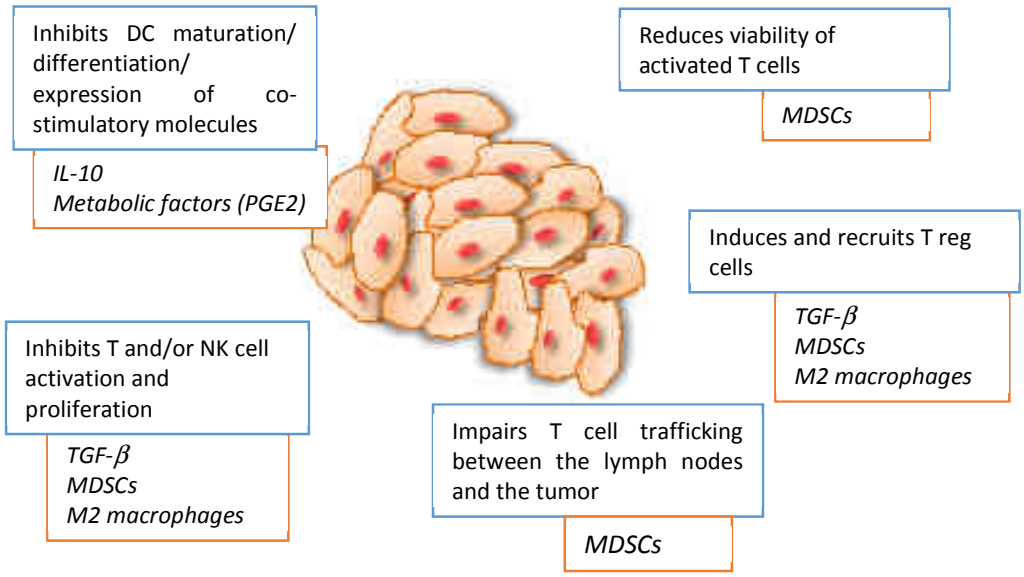
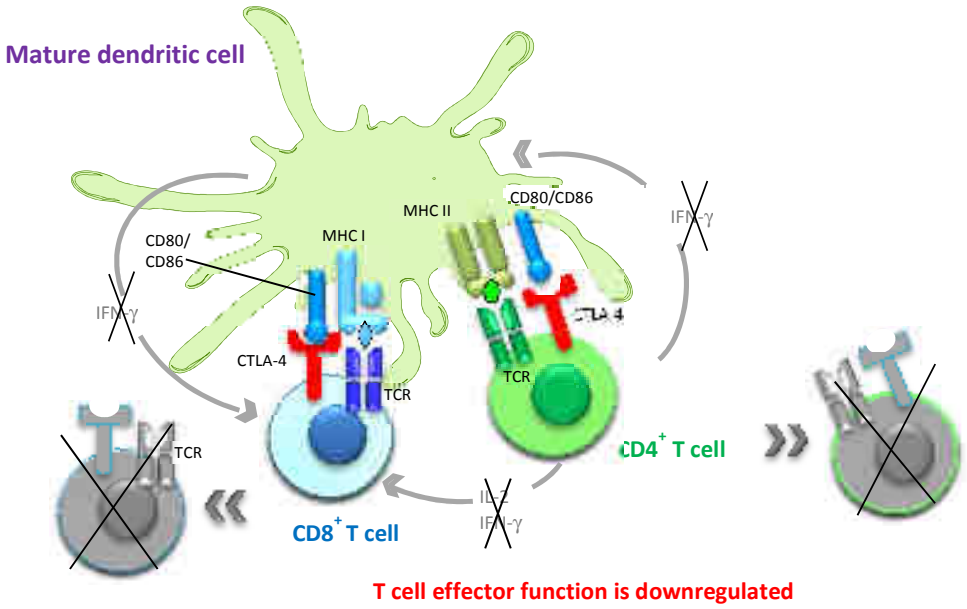


Figure 15 : The effect of the inhibitory microenvironment on the tumor-specific immune response. *Depicted are examples of the role of the tumor microenvironment in impairing immune responses. The tumor induces an inhibitory microenvironment through the production of inhibitory cytokines (IL-10, TGF-β) and metabolic factors such as Prostaglandin E2 (PGE2). This microenvironment exerts direct effects on NK cells and CTLs, and indirect effects by impairing DC maturation and recruiting or inducing inhibitory cells. M2 macrophages, MDSCs, and Treg cells coordinate to inhibit the tumor-specific immune response. MDSC: myeloid-derived suppressor cells.*

3.4. The cancer-specific immune response is also modulated by inhibitory loops

Modulation of the tumor specific immune response may occur as a result of negative regulation following immune exhaustion. Indeed, at the time of the initial response to antigens, T lymphocytes express CD28 that binds to its partners, B7.1 and B7.2 molecules, expressed on DCs. CD28/B7 interaction provides co-stimulation to the TCR signaling and results in the activation and differentiation of T cells. However, after a certain number of cycles, CD28 is downregulated, and is replaced by the Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4). CTLA-4 binds to B7.1 and B7.2, thus providing a negative feedback that downregulates effector activity, and prevents over-activation of the immune system and bystander damaging effect on healthy tissues. It is part of what is called "Immune checkpoints" (Pardoll, 2012; Teng *et al.*, 2015) (figure 16). In the case of cancer, continuous stimulation provided by tumor cells induce the upregulation of CTLA-4, thereby mediating the evasion of



tumor cells from immune destruction.

Figure 16. The cancer specific immune response is downregulated by immune checkpoints, such as the Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4)*. At the time of the initial response to antigens, T lymphocytes express CD28 that binds to its partners, CD80 and CD86 (B7) molecules, expressed on DCs. CD28/B7 interaction provides co-stimulation to the TCR signaling and results in the activation and differentiation of T cells. However, after a certain number of cycles, CD28 is downregulated, and is replaced by CTLA-4. CTLA-4 binds to B7.1 and B7.2, thus providing a negative*

feedback that downregulates effector activity, and prevents over-activation of the immune system and bystander damaging effect on healthy tissues.

Another immune checkpoint that is hijacked by the tumor cell is the PD1/PD-L1 pathway. PD-1 is an inhibitory receptor that is upregulated on activated T cells upon the engagement of the TCR by a cognate antigen. PD-1 has 2 ligands, PDL-1 and PDL-2. While PD-L2 expression is restricted to DCs and macrophages, PDL-1 is additionally expressed on B cells and on cancer cells. PD-L1 expression on cancer cells is upregulated by inflammatory signals. Binding of PD-1 to its ligands generates an inhibition of the T cell activity. PD1/PDL-1 interactions occur in the effector phases of the T cell response in the periphery and aims to prevent tissue damage from excessive inflammation and to maintain self-tolerance. However, by upregulating PDL-1 expression, cancer cells hijack this pathway to evade immune destruction (Pardoll, 2012; Ribas, 2015; Teng *et al.*, 2015). It is reported that in some tumors, constitutive oncogenic signaling can induce PD-L1 expression regardless of the inflammatory signals of the microenvironment (Pardoll, 2012).

Despite its central role in tumor specific immune defense, IFN-γ may be a double-edged sword. Indeed, it induces the production of indolamine 2’3’-dioxygenase (IDO) (Ribas, 2015), an inhibitory metabolic enzyme, as well as the upregulation of 1 PD-L1 on tumor cells and tumor-associated myeloid cells.

3.5. Cancer progression: the three “Es” of tumor development

Immunosurveillance and immune escape processes appear to co-exist in a dynamic equilibrium that tips towards tumor evasion with time. When immunosurveillance is fully effective, the immune system can basically destroy all immunogenic cancer cells. This is the “elimination” phase. When it is incomplete, tumor cells continue to exist, yet, in a dynamic “equilibrium” phase where the immune system can keep the tumor in a dormant state. Progressively, the tumor is edited by a number of genetic and epigenetic mutations that arise and accumulate during tumor proliferation, as well as by certain aspects of the immune reaction itself. In this case, malignant cells may still be visible to the immune system, however the selective pressure of the immune response originally aiming at eliminating them drives them to spontaneously develop additional escape mechanisms. Cells with phenotypes that favor tumor escape or resistance are maintained. Progressively, the immunosurveillance

mechanisms fade, the immune system has less control over the tumor, and the tumor enters the "evasion" phase (Dunn *et al.*, 2002).

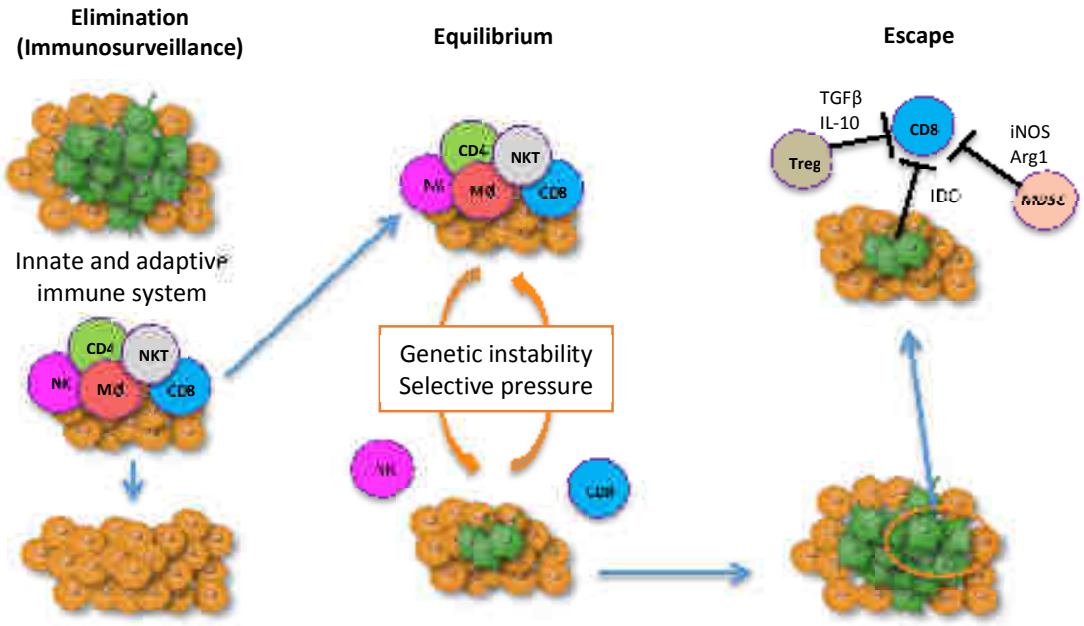


Figure 17: The three phases of cancer development. *During the elimination phase, innate and adaptive immunity cells cooperate to destroy malignant cells. With time, genetic instability of tumors leads to multiple tumor variants that will be naturally selected, during the equilibrium phase, by the selective pressure of the immune response for those with low immunogenicity. The resulting tumor cells propagate and progressively inhibit the adaptive immune response through multiple mechanisms, such as the production of indolamine 2'3'-dioxygenase (IDO), an inhibitory metabolic enzyme, and the induction and recruitment of regulatory T cells and Myeloid Derived suppressor cells (MDSCs). In turn, these inhibitory cells negatively affect the function of effector CD8⁺ T cells and the tumor escapes the immune response (the escape phase).*

4. Therapeutic cancer vaccines

In order to restore tumor specific immunity in cancer patients, multiple strategies aim to subvert the tumor-induced immunosuppression. In theory, each of the above mentioned steps of immune response can be targeted, either passively or actively. Active cancer immunotherapy relies heavily on therapeutic tumor vaccination. It is particularly interesting because it reprograms the early steps of adaptive immune response to better fight tumors. This can be achieved by reactivating pre-existing tumor-specific lymphocytes and by inducing new ones. Cancer vaccines may alternatively skew the immune response by reprogramming lymphocytes from a non-protective towards a protective profile (figure 18).

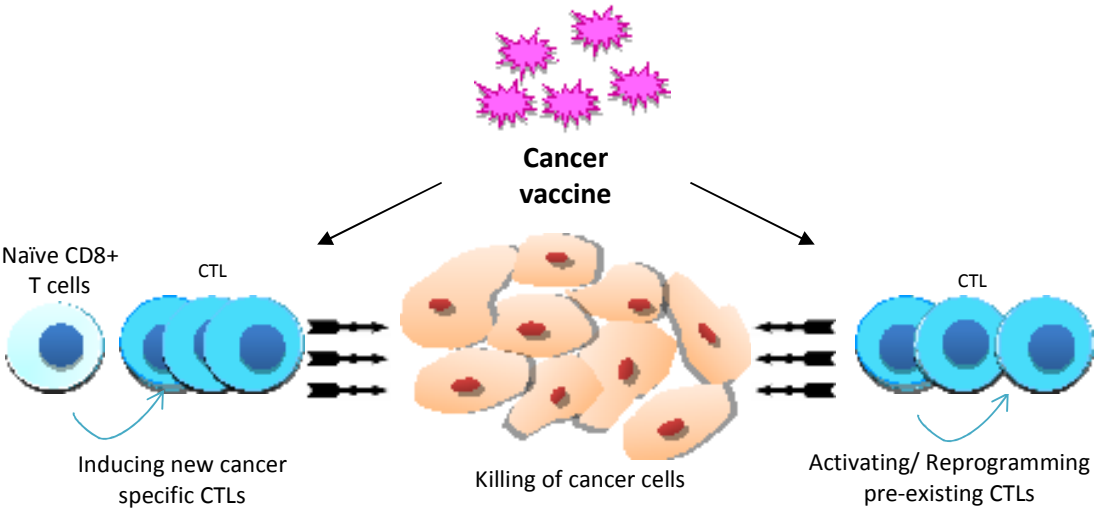


Figure 18: Cancer vaccines are intended to optimize the amplitude and the quality of the tumor-specific immune response. *They either induce new cancer specific CTLs, or activate or reprogram preexisting ones, thus mediating cancer cell killing.*

4.1. Cancer vaccine requirements

Cancer vaccines are intended to destroy tumor cells, while sparing normal tissues, and to induce an immunological memory that would protect the host from subsequent tumor initiation. Therefore, they aim to improve both the amplitude and the quality of tumor-specific responses. The amplitude of a response is defined by the number of activated immune cells, whereas its quality is defined by the profile of secreted chemokines, cytokines and mediators. It is widely accepted that Th1 and CTL responses are the most protective effectors against cancer. The benefit of B-cells and Th2 responses is by far less frequently evidenced (Fridman *et al.*, 2012).

Cancer vaccines should be able to efficiently reach large numbers of APCs, and particularly DCs, to activate them by signaling *via* engagement of co-receptors and to turn them into immunostimulatory DCs, which would present key protective cancer epitopes to B and T lymphocytes. This requirement is based on two independent factors, namely the composition of the vaccine and its delivery route:

i) the vaccine should either include, or induce the expression of $CD4^+$ T cell epitopes in combination with TAA-derived $CD8^+$ epitopes. Additionally, an immunostimulatory molecule,

such as a PRR ligand, is absolutely needed to provide danger signals necessary for the maturation of the DC;

ii) the delivery route is crucial because it dictates which types of DCs are targeted. This will be discussed in chapter 3.

4.1.1. Cancer vaccines need immunostimulatory molecules

4.1.1.1. Adjuvants: conventional immunostimulatory molecules

Conventional immunostimulatory molecules used in conventional vaccines are adjuvants that come in the form of colloids or emulsions of mineral oils. Conventional adjuvants exert their effect through their ability to 1) form a depot, thus ensuring slow sustained release of antigens, 2) to favor uptake by DCs and 3) to provide danger signals to the DCs.

The traditional adjuvant used for human vaccination for more than 80 years is alum (Brewer, 2006). It contains a mixture of colloidal aluminum salts, mainly aluminum hydroxide but also aluminum phosphate, in proportions that vary according to different manufacturers (Lambrecht *et al.*, 2009). Adsorption of soluble antigens to alum results in a particulate form that is more efficiently internalized by APCs. After years during which the exact mode of action of alum was not fully understood (Brewer, 2006), it was recently found that it activates components of the inflammasome complex, by signaling through the NALP3, leading to the release of pro-inflammatory cytokines such as IL1-β and IL-18 (Lambrecht *et al.*, 2009).

Mineral oil-based adjuvants, such as complete and incomplete Freund's adjuvants, are extremely reactogenic therefore, they are not used in human vaccines. Recently, the base material of this class of adjuvants has been highly refined to decrease their reactogenicity. Incomplete Freund's adjuvant, termed montanide in its clinical grade, is currently being added to therapeutic cancer vaccines (Leroux-Roels, 2010).

4.1.1.2. New-generation immunostimulatory molecules

Alternatively, modern vaccines tend to replace the conventional adjuvants with well-defined synthetic immunostimulatory ligands, such as TLR agonists (Jalali *et al.*, 2012; Roth *et al.*, 2004; Thomann *et al.*, 2011). Currently used TLR agonists are usually bacteria-derived. They can be lipopolysaccharides, such as monophosphoryl lipid A (MPLA), a TLR 4 agonist, or lipopeptides

such as dipalmitoyil cysteine-alanyl-glycine (Pam_2CAG), a TLR 2/6 agonist, tripalmitoyil cysteine-alanyl-glycine (Pam_3CAG), a TLR2/1 agonist, or DNA motifs such as unmethylated-CPG, a TLR9 agonist, to name a few.

MPLA is currently one the leading innovative vaccine adjuvants. It is a chemically detoxified form of the lipid A, the anchor moiety of lipopolysaccharides (LPS), a highly immunostimulatory structure found on the outer cell surface of Gram negative bacteria (Alving and Rao, 2008). MPLA is insoluble and prone to aggregation. Therefore, it is frequently administered integrated into formulations that increase its efficacy and availability; for example, it may be adsorbed on alum, or incorporated in oil-in-water (o/w) emulsions or even integrated into a liposome (This will be detailed in chapter 2). These associations form what is currently known as adjuvant systems (AS). They were developed by GlaxoSmithKline (Alving and Rao, 2008; Alving *et al.*, 2012a; Didierlaurent *et al.*, 2009; Garçon *et al.*, 2007). For instance, AS04 contains MPLA adsorbed on alum, and is currently used in two licensed vaccines, namely Cervarix, a vaccine against the Human Papilloma Virus (HPV), and Fendrix, a vaccine against the Hepatitis B virus (Didierlaurent *et al.*, 2009). AS02 contains MPLA in addition to a purified fraction of Quil A Saponin (QS-21, *Quillaja saponaria* Molina fraction 21; Antigenics Inc, a subsidiary of Agenus Inc., Lexington, MA, USA). AS01 contains MPLA and QS-21 formulated into a liposome. The most potent is the AS15 comprising, in addition to the AS01, the CpG adjuvant. It is currently in clinical development where it is being explored in active immunotherapy of non-small lung cancer and melanoma (Leroux-Roels, 2010).

The formulation in which MPLA is presented to the DCs was found to orient the immune response. When solubilized in water, it was found to skew the immune response towards humoral responses, whereas when in liposomes or oil-in-water (o/w) emulsions, it triggered preferably T cell responses. More specifically, a larger particle size within the formulation induced a stronger CTL response. For instance, the AS01 adjuvant system was found to be more potent than AS02 in eliciting CTL responses (Alving *et al.*, 2012b).

Pam_2CAG is a diacetylated lipopeptide derived from the N-terminal moiety of E-coli lipoprotein. This molecule was found to induce the maturation of human monocyte-derived DCs (MDDCs) *in vitro*, as indicated by the expression of CD80, CD83, CD86 and HLA-DR molecules (Espuelas *et al.*, 2005). When incorporated into liposomes with a $CD4^+$ T cell

epitope, Pam_2CAG was capable of inducing a humoral as well as a cytotoxic immune response. The CTL response was also protective against tumor growth in mice (Heurtault *et al.*, 2009; Thomann *et al.*, 2011).

As previously described in this manuscript, TLR agonists function as danger signals and are responsible of DC maturation, a pivotal event for the induction of a specific immune response. They induce the expression of co-stimulatory molecules and cytokines, thereby improving the quality and amplitude of immune responses.

4.2. Recent advances in cancer vaccination

The development of efficient cancer immunity is hampered by the extreme diversity of tumor origins, types and molecular characteristics and by the genetic diversity of patients. Each histological type comprises multiple subtypes differing by the mutations that drive tumor development, leading to various phenotypes and differences in response to treatment. Despite the challenges, therapeutic cancer vaccines have proven their worth and achieved a clinical proof-of-concept. One vaccine is already on the market, namely Sipuleucel-T (Provenge®, Dendreon Corporation); it is used for the treatment of prostate cancer. Several strategies for therapeutic cancer vaccination are currently being evaluated in clinical trials, mainly related to the nature of the cancer vaccine itself, or to its administration route (the administration route will be discussed in chapter 3). Cancer vaccines can be broadly assigned to 4 groups, namely tumor cell-based, nucleic acid-based, DC-based and protein/peptide-based vaccines, and will reviewed herein (figure 19).

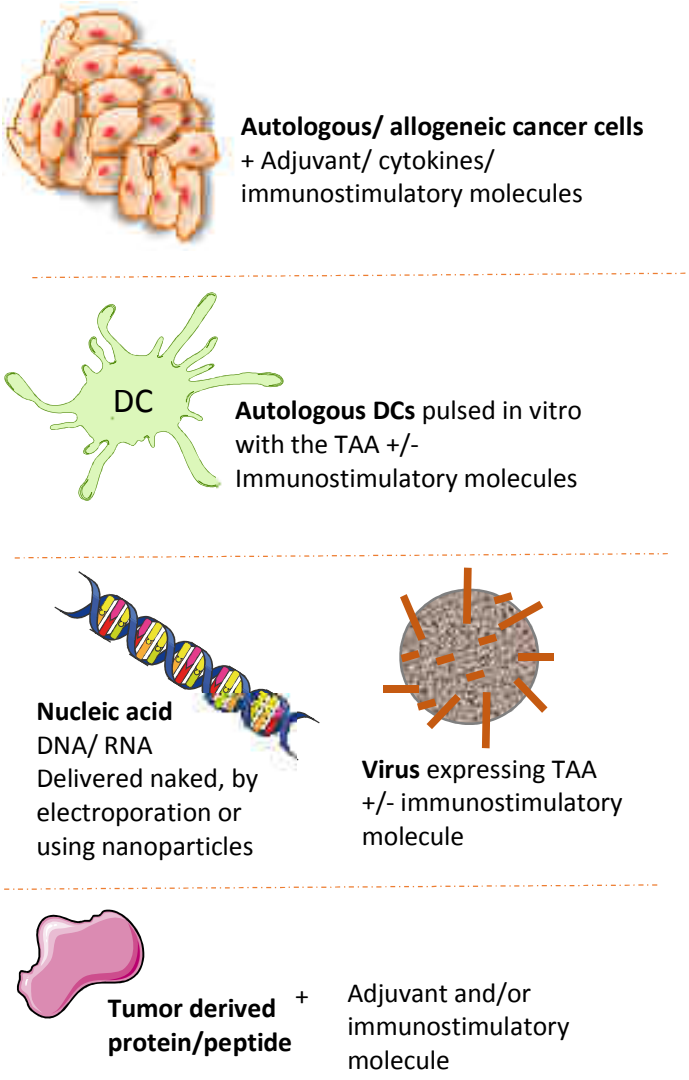


Figure 19 : Cancer vaccine approaches. *Recent advances in cancer vaccination led to the development of multiple types of cancer vaccines that deliver TAAs and immunostimulatory molecules to the immune system by different approaches, broadly assigned into 4 groups: irradiated autologous or allogeneic tumor cells, autologous DCs pulsed in vitro with TAA, nucleic acid-based vaccines (DNA, RNA and engineered viruses), in addition to whole proteins or TAA-derived peptides.*

4.2.1. Tumor cell-based vaccines

Tumor cell-based vaccines are the homologs of classic attenuated or inactivated whole microorganism vaccines, since they rely on the use of "inactivated cancer cells" to elicit anti-tumor immunity. This strategy bypasses the need to define specific protective TAAs. In these vaccines, a wide array of TAAs are provided by whole cancer cells, which are irradiated to stop their proliferation yet retain their full antigenicity. Tumor cell-based cancer vaccines are said to be autologous when the patients' own cancer cells are harvested and expanded, or

allogeneic when pre-established cancer cell lines are used instead. Irradiated cancer cells are injected into the patient, combined to an immunostimulatory molecule. However, tumor cell-based vaccines can be very costly, and, in the case of autologous vaccines, it is difficult to ensure adequate specimens of tumor cells (Disis, 2014): these cells may not only be technically challenging to harvest in sufficient numbers, but they may also be poorly immunogenic, either because the expression of neoantigens is reduced or totally suppressed, or because they lack MHC class I and co-stimulatory molecules. Among the multiple strategies used to increase the immune response to the vaccine, the most common one is tumor cell transduction to express either the tumor antigen, or a costimulatory molecule, or an immunostimulatory mediator.

To date, one of the most successful genetically engineered tumor cell-based vaccines is GVAX that was developed by Somatix in 1993. It consists of transfected tumor cells that express GM-CSF. The broad platform vaccine is based on either autologous or allogeneic tumor cells. GM-CSF is expressed either by the tumor cells themselves, or by bystander cells mixed with them. GVAX was evaluated in phase 1 and phase 2 clinical trials for a number of tumors like prostate cancer and non-small-cell lung carcinoma. Upon binding to its broadly expressed receptor on DCs, GM-CSF induces upregulation of key cytokines and costimulatory molecules. GVAX has resulted in an enhancement of lymphocytes infiltration of the tumor in most patients (Wong *et al.*, 2016), and even occasional clinical responses in a number of solid tumors. Indeed, in prostate cancer patients, monthly injections of a GVAX vaccine comprising 3 irradiated allogeneic prostate cell lines increased median survival by 38 weeks (Michael *et al.*, 2005). Another GVAX variant, comprising a mixture of irradiated autologous tumor cells and GM-CSF producing “bystander cells”, was tested in patients with advanced chronic lymphoid leukemia. Prior to vaccination, bone marrow-depleted patients were reconstituted with allogeneic hematopoietic stem cells. Vaccine injection resulted in an increase in the number of tumor-reactive $CD8^+$ T cells (Burkhardt *et al.*, 2013). In a phase I/II clinical trial in non-small-cell lung carcinoma patients, a similar “bystander” GVAX platform induced anti-tumor immune activation, however, unlike the original GVAX platform (where autologous or allogeneic cancer cells are genetically modified to secrete GM-CSF), it failed to induce objective tumor response (Nemunaitis *et al.*, 2006).

4.2.2. Nucleic acid-based vaccines

Nucleic acid-based vaccines use different strategies to deliver genetic material coding for antigens and/or immunostimulatory molecules into the patients' DCs in situ. The intended goal of these strategies is the transfection of the DCs with plasmid DNA or RNA or on their infection with recombinant non virulent viruses, in order to induce expression of the encoded proteins. For example, when DCs are transfected with a recombinant virus expressing TAAs and immunostimulatory molecules they express these molecules, in addition to viral products (including danger signals). Endogenous expression of the TAAs results in their presentation on MHC class I molecules, thus favoring cross-presentation, while virus induced danger signals and co-stimulatory molecules enhance DC maturation, resulting in an improved T cell cross-priming.

Despite encouraging preclinical trials, clinical results of DNA vaccines have been less effective than anticipated. Therefore, development of new technologies that aim to enhance their efficiency is ongoing (Disis, 2014). RNA vaccines are thought to be safer than DNA vaccines, since they do not risk integrating the human genome. Early phase I/II trials of RNA vaccines conducted in patients with melanoma, prostate cancer and non-small-cell lung cancer proved them to be well-tolerated and immunogenic (Disis, 2014; Kübler *et al.*, 2015).

The development of virus-based vaccines is more advanced. Commonly used viruses are retroviruses, poxviruses, adenoviruses and herpesviruses. A promising virus-based vaccine, PROSTVAC-TRICOM, designed to treat advanced castration-resistant prostate cancer patients, is currently under development. The initial formulation comprised two different Prostate Specific Antigen (PSA)-expressing viruses, and was tested in a prime/boost regimen including vaccinia virus for prime immunization and fowlpox virus for recall boosters. It was well tolerated and resulted in encouraging clinical responses (Kaufman *et al.*, 2004). The vaccine was then improved by engineering the vectors to additionally encode three T co-stimulatory molecules. The improved vaccine was named PROSTVAC-TRICOM. In a phase II clinical trial, this vaccine increased overall survival time by 8-months (Singh *et al.*, 2015; Wong *et al.*, 2016) It is currently in a phase III clinical trial, expected to be completed in 2018.

4.2.3. DC-cell based vaccines

These vaccines rely on the harvesting of the patient's own DC and their culture in vitro in the presence of the vaccine prior to their reinjection to the patient. The intended key immunological events are vaccine uptake by DCs and their activation and maturation to trigger the cancer immune response cascade. In order to reach this goal, a new tumor vaccine approach was recently developed and is being extensively explored in clinical trials of active cancer immunotherapy. In this approach, the patient's monocyte-derived DCs are either pulsed i*n vitro* with tumor antigens or infected with viral vectors encoding these antigens. One such vaccine, Sipuleucel-T (Provenge®), manufactured by Dendreon, is the first therapeutic cancer vaccine approved by the US Food and Drug Administration (FDA) in 2010 for clinical use. It ensures a 4 month-extension in overall survival of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer patients. This vaccine increases recruitment of T cells to the tumor, and it could occasionally prolong PSA doubling time, a parameter used to evaluate tumor progression. In this patient-tailored vaccine, autologous PBMCs are collected by leukapheresis from the patient's peripheral blood. Monocyte-derived DCs are then incubated with a fusion protein, consisting of prostatic acid phosphatase (PAP) and GM-CSF. GM-CSF helps overcoming the tumor induced immunosuppression by recruiting new DCs to the tumor site and inducing their maturation (Johnson *et al.*, 2015; Rehman *et al.*, 2016). It was also found to increase tumor infiltration with vaccine-induced CTLs through the induction of two homing molecules (Clancy-Thompson *et al.*, 2013). PAP is a highly prostate cancer-specific TAA expressed in more than 95% of prostate adenocarcinomas. Mature APCs, known as APC8015, are then reinfused into the patient (Graff and Chamberlain, 2015).

Despite their efficacy, DC-cell based vaccines are too costly and technically complex to be produced on a large scale. Therefore, delivery of protein/peptide-based vaccines to the DCs *in vivo* with a comparable efficacy are highly preferable.

4.2.4. Protein/peptide-based cancer vaccines

Protein/peptide-based cancer vaccines typically contain TAA-derived sequences, which vary in size from whole proteins to single epitope peptides, associated with adjuvants. The rapid expansion of the list of well characterized TAAs, in addition to our improved understanding of tumor antigen presentation, and of the requirements of T cell activation facilitated the

development of this class of vaccines. Being highly specific, they exert a low risk of inducing adverse reactions and are therefore very desirable. However, they are devoid of danger signals, which implies a careful choice of strong adjuvants, in order to elicit a sufficiently potent immune response (Guo *et al.*, 2015).

One of the targets of peptide/protein-based vaccines is Melanoma Antigen E (MAGE)-A3, a cancer-testis antigen expressed in a number of tumors, including non-small-cell lung carcinoma and melanoma (Esfandiary and Ghafouri-Fard, 2015). The importance of the adjuvant in peptide/protein-based vaccines was illustrated in a phase I clinical study, in which patients were immunized either with full-length MAGE-A3 protein alone, or with full-length MAGE-A3 protein combined to a saponin-based adjuvant containing MPLA (AS02B). The majority of patients receiving the adjuvanted vaccine, termed MAGE-A3 Immunotherapeutics, elicited strong immune responses. In contrast, only a very small proportion of those receiving the non adjuvanted vaccine responded. Three years later, when all of these patients subsequently received booster injections of MAGE-A3 Immunotherapeutics, only those who were primed with the same vaccine could quickly re-establish high titer antibodies and vaccine specific $CD4^+$ and $CD8^+$ T cells. In contrast, only 2 of 7 patients primed with the non-adjuvanted MAGE-A3 protein elicited vaccine-specific antibodies, with a few vaccine specific-$CD4^+$ T cells and no $CD8^+$ T cells (Atanackovic *et al.*, 2008). MAGE-A3 Immunotherapeutics was also shown to elicit antibody responses in patients with resected non-small cell lung carcinoma (Vansteenkiste *et al.*, 2013) and with melanoma. In the latter, it also exhibited a beneficial clinical activity. Indeed, in a phase II study, adjuvanted full-length recombinant MAGE-A3 was found to extend the overall survival, the disease free survival and to elicit complete responses (Kruit *et al.*, 2013). Despite these encouraging results, a large phase III clinical trial conducted on non-small lung carcinoma patients was disappointing. In this trial, MAGE-A3 Immunotherapeutics did not yield any improvement in disease-free survival (Vansteenkiste *et al.*, 2016). Thus, the development of this vaccine was interrupted for NSCLC. However, research is ongoing for other tumors.

Attempts for vaccination against MAGE-A3 expressing tumors also involved the development of MAGE-A3-derived peptide-based vaccines. One of the first attempts was conducted using a single $CD8^+$ T cell epitope. This vaccine induced disease regression in 3 out of 25 patients, although no CTL response was detectable against the vaccine peptide (Marchand *et al.*, 1999).

Other attempts involving the incorporation of both MHC class I and MHC class II epitopes resulted in the induction of vaccine specific T lymphocytes. However, these lymphocytes were found to exhibit a regulatory activity (François *et al.*, 2009). Lately, an improved MAGE-A3 peptide-based vaccine was developed by including in the peptide sequence a cell-penetrating peptide derived from the HIV virus (HIV-TAT). This strategy aims to facilitate the vaccine penetration to the endoplasmic reticulum and Golgi apparatus of DCs and thus to increase the formation of MHC-peptide complexes and enhance presentation. Moreover, the epitopes were linked *via* cleavable linkers that allow the release of individual peptides in the Golgi apparatus. Finally, in addition to montanide, a strong adjuvant, the vaccine comprised GM-CSF as a positive immunomodulatory (Zandberg *et al.*, 2015). In a phase I clinical trial, this vaccine elicited cellular and antibody responses in recurrent/metastatic squamous cell carcinoma of the head and neck (Zandberg *et al.*, 2015).

Chapter 2: Liposomes as systems for the delivery of protein/peptide-based vaccines to DCs

Except for tumor cell-based and DC-based vaccines, cancer vaccines are generally based on the delivery of small molecules, such as proteins, peptides or nucleic acids to DCs in vivo. The therapeutic effect of these molecules depends heavily on their ability to cross biological barriers in sufficient amounts. Nucleic acids need to be incorporated into the nucleus of the target DC and to be transcribed and translated in order to induce the cellular expression of the desired molecules (peptides, proteins, co-stimulatory molecule…). Proteins and peptides need to be internalized by the appropriate DCs and to be presented to cancer-specific lymphocytes in order to induce immune activation. In this chapter, we will focus on the delivery of protein/peptide-based vaccines.

Recent advances in nanoparticle development have yielded several types of synthetic particles with well-characterized biological functions that can serve as delivery vehicles of vaccines. Liposomes are among the most investigated and the most promising examples of such nanoparticles. It is now established that they can successfully deliver peptides or proteins, along with immunostimulatory molecules, to target cells *in vivo*.

1. Generalities

1.1.A brief insight into liposomes

Liposomes were first discovered by Alec D Bangham and his colleagues in the 1960s (Bangham and Horne, 1964; Bangham *et al.*, 1974) during their research about lipid bilayers of plasma membranes. The term liposome is composed of the 2 Greek words: “Lipos” meaning *fat* and ‘Soma” meaning *body*. Structurally, liposomes are concentric vesicles or capsules in which an aqueous core is enclosed by one or more lipid bilayers. Due to this architecture, they belong to the family of nanocapsules, which, in parallel with nanospheres, constitute the nanoparticle family. Unlike nanocapsules, nanospheres are entirely composed of a matrix of polymers or solid lipids. Both nanocapsules and nanospheres are dispersed in a medium, and therefore, lead to colloidal suspensions.

1.1.1. Liposomes assemble from building blocks

Liposomes are mainly made of phospholipids that, upon dispersion in water, auto assemble in closed structures or sacs. Due to their amphipathic nature, their hydrophilic heads are attracted by the surrounding aqueous solvent, thus forming a surface or layer, while their long hydrophobic tails, formed by acyl chains, line up and interact together. When the lipid layer is formed by two surfaces of phospholipids, the tails of the two opposed layers, repelled by water, face each other, thus forming an inner hydrophilic compartment (Bozzuto and Molinari, 2015). Altogether, these rearrangements form the lipid bilayer that constitutes a relatively impermeable barrier preventing the passage of molecules from the aqueous core towards the outer medium, and vice versa (Bozzuto and Molinari, 2015) (figure 20). When the lipid layer is formed by a single surface of polar heads, the lipid chains are oriented towards the core of the particle, thus forming micelles instead of liposomes, and the inner core is hydrophobic

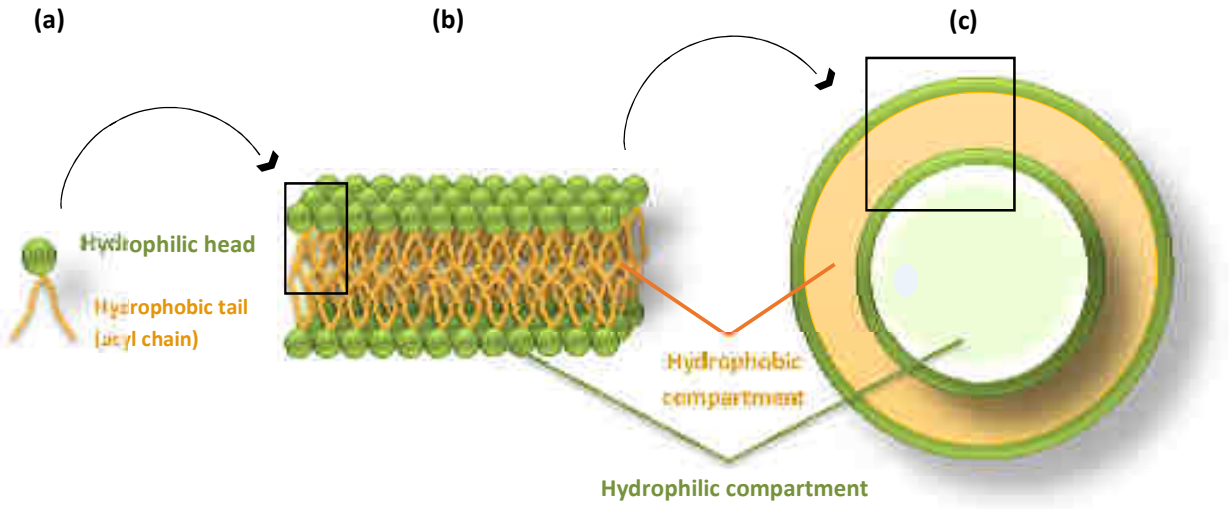


Figure 20: Representation of a phospholipid (a), the steric organization of a lipid bilayer (b) and a liposome (c). *Due to their amphipathic nature, phospholipids assemble in lipid bilayers, where their hydrophilic heads are attracted by the surrounding aqueous solvent and their long hydrophobic tails, formed by acyl chains, line up and interact together. Lipid bilayers form spherical structures enclosing an aqueous core, called liposomes.*

1.1.2. Liposomes structure affects their stability

Besides hydrophobic interactions that form the lipid bilayers, the structure of liposomes is maintained by Van der Waals forces that assemble the hydrophobic acyl chains in the inner compartment of the lipid bilayer, and by hydrogen bonds and polar interactions between the

polar heads and the aqueous environment (Bozzuto and Molinari, 2015). The length of the hydrophobic tail and its type affect the packing of the lipid bilayer and, thus, its stability. The longer the tails are, the more space they have to interact with each other, and the less fluid and more stable is the lipid bilayer. The type of the hydrophobic tail, specifically the degree of saturation in the chain, predicts how the lipids bind together. Unsaturated double bonds create a free space within the layer, allowing additional flexibility of the adjacent chains (Hosta-Rigau *et al.*, 2012).

It should be noted that these factors, namely the length and the type of the hydrophobic tail, affect the fluidity of a lipid bilayer by affecting its transition temperature (Tc). At low temperatures, the acyl chains are in a lamellar "solid" gel phase where they are preferentially aligned and lateral diffusion is very slow. Therefore, the lipid bilayer is "rigid". When the temperature is raised and reaches Tc, the membrane undergoes a transition into the fluid liquid crystalline phase, a disordered state in which the lipids are free to diffuse laterally. In this case, the lipid bilayer is excessively fluid (Pentak, 2014). Therefore, the structure of liposomes is far from being perfectly stable.

1.1.3. Cholesterol role in the stability of the lipid bilayer

Liposome fluidity can be modified by cholesterol addition to the lipid bilayer. On fluid bilayers, cholesterol acts as a stabilizer: its rigid part intercalates between the hydrophilic heads, and also between hydrophobic tails, thus partially reducing their flexibility and stabilizing the membrane. Cholesterol also increases the degree of orientation of the apolar tails, thus reducing the mobility of the lipid membrane (Hosta-Rigau *et al.*, 2012). In contrast, the addition of cholesterol to gel phase bilayers disrupts local packing orders, thus decreasing the membrane stability.

1.1.4. Liposomes vary in composition and structure

While the first liposomes were solely composed of natural lipids, other components were later integrated into their structure, such as synthetic lipids, surfactants, or even ethanol. The resulting variants of liposomes will be further discussed in the chapter 3, section 4.4.4. The phospholipid and cholesterol composition of classic liposomes makes them similar to biological membranes and devoid of toxicity. Therefore, they are desirable for the delivery of biologically active molecules.

Liposomes can be unilamellar, when a single lipid bilayer surrounds their aqueous core, multilamellar when they have multiple concentric bilayers, or multivesicular when multiple vesicles co-exist side by side in the core of a bigger one (figure 21). Their size ranges from the micrometric (1-5 µm) to the nanometric (30-100 nm) scale. Since nanoparticles are defined as ones having at least one of their dimensions < 100 nm, liposomes whose diameter is between 100 and 1000 nm are designated as sub-micrometric particles instead of nanoparticles. Liposomes used in the medical field usually range from 50 to 450 nm (Bozzuto and Molinari, 2015). The liposomes that were formulated in this project were all nanometric, and therefore, all the resulting liposome-based vaccine constructs are nanoparticular.

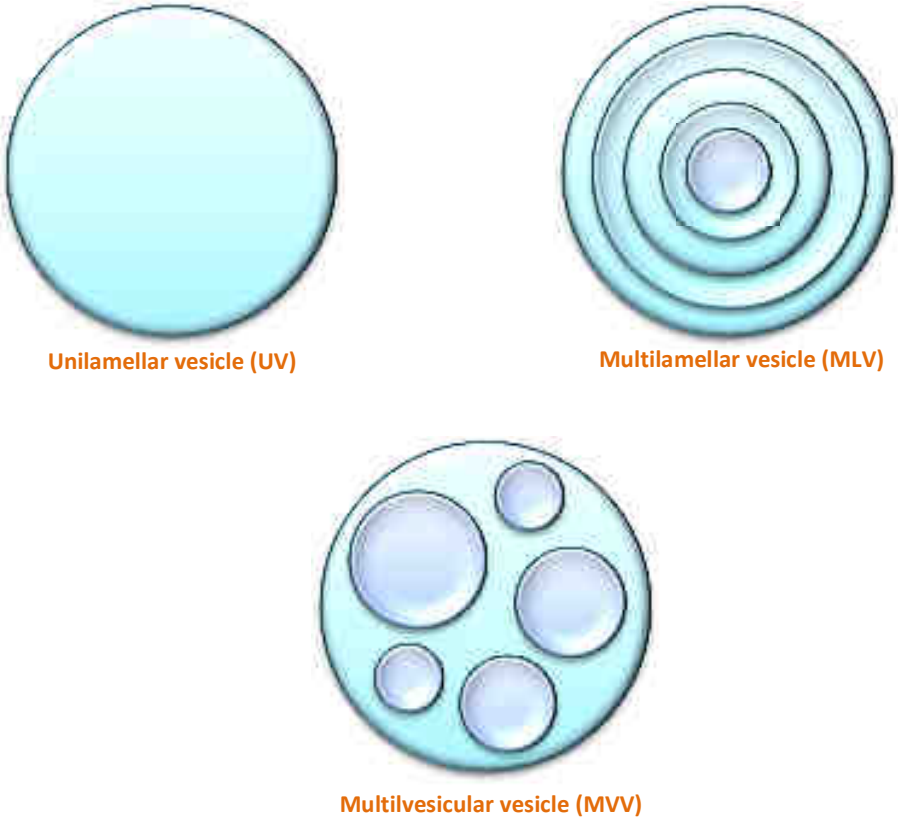


***Figure 21: Types of liposomes**. Liposomes can be composed either of a single lipid bilayer, and are named unilamellar vesicles (UV), or of multiple concentric lipid bilayers, and are named multilamellar vesicles (MLV). Finally, a less exploited type of liposomes is the one composed of multiple vesicles enclosed in a large liposome, named multivesicular vesicles (MVV).*

1.2. Liposome formulation techniques

Several methods can be used for liposome preparation, greatly affecting the characteristics of the resulting vesicles, especially their size and lamellarity. Typically, a first step involves the formation of large vesicles, which are often multilamellar. Post-formation energetic processing is then necessary to break down the vesicles into homogeneous small oligolamellar or small unilamellar vesicles (SUV).

The thin film hydration, or Bangham method (Bangham *et al.*, 1967, 1974) figures among the most widely used conventional methods for liposome preparation and will be used throughout this work. It is relatively simple to implement and does not require sophisticated equipment. Lipids are first dissolved in an organic solvent, forming a thin lipid film after solvent evaporation. The lipid film is then hydrated in an aqueous medium resulting in the dispersion of large heterogeneous MLVs (Bozzuto and Molinari, 2015; Patil and Jadhav, 2014). Other MLV preparation methods involve for instance the reverse-phase evaporation and solvent injection methods. In these methods, the aqueous solvent and the organic phase are injected one into the other, before the organic solvent is finally eliminated by evaporation (Heurtault *et al.*, 2009; Patil and Jadhav, 2014).

Large vesicle breaking methods usually involve sonication or extrusion. Sonication is a convenient and practical method. It consists in applying an ultrasonic irradiation that disrupts MLVs into SUVs. Titanium dust resulting from the sonicator probe is easily removed through a centrifugation step. Extrusion is also widely used. It consists in forcing the MLVs several times through a membrane filter with a defined pore size, and yields homogeneous populations (Bozzuto and Molinari, 2015). Both methods were used in our work.

Multiple technologies and variations were introduced in the process on liposome preparation during the last years, such as microfluidics, especially for scaling up for industrial production, with the advantage of increasing the control over the size, lamellarity and homogeneity of the produced liposomes (Bozzuto and Molinari, 2015).

1.3. Physicochemical characteristics of liposomes

The physicochemical characteristics of liposomes, including their shape, lamellarity and most importantly, their size and charge (zeta potential) largely influence their biological behavior.

Therefore, a strict control over these parameters and their systematic characterization are crucial for the development of liposome-based constructs.

1.3.1. Liposome size

The liposome size, or diameter, is mostly defined by the post-formation processing method, such as the duration of MLV sonication and the number of passages through the membrane filter during extrusion.

Several techniques are used for size determination, such as the dynamic light scattering (DLS) method, also known as photon correlation spectroscopy. Particles in suspension undergo a Brownian movement resulting from their collision with the solvent molecules. Briefly, when these particles are hit by a laser beam, the random particle movement causes fluctuations in the scattered light. The measured intensity of these fluctuations allows the determination of a mean size of the liposomes and informs us about the size distribution within the liposomal population. The homogeneity of the liposomal preparation is defined by an index, termed polydispersity index (PDI) that indicates if the liposomes are mono- or polydispersed (Bozzuto and Molinari, 2015). This technique is accurate and simple to perform, and was used for size determination of our liposomal formulations in this work.

Other techniques involve electron microscopy and atomic force microscopy. These techniques provide accurate determination of the size and shape of the liposomes, however, they are very expensive and require highly specific equipment.

1.3.2. Liposome zeta potential

The zeta potential of a liposome is determined by the charge of its constituents. Whereas cholesterol is neutral, phospholipids can be anionic, neutral, or cationic. For instance, liposomes prepared with phosphatidylcholine, which is neutral, and phosphatidylglycerol, which is negatively charged, in addition to cholesterol, are anionic.

The zeta potential of liposomes influences their stability in suspension and their interactions with active molecules (adsorption on their surface) or biological components such as negatively charged cell membranes. It has to be measured for each sample. The method of zeta potential measurement uses DLS as for size measurement but with an applied electric field (electrophoresis).

Cationic liposomes were reported to induce the maturation of DCs (Soema *et al.*, 2015). They were also reported to induce apoptosis and toxicity, and to exert a strong pro-inflammatory effect by inducing the expression of pro-inflammatory mediators by DCs and macrophages, such as reactive oxygen species (ROS) (reviewed by (Lonez *et al.*, 2012). Besides exerting an adjuvant effect, this pro-inflammatory role may induce non-desired off-target effects. Therefore, anionic liposomes, lacking this effect, are desirable in this context. For this reason, we chose them for the development of our liposomal constructs.

The possibility of controlling the physico-chemical properties of the liposomes confers them a great versatility. Their size and charge can greatly influence their behavior. Therefore, using the multiple formulation techniques and varying the composition of the liposomes will allow a controlled modification of these characteristics, thus permitting the study of their influence in a biological context.

2. Liposomes as vaccine delivery systems

Due to their physicochemical properties, such as their colloidal nature, size, versatility, ease of preparation and capacity to carry a relatively big cargo of bioactive molecules, liposomes are widely investigated as delivery vehicles.

Beyond their potential for drug delivery, liposomes rapidly gained attention for vaccine delivery for several reasons. Their similarity to biological membranes shed the light on their tolerability. Their lack of intrinsic immunogenicity is particularly interesting because they don't risk to hijack the vaccine-specific immune response or induce off-target responses. Moreover, they can be designed to include the pathogen key components and other needed molecules for induction of an immune response, through the incorporation of both proteins/peptides and danger signals (as detailed in chapter 1).

The components to be delivered by liposomes can be either adsorbed on the lipid bilayer, or incorporated in their structure, or even anchored on their surface (figure 22).

- **For adsorption**, a simple physical mixture of the compound with the liposomes is performed taking advantages of Van der Waals or electrostatic interactions.
- **If a molecule is to be incorporated into the liposome**, it usually has to be added at some stage during their preparation. Hydrophilic molecules are added in the hydration fluid and are entrapped in the aqueous core, whereas hydrophobic or liposoluble molecules are added to the organic lipid mixture, and thus, are incorporated in the

lipid bilayer. In our liposomal constructs, lipophilic immunostimulatory molecules, namely TLR agonists, are incorporated into the lipid bilayer (Heurtault *et al.*, 2009; Roth *et al.*, 2004).

- **To anchor peptides on the liposome surface**, an amphiphilic anchor, which is able to chemically react with the peptide, is added to the composition of the lipid membrane. This is the case of our liposomal constructs, where a functionalized lipid anchor developed in our laboratory (Heurtault *et al.*, 2009; Thomann *et al.*, 2011) was first incorporated into the lipid bilayer. After liposome formation, a coupling step served to conjugated peptides through a covalent bond with the lipid anchor. Thus, the resulting liposomal formulations display on their surface peptides that are conjugated to a lipid anchor, which is itself inserted in the lipid bilayer.

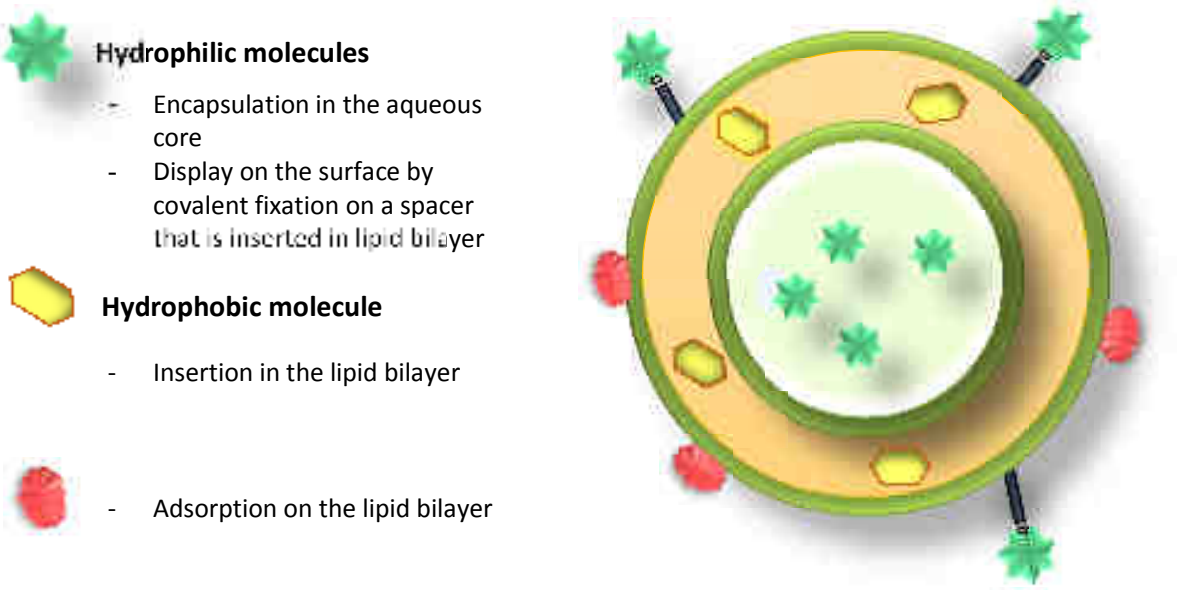


Figure 22: Interaction of vaccine components with the liposome. *Due to the physicochemical properties of liposomes, vaccine compounds can either be incorporated in their core, or included in their surrounding layer(s), or attached to or adsorbed on their surface*

An additional application for liposomes is their use for tracking purposes. To this end, fluorescent molecules can be added either in their aqueous core, and fluorescence can be detected when the liposome opens and releases its content, or in their lipid bilayer, and the liposome can be detected when it is intact.

2.1. Liposomes improve vaccine immunogenicity

2.1.1. Encapsulation protects molecules and increases their bioavailability

Beyond the simple delivery of vaccines, liposomes can protect them from their environment. Whereas protein antigens and certain adjuvants risk rapid degradation, liposomes can shield them from their microenvironment, thus ensuring that increased amounts are delivered to DCs, yielding higher immune responses (figure 23).

For example, microbial CpG DNA is a short single-stranded DNA molecule that acts as a TLR9 agonist when it is unmethylated and therefore, risks rapid degradation by nucleases (Malyala *et al.*, 2009). Its encapsulation into liposomes was shown to protect it, thus harnessing its full immunostimulating potential. Indeed, liposomes presenting ovalbumin peptides and encapsulating unmethylated CpG were found to increase IFN-γ and IL-6 secretion, Th1 cytokines and chemokines gene transcription, in addition to a cell-mediated ovalbumin (Ova) specific immune response, as compared to the free CpG form (Erikçi *et al.*, 2011).

2.1.2. Membrane display preserves epitopes in natural conformation

When cell-surface proteins or peptides are administered as soluble molecules, they are not in their natural membrane environment. Therefore, they may lose important epitopes that might be involved in protective humoral immunity. For instance, peptides that are normally found close to transmembrane domains interact with the lipid membrane and adopt accordingly a given specific conformation that is not preserved in the soluble molecule. An example is the membrane proximal external region (MPER) of the glycoprotein gp41 of the human immunodeficiency virus (HIV). When administered in oil emulsions or alum, MPER peptides fail to induce a humoral response, even in the presence of a TLR agonist. However, when anchored on the surface of a liposome, these peptides induce high-titer specific antibodies (Hanson *et al.*, 2015). For most cancer vaccines, conformation loss is not an issue, as protection is mainly mediated by adaptive cellular immune responses against short linear peptides. However, in some cancers, humoral immune responses are desirable. One such target is the MUC-1, a protein that is highly expressed and aberrantly glycosylated in a number of cancers, including adenocarcinomas. Vaccination with MUC-1 peptides fails to induce immune humoral responses, whereas the display of these peptides on the surface of a liposome confers them a conformation that induces potent humoral responses (Guan *et al.*, 1998).

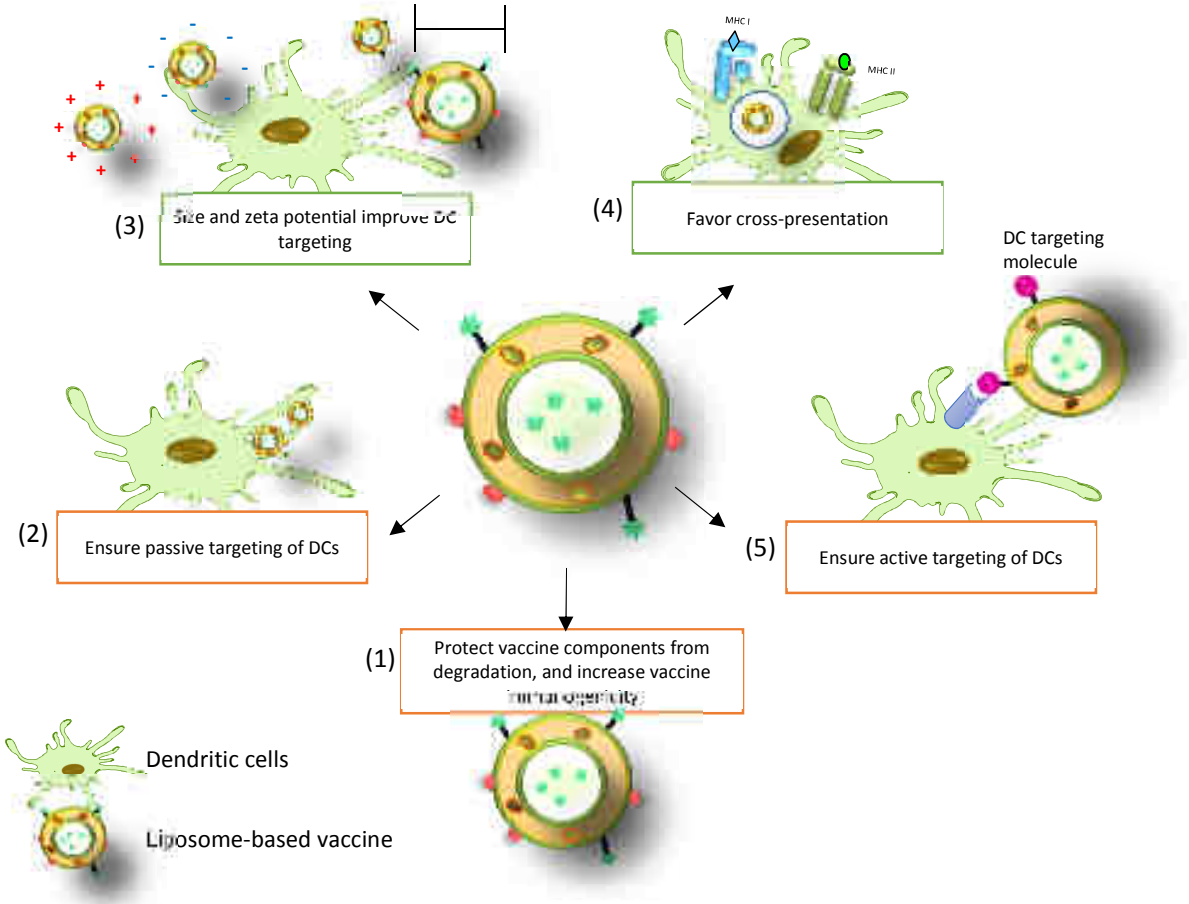


Figure 23: Mechanisms by which liposomes favor the induction of immune responses. *Liposomes immunostimulatory activity depends on multiple factors. (1) They protect the vaccine from degradation and exert a depot effect. (2) They ensure passive targeting of DCs. (3) Their size and charge can modulate their interaction with DCs. (4) Their particulate nature and composition enhances cross-presentation by DCs. (5) Addition of DC targeting molecules can increase their binding and uptake by DCs.*

2.2. Versatility of liposomal vaccines is crucial for tumor vaccination

An undeniable key advantage of liposomal carriers is their versatility and plasticity. Multiple parameters can be easily controlled to tailor their properties in view of an optimal immune response. For instance, various formulation techniques yield liposomes of different sizes. Depending on their composition, size, charge, and receptor interactions (which can all be strictly controlled), their stability and behavior in the biological environment may also be modified, thus controlling their distribution within the administration site, their retention and trafficking, their uptake by DCs and finally, the subsequent immune response (figure 23).

2.2.1. Size and zeta potential control for a better vaccine: small changes make big differences

The size of a liposomal vaccine, its structure as well as its zeta potential influence the way it interacts with biological barriers, its stability and persistence at the administration site and its immunostimulating activity. Studies conducted on liposomes are not abundant; therefore, the data is mostly generated from studies conducted on various nanoparticles.

Particle size influences uptake by DCs, the ultimate recipient of cancer vaccines. Particles ranging between 40 and 200 nm (Foged *et al.*, 2005; Xiang *et al.*, 2006; Zhao *et al.*, 2014), or even 500 nm are most efficient for uptake by DCs, with no significant differences in uptake efficiency within this range (Chang *et al.*, 2017). Particles larger than 500 nm are preferentially taken up by macrophages instead of DCs (Xiang *et al.*, 2006). Macrophages are not capable of cross-presentation, and therefore, cannot induce tumor-specific CTL responses. The optimal size for uptake by DCs seems similar to that for efficient draining. Smaller particles are drained too fast and, therefore, do not interact with DCs, whereas larger ones are poorly drained to lymph nodes (Fan and Moon, 2015).

Concerning the surface charge, or zeta potential, some authors found no difference in uptake efficiency between cationic and anionic nanoparticles (Fromen *et al.*, 2016), while others reported that silica cationic nanoparticles exhibit increased internalization (Jambhrunkar *et al.*, 2014; Osaka *et al.*, 2009) due to the electrostatic interactions between their positive surface charge and the negatively charged cell membranes. However, other authors found that this was only true for larger particles: 1 µm polystyrene or PLGA particles were more efficiently taken up when positively charged, while, for polystyrene particle < 500 nm, the degree of internalization is independent of the surface charge (Foged *et al.*, 2005; Thiele *et al.*, 2003; Wischke *et al.*, 2006).

The most optimal size range and charge for uptake by DCs and induction of an immune response remain a matter of debate, thus indicating a certain degree of flexibility and an influence of multiple parameters.

2.2.2. Targeting of liposomes to DCs for enhanced induction of tumor-specific responses

2.2.2.1. Liposomes ensure passive targeting of DCs

Despite statements in old reports based on *in vitro* studies (Pagano and Weinstein, 1978), liposomes do not fuse with cell membranes, do not exchange lipids with them, nor do they stably adsorb on them. After administration, liposomes are rapidly internalized by the phagocytes, especially by DCs. Their internalization mechanism involves endocytosis, via multiple pathways (endocytosis, micropinocytosis). Liposomes are rapidly coated by opsonins, such as immunoglobulins and fibronectin, that help phagocytes to recognize and eliminate them (Ishida *et al.*, 2001). This may be deleterious for drug delivery, however, this property is highly desirable for vaccine delivery since it ensures passive targeting that increases vaccine uptake by DCs.

Additionally, the particulate nature of liposomes, as well as other nanoparticles, favors uptake by DCs. Indeed, it leads to a depot effect that provides slow sustained release of the vaccine antigens at the vaccination site, thus giving time for DCs to uptake these antigens.

2.2.2.2. Liposomes can be modified to actively target DCs

Due to their properties (structure, composition), liposomes can be "decorated" with a variety of targeting molecules that enhance their uptake by DCs, thus optimizing DC activation and sparing the host from off-target effects. Liposome targeting can also be controlled to deliver the vaccine cargo to specific DC subsets.

One approach consists in displaying, on the liposome surface, ligands or monoclonal antibodies specific for endocytose receptors, such as C-type lectin receptors or carbohydrate receptors, expressed on DCs. Targeting of these receptors has been investigated by several groups including ours. For instance, liposomes targeted to the C-type lectin receptor DCIR by a monoclonal antibody (mAb) and delivering a TLR7 agonist were shown to induce potent secretion of IL-12p70, IFN-α2a, and IFN-γ, all involved in protective tumor specific responses (Klauber *et al.*, 2017).Surface-modified liposomes expressing a glycan which is highly specific for the C-type lectin receptor DC-SIGN were efficiently taken up by human monocyte-derived DCs. Furthermore, they induced their maturation and the production of TNF-α and IL-6 (Boks *et al.*, 2015) and mediated antigen cross-presentation to $CD4^+$ and $CD8^+$ T cells (Fehres *et al.*, 2015a).

In previous works, our team has developed mono, di and tetramannosylated ligands for anchoring in the liposome bilayer. An *in vitro* evaluation of liposomes incorporating these ligands showed increased endocytosis by human DCs as compared to plain liposomes. The difference was the most dramatic with the di- and tetramannosylated ligands that showed a similar efficacy (Espuelas *et al.*, 2003, 2008). Therefore, the di-mannosylated ligands were incorporated into a liposomal vaccine carrying a TLR agonist, in addition to a $CD8^+$ T cell epitope and a CD4+ T cell epitope. Interestingly, di-mannose addition made it possible to decrease the adjuvant amount by 100-fold without decreasing the vaccine efficacy (Thomann *et al.*, 2011).

Another approach consists in targeting liposomes to the Fcγ receptors that are expressed on phagocytes and DCs. To this end, Fc fragments or IgG antibodies (Allen *et al.*, 1995)(176) are conjugated to a lipid anchor inserted in the liposome bilayer. These targeting strategies were proven to be efficient, since liposomes incorporating IgG exhibited an increased uptake by DCs and elicited an immune response against the model antigen Ovalbumin (Ova) (Kawamura *et al.*, 2006)(175). Similarly, Cruz *et al.* showed that liposomes expressing Fc fragments and containing peptides derived from NY-ESO-1 (a cancer testis antigen), tetanus toxoid and an adjuvant were shown to induce a potent immune response (Cruz *et al.*, 2014). The same research group pursued the development of similarly targeted vaccines against the Luteinizing-Hormone-Releasing Hormone (LHRH). The results consistently indicated increased DC maturation, cytokine production and subsequent lymphocyte activation (Rueda *et al.*).

2.2.3. Liposomes favor cross-presentation

2.2.3.1. Cross-presentation by conventional liposomes

Liposomes were found to induce $CD8^+$ T cell responses (Alving *et al.*, 2016; Chikh and Schutze-Redelmeier, 2002; Filskov *et al.*, 2017; Thomann *et al.*, 2011), indicating that the delivered components undergo cross-presentation on MHC class I molecules (figure 24). Among the multiple studies investigating this phenomenon, confocal laser scanning microscopic analysis revealed that when liposomes made of unsaturated fatty acids are internalized by macrophages, ovalbumin peptides bound to their surface are associated to both MHC class I and MHC class II molecules (Tanaka *et al.*, 2010; Taneichi *et al.*, 2006). *In vivo* evaluation of the potential of these liposomes showed that they induce CTL responses and eradication of tumors expressing the immunizing peptide (Taneichi *et al.*, 2006).

A phenomenon of endosomal escape of conventional liposome-delivered antigens from endosomes to the cytosol is speculated, however the molecular mechanisms that mediate this escape remain poorly understood. Some studies also suggested that the nanometric size of particles is crucial to prevent excessive acidification of the endosomes or phagosomes, as compared to micrometric particles and soluble antigens. The slightly acid pH preserves peptides from excessive degradation, thereby promoting cross-presentation (Chang *et al.*, 2017; Seydoux *et al.*, 2014).

2.2.3.2. pH-sensitive liposomes enhance cross-presentation

In order to enhance cross-presentation through the cytosolic pathway, "intelligent" pH-sensitive liposomes have been designed to further favor the endosomal escape process. These liposomes keep their cargo in physiological conditions until they reach the acidic endocytic vacuoles, where, depending on their composition, they either fuse with these vacuoles or disrupt them, thereby releasing the encapsulated antigen in the cytosol (Fan and Moon, 2015; Hu *et al.*, 2015) (figure 24).

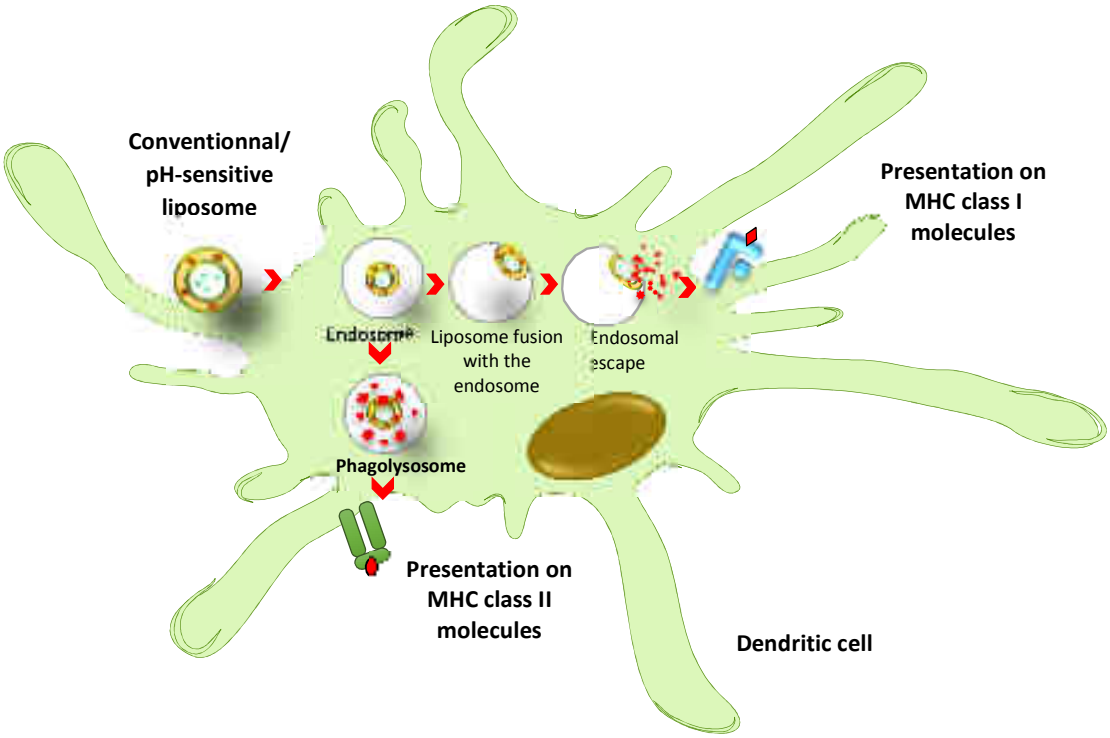


Figure 24: Liposomes favor cross presentation. *Expected mechanism by which liposomes promote cellular immune responses. Liposomes are taken up by endocytosis. Conventional liposomes prevent excessive acidification of the endosome, favoring endosomal escape and cross-presentation. pH-sensitive liposomes further promote this phenomenon: in the weakly acidic endosome environment, fuse with and/or destabilize endosomes and release their cargo into the cytosol, which results in antigen cross-presentation via MHC class I and II molecules and induction of a cytotoxic T lymphocyte (CTL) response.*

pH-sensitive fusion-active liposomes: To render them sensitive to low pH, liposomes can be modified with either pH-sensitive polymers, such as linear or hyperbranched 3-methylglutarylated poly(glycidol), or with pH-sensitive biodegradable polysaccharides, such as the polysaccharide-based 3-methylglutarylated dextran derivative. When the endosomal pH drops below 6, these liposomes are protonated, turning from hydrophilic to hydrophobic, fusing with the endosome and releasing their cargo into the cytosol. As an example of the validity of this strategy, fusion-active liposomes encapsulating ovalbumin (Ova) generated a greater CTL response, as compared to unmodified liposomes, and protected mice from challenge with an OVA-expressing tumor (Yuba *et al.*, 2013, 2014). When these liposomes were further modified to incorporate a cationic lipid, they were found to exhibit increased sensitivity to pH variation and improved interaction with DCs (Yoshizaki *et al.*, 2014).

pH-sensitive pore-forming liposomes: pH-sensitive pore-forming liposomes are based on encapsulation of listeriolysin O, a member of the cytolysin family. Cytolysins mediate the virulence of certain pathogens through the formation of pores in cell membranes, their degradation or their solubilization. These functions provide the pathogen access to the cytosol of the infected cell or mediate its escape from phagosomes. Listeria monocytogenes, for instance, escapes immune defenses by lysing the phagosomal membrane, using listeriolysin O. When listeriolysin O was co-encapsulated with ovalbumin into pH-sensitive liposomes, it was found to promote endosomal escape of the antigen to the cytosol in primary cultures, to increase CTL induction *in vivo* as compared to conventional liposomes and to confer protection to mice against antigen-expressing tumors (Mandal and Lee, 2002).

2.2.4. Liposomes can deliver vaccines through multiple routes

The physicochemical properties of liposomes can also be manipulated to adapt them to different delivery routes, including unconventional ones such as the mucosal and the transcutaneous (TC) routes, which are increasingly investigated in novel vaccination strategies. Indeed, because of their immune potential, these routes provide attractive alternatives to conventional subcutaneous and intramuscular vaccination. The skin and the mucosa are rich in immune cells, especially in DCs that can internalize antigens, migrate to draining lymph nodes and induce adaptive immune responses. Currently, attempts are being made to harness the immune potential of the skin through TC immunization. The specialized

DCs of the skin are Langerhans cells (LCs) and dermal DCs (dDCs). Various strategies are being developed to target them using vectors specialized in skin delivery, for microbial and cancer vaccination.

Conventional liposomes were reported to mediate TC passage of vaccines. For instance, it was reported that TC administration of saponin-containing liposomes encapsulating ovalbumin induced high titers of anti-OVA antibodies (Zhang *et al.*, 2017). Additionally, it has been shown that unsaturated lipid chains in the hydrophobic regions of phospholipids also mediate the TC passage (Yokomizo and Sagitani, 1996). Variants of liposomes which are more adapted to the TC route are developed, such as ultradeformable liposomes, or transfersomesTM. Their lipid bilayers are designed either to transiently disrupt the architecture of the skin barrier, or to squeeze into pores smaller than their size, in order to cross the skin barrier (Benson, 2006; Cevc and Blume, 1992; Cevc *et al.*, 1998; Rattanapak *et al.*, 2012)

Chapter 3. Delivering cancer vaccines to relevant DCs: the transcutaneous route

Transcutaneous (TC) cancer vaccination using liposomes

In the search for new and effective vaccine delivery strategies, special attention is given nowadays to the transcutaneous route (TC), for its potential to induce immune activation. TC vaccination, also known as epicutaneous or transdermal immunization, consists in a minimally invasive application of the vaccine on intact or barrier-disrupted skin.

TC vaccines were first designed in the context of anti-infectious vaccination. Multiple studies have since demonstrated their capacity to induce $CD4^+$, $CD8^+$ and B cell responses (Eypper *et al.*, 2013; Levin *et al.*, 2015; Ng *et al.*, 2012; Vassilieva *et al.*, 2015) which are comparable to those elicited by the traditional intramuscular or subcutaneous routes (Rouphael *et al.*, 2017). Being needle-free, pain-free, self-administered and suitable for children and elderly immunization, TC vaccines are highly desirable, especially during epidemics. Also, the TC route, in contrast to injections, provides sustained presence of antigen and adjuvant at the immunization site, thus ensuring a prolonged presentation of the antigen to immune cells. This effect is particularly marked with skin patch-based vaccines that can ensure the vaccine's persistence for several hours. Additionally, TC immunization offers the opportunity to target DC subsets that are different from those targeted during intramuscular or subcutaneous immunization (Karande and Mitragotri, 2010).

Shortly after its investigation for anti-infectious vaccines, the perspectives for the TC route widened considerably, as its ability to induce systemic CD4+ and CD8+ T cell immunity (Combadiere and Liard, 2011; Engelke *et al.*, 2015; Ita, 2016; Levin *et al.*, 2015) drew the attention to its potential in cancer vaccination. Several preclinical and clinical findings prompted further search for improved vaccine design for TC vaccination, for optimal strategies that maximize the effectiveness of TC vaccine delivery and for a better understanding of the role of different skin DC subsets.

ADP-ribosilating toxins, such as cholera toxin and E-coli thermolabile enterotoxin, were found to be the most potent adjuvants for the TC route (Engelke *et al.*, 2015; Partidos and Muller, 2005; Partidos *et al.*, 2004). However, the use of active toxins in humans implies safety

concerns that prohibit its application. Indeed, an attempt was made to vaccinate healthy volunteers with a Listeria monocytogenes vaccine adjuvanted with cholera toxin. In this trial, the vaccine did not induce generalized serious side effects, however, it induced localized side effects in all participants (Eypper *et al.*, 2013). Therefore, in order to develop the TC route in humans, finding an alternative adjuvant system that increases skin permeability and induces a robust CTL response is a must.

In this section comprising the **review article** "Enhancing Tumor-Specific Immune Responses by Transcutaneous Vaccination", we will discuss the immunological features of the skin, highlight the potential of TC vaccination in improving therapeutic cancer vaccines and provide a comprehensive review of the technologies that make this vaccination possible, including nanotechnology. A particular attention is dedicated to new liposome-based vaccine formulations and the opportunity they provide for targeting potent skin DCs through the TC route. Finally, we report the latest advances in clinical development that drive forward transcutaneously delivered cancer vaccines.

Review article

Enhancing Tumor-Specific Immune Responses by Transcutaneous Vaccination

Accepted in Expert Review of Vaccines, September 2017

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Abstract:

Introduction:

Whereas our understanding of the immune system involvement in cancer control has increased over recent years, the development of cancer vaccines intended to reverse tumor-induced immune tolerance remains slow as most current vaccine candidates exhibit limited clinical efficacy. The skin is particularly rich with multiple subsets of dendritic cells (DCs) that are involved to varying degrees in the induction of robust immune responses. Transcutaneous administration of cancer vaccines may thus harness the immune potential of these DCs, however, this approach is hampered by the impermeability of the *stratum corneum*. Innovative vaccine formulations including various nanoparticles, such as liposomes, are therefore needed to properly deliver cancer vaccine components to skin DCs.

Areas covered:

The recent insights into skin DC subsets and their functional specialization, the potential of nanoparticle-based vaccines in transcutaneous cancer vaccination and, finally, the most relevant clinical trial advances in liposomal and in cutaneous cancer vaccines will be discussed.

Expert commentary:

To define the optimal conditions for mounting protective skin DC-induced anti-tumor immune responses, investigation of the cellular and molecular interplay that controls tumor progression should be pursued in parallel with clinical development. The resulting knowledge will then be translated into improved cancer vaccines that better target the most appropriate immune players.

Keywords:

Cancer vaccine, liposome, nanoparticle, skin dendritic cell, transcutaneous vaccination

1. Introduction:

The last decades have witnessed a gradual shift in cancer management from conventional therapy (surgery, radiation therapy, chemotherapy and endocrine therapy) to immunotherapy, mainly with monoclonal antibodies specific for tumor antigens. More recently, targeted immunotherapies, intended to break the immune tolerance induced by tumors or to actively stimulate the patient's immune system against cancer cells, have emerged. These approaches stem from our understanding that despite being antigenic and often also immunogenic, most tumors fail to induce protective immunity because of their immunosuppressive microenvironment. Immunotherapy is therefore intended to reverse this microenvironment effect, thus harnessing the immune system to attack cancer cells.

2. Cancer immunity: challenges and vaccine design requirements

2.1. Protective tumor-specific immune response

A protective adaptive immune response against tumor cells should consist of several key steps, including **1)** Tumor Associated Antigen (TAA) expression by tumor cells, and release of these antigens by dying cells. **2)** Release of damage-associated molecular patterns (DAMPs) that provide danger signals to dendritic cells (DCs) inducing their maturation. DAMPs are recognized by specific receptors on DCs named Pattern Recognition Receptors (PRR). **3)** Cross-presentation of tumor antigens by mature DCs, on MHC class I and class II molecules, to tumor-specific $CD8^+$ and $CD4^+$ T cells respectively. **4)** Priming of tumor-specific T cells resulting in cytotoxic T lymphocyte (CTL) differentiation. **5**) Migration of effector T cells and infiltration of the tumor and, finally, **6**) recognition and killing of tumor cells by effector CTLs. Optimal CTL differentiation requires, in addition to mature DCs, the presence of $CD4^+$ IFN-γ -producing T helper cells, named Th1 (figure 1).

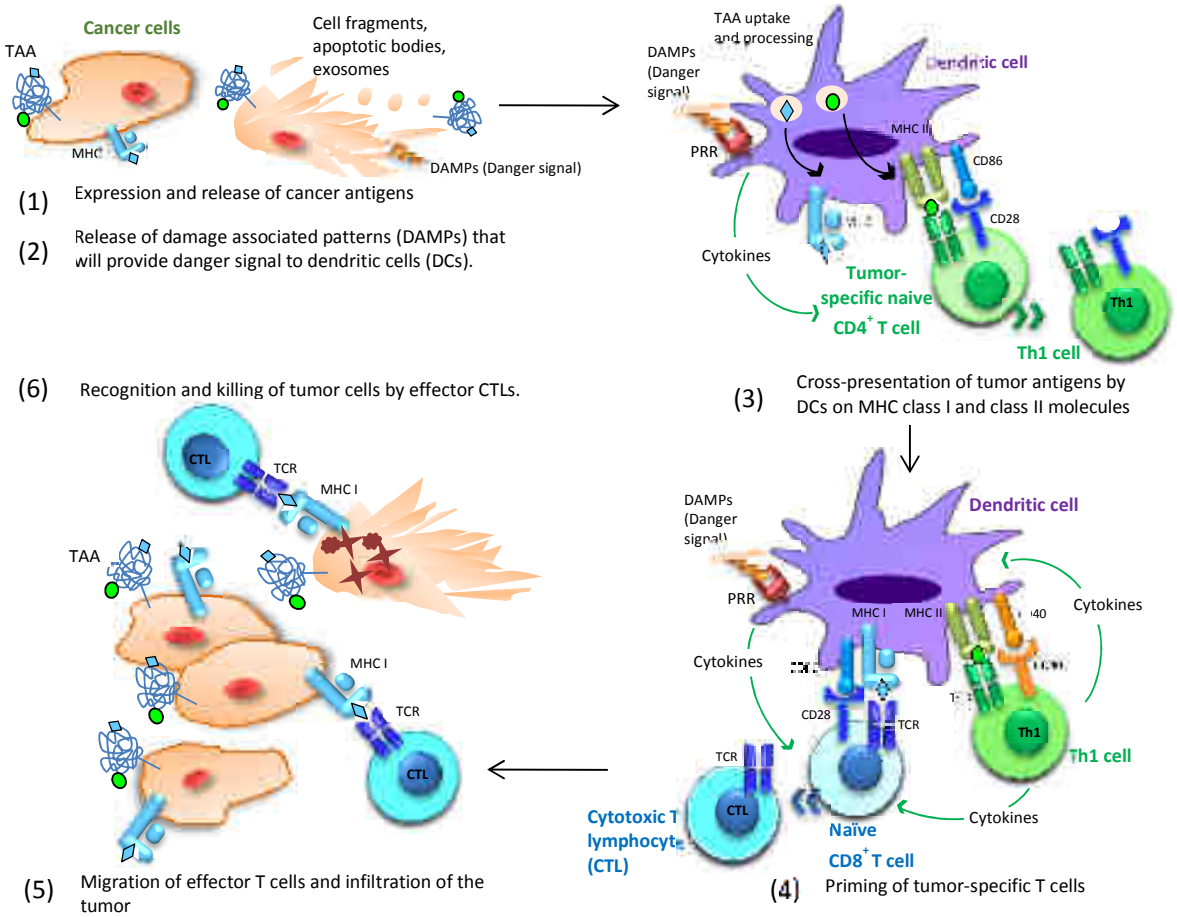

Figure 1: Key steps of a protective adaptive tumor-specific immune response. **(1)** Tumor associated antigens (TAAs) are expressed by tumor cells, presented on MHC class I molecules and released from dying cells. **(2)** Damage-associated molecular patterns (DAMPs) are also released from stressed or dying cells, and meet their specific receptors on DCs**. (3)** TAAs are internalized by DCs and cross-presented on MHC class I and class II molecules. **(4)** TAA-specific CD4+ and CD8+ T cells are primed by DCs. CD4+ T lymphocytes differentiate into Th1 cells and primed CD8+ lymphocytes differentiate into CTLs. **(5)** Effector cells migrate to the tumor where **(6)** CTL recognize and kill TAA-expressing cells. MHC: major histocompatibility complex, TAA; Tumor associated antigen, DAMP: damage-associated patterns, DC: dendritic cell, PRR: pattern recognition receptor, TCR: T cell receptor, CTL: Cytotoxic T lymphocyte.

2.2. Kinetics of tumor development and escape from immune response

During the initial tumor development stage, the tumor-specific immune response is capable of eliminating all immunogenic cancer cells. Progressively, mutations decrease tumor cell immunogenicity resulting in a dynamic “equilibrium phase”, where the immune system cannot destroy all cancer cells, but only most of them, to keep the cancer in a dormant state. This state will progressively fade, as specific tumor escape mechanisms, along with the exhaustion

of lymphocytes, will render this immune response inefficient. At this stage, the balance between the effector and regulatory immune compartments is seriously broken and the tumor enters the “evasion phase” and develops more rapidly [1].

Tumor escape mechanisms were divided by Teng *et al* (2015) [2] into three major categories (table 1). First, under the selective pressure of the immune system, a myriad of genetic and epigenetic alterations occurs, resulting in several events referred to as immunoediting. They include inhibition of antigen presenting machinery, expression of new TAAs, and downregulation or loss of highly immunogenic TAAs and co-stimulatory molecules. Second, tumor cells survival and resistance to apoptosis and to cytotoxic effectors of immunity is enhanced. Third, tumors establish an immunosuppressive microenvironment by favoring the induction and recruitment of immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC). Therefore, the pattern of tumor infiltrating lymphocyte subsets is a key criterion that drives disease progression.

In addition, chronic antigen exposure causes a continuous ligation of inhibitory receptors on immune effector cells. This leads to an “exhausted” [2], characterized by Wherry *et al* as “a poor effector function, a sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells” [3]. Under normal physiological conditions, the immunosuppressive pathways described above are crucial for the prevention of excessive immune responses and thus, the maintenance of self-tolerance by ensuring a balance between inhibitory and co-stimulatory signaling. In the case of cancer, however, these mechanisms shift the balance towards an inhibitory state [2].

Table 1: Tumor cells escape mechanisms according to Teng *et al*, 2015 [2].

Loss of tumor cell immunogenicity	- Downregulation/ loss of strong antigens - Downregulation /loss of co-stimulatory molecules - Downregulation of MHC-I expression
Tumor-cell resistance to apoptosis	- Upregulation of immune cytotoxicity resistance molecules (STAT-3) - Upregulation of prosurvival factor genes (Bcl-2, Her2…)
Establishment of an immunosuppressive microenvironment	- Production of immunosuppressive cytokines (IL-10, TGF-β) - Production of immunosuppressive metabolic factors (PGE-2) - Induction and recruitment of Tregs and MDSCs - Adaptive immunity blockade by induction of exhaustion in T cells

MHC: Major histocompatibility complex; STAT-3: Signal Transducer and Activator of Transcription-3; Bcl-2: as B-cell lymphoma-2; Her2: Human epidermal growth factor receptor 2; IL-10: Interleukin-10; TGF- β: Tumor growth factor-β; PGE-2: prostaglandin E2; Treg: Regulatory T cells; MDSC: myeloid-derived suppressor cells.

2.3. Therapies based on reversal of immune tolerance

In theory, in order to reverse tumor-induced immune tolerance, the above-mentioned key steps can be targeted using two different therapeutic approaches. On one hand, administration of antagonists of inhibitory signals or agonists of co-stimulatory ones can be used to inhibit immunosuppressive mechanisms and amplify antigen-specific T cell responses. On the other hand, therapeutic cancer vaccines are intended to induce active cancer immunity either by activating pre-existing host antitumor immune cells or by inducing the differentiation of new ones.

Cancer vaccines are therapeutic preparations intended to enhance both the number and the function of tumor-specific CTLs. They should therefore contain $CD8^+$ T cell epitopes derived from TAA of the targeted tumor type, as well as $CD4^+$ T cell epitopes and a potent adjuvant. The adjuvant, which is usually a PRR agonist, plays the role of a danger signal that activates and drives maturation of DCs. Following uptake and epitope cross-presentation, mature DCs would induce Th cells and tumor-specific CTLs. Besides its composition, the delivery route of the vaccine is also crucial as it dictates the amount and type of DCs to be targeted. It may also contribute to vaccine-induced inflammation that plays a role in DC maturation.

The most popular vaccination routes are the intramuscular (IM) and the subcutaneous (SC) ones, mainly for their ease of administration, despite the scarcity of DCs in muscles and their virtual absence in the hypodermis. Recently, the transcutaneous approach has been considered because of the abundance of DCs in the skin.

3. Vaccination via the skin

The skin is the main barrier that protects the body from the external environment, and therefore, is continuously challenged by microbes, physical and chemical aggressions and injuries. To face these challenges, it harbors a specialized, highly complex innate and adaptive immune network, capable of mounting adequate immune responses. This 'skin immune system' (SIS) consists of specialized skin-resident immune cells, along with immunocompetent skin-trophic lymphocytes and DCs that constantly recirculate between the skin, the lymphatic vessels, the skin-draining lymph nodes and, in the case of lymphocytes, the bloodstream.

3.1. The skin immune system

In many species, including humans and mice, the skin is anatomically composed of 3 layers, namely, from the outer to the inner side, the epidermis, the dermis and the hypodermis. The epidermis is comprised mainly of keratinocytes. Its outermost layer is the *stratum corneum*, or horny layer, which is composed of 4-20 layers of dead corneocytes and confers the barrier function of the skin. The immune cells of the epidermis are Langerhans cells (LCs) and effector and memory $CD8^+$ cytotoxic T cells. The dermis is a connective tissue composed of a fibroblast-rich network of collagen and elastin fibers embedded in proteoglycans, providing strength and elasticity to the skin [4]. It contains dermal dendritic cells (dDCs), natural killer (NK) cells, memory B and T cells as well as mast cells and macrophages [4,5]. The hypodermis is also called the subcutaneous (SC) tissue or adipose tissue. This layer of white fat is composed mainly of fibroblasts and adipocytes and plays a role in fat reserve and thermal isolation. Unlike the epidermis or the dermis, the hypodermis naturally lacks resident immune cells [4]. Finally, the skin contains appendages like hair follicles and sebaceous glands that together, form pilosebaceous units. Hair follicles originate from the dermis, are surrounded by an epidermal sheath [4] and are connected with a network of blood capillaries and nerve endings. The epidermal sheath surrounding the follicle is a stratified epithelium that is continuous with the epidermis. However, it is discontinued at the entrance of the sebaceous gland duct to the

hair canal [6]. Thus, hair follicles represent a potential entry port for pathogens and chemicals (figure 2).

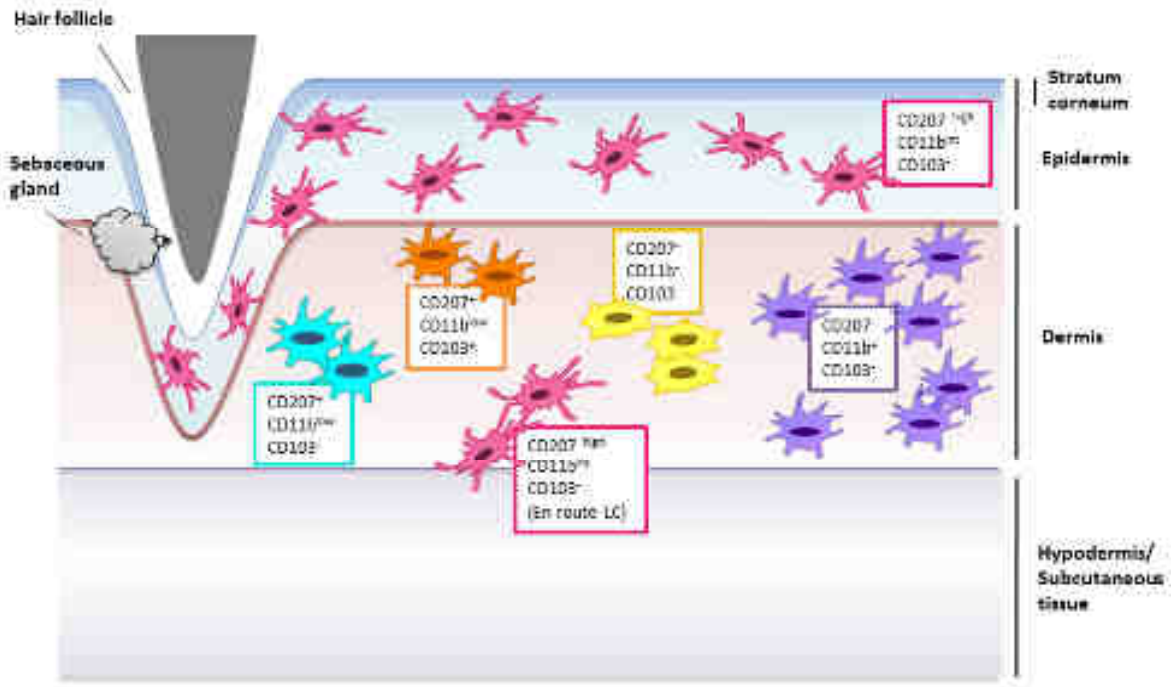

Figure 2: Skin layers, skin dendritic cells and their corresponding markers.

3.2. Skin DCs subsets

The skin contains a large number of DCs. These immune sentinels exhibit potent phagocytic, macropinocytic and endocytic activity, thereby internalizing microorganisms, cell debris, pathogen constituents and soluble molecules from their surroundings. Their role is to constantly sample their microenvironment, process antigens and present them to T lymphocytes.

3.2.1. Langerhans cells:

LCs are the only DC subset in the epidermis, accounting for 2-5% of all epidermal cells [4,7]. They are characterized by a high expression of langerin (CD207) and MCH class II, an intermediate expression of CD11b (CD11b^{int}) and the absence of the integrin alpha E chain (CD103^{-}) [8]. Overall, 2–3 % of LCs circulate naturally and continuously from the epidermis to the lymph nodes, across the dermis [4].

LCs are specialized in epidermal immunosurveillance. Despite their scarcity, their extensive network of dendrites covers the epidermis entirely and extends and retracts in a rhythmic manner allowing them to sample the fluid in the intercellular spaces between keratinocytes [7,9]. This behavior is termed ‘‘dendrite surveillance extension and retraction cycling habitude’’ (dSEARCH) [7]. Activated LCs can migrate to draining lymph nodes to prime antigen-specific T lymphocytes, thus initiating humoral and cellular immunity.

Additionally, upon sensing inflammatory signals, they can provide skin surface immunosurveillance by increasing their dSEARCH motion and projecting their dendrites through tight junctions between keratinocytes towards the stratum corneum [9,10]. Thereby, they are able to collect extra-tight junctions pathogens/particles that have not yet breached the epidermal barrier. Ouchi *et al* [11] have shown that after patch immunization of mice with *S. aureus*-derived toxin, a high molecular weight molecule unable to cross the *stratum corneum* barrier, a protective IgG1 antibody response was detectable in their sera. Similarly, confocal microscopy experiments performed on immunostained human epidermal sheets, showed that the dendrites of activated LCs, extend above the tight junctions [9,10], and internalize topically applied proteins *via* endocytosis [10].

Whether LCs are capable of immunosurveillance of the dermis is still debatable. Using a mouse model of dermal melanocytosis, Hemmi *et al.* suggested that epidermal LCs could not reach down to the dermis as they failed to uptake melanocyte granules [12]. However, more recently, Flacher *et al.* showed that monoclonal antibodies (mAb) targeting endocytic receptors were efficiently taken up by LCs in human and mouse skin explants [13]. Moreover, using *in vivo* experiments, they showed that these monoclonal antibodies are subsequently transported by LCs to the draining lymph nodes [14]. To explain these results, two scenarios can be proposed. It is most probable that the mAb have diffused across the basement membrane separating dermis and epidermis. Yet, it cannot be excluded that LCs have reached “down” to the dermis where they internalized them.

3.2.2. Dermal dendritic cells:

Dermal DCs (dDCs) are heterogeneous. Their markers vary between mice and humans. In 2005, Kissenpfennig *et al* showed [15] that langerin/CD207, first thought to be restricted to LCs, was also expressed by some dDCs subpopulations. Based on the expression of CD207,

CD11b and CD103, Henri *et al* [8] identified in 2010 four distinct sub-populations of DCs from digested skin: $CD207^{high}$ $CD11b^{int}$ $CD103^{-}$ cells corresponds to the epidermal LCs "en route" towards lymph nodes, while the three remaining subsets are dermal resident subsets, including $CD207^{+}$ $CD11b^{low}$ $CD103^{+}$, $CD207^{-}CD11b^{+}$ $CD103^{-}$ and $CD207^{-}$ $CD11b^{-}$ $CD103^{-}$.

Similar to LCs, dDCs are all $MHCII^{high}$. They can present antigens to T cells following uptake, maturation and migration to draining lymph nodes. Dermal DCs were shown to carry *Leishmania major* antigens [16] or locally applied ovalbumin antigens [17] to draining lymph nodes, where they induced antigen-specific T cell proliferation.

3.3. Antigen presentation potential of skin DCs

3.3.1. Endocytic receptors of skin DCs

Skin DCs are equipped with a panel of receptors that mediate pathogen/vaccine uptake and tailor vaccine-induced immune responses. Among these, endocytic receptors of the C-type lectin superfamily recognize pathogen-specific carbohydrate structures [18]. They therefore offer the opportunity of targeting the endocytic pathway *via* their specific ligands. Examples of endocytic receptors are DC-SIGN/CD209, Langerin/CD207, Clec9A/DNGR and the mannose receptors family, including the mannose receptor MR/CD206, DEC-205/CD205, Endo180, and the M-type phospholipase A2 receptor [19].

3.3.2. Skin DC function in cellular immune response activation: relevance to cancer vaccination?

Protective immunity against cancer cells requires cross-presentation of exogenous antigenic peptides on both MHC class I and class II to $CD8^{+}$ and $CD4^{+}$ T cells respectively, in order to drive CTL differentiation. While it is established that under inflammatory conditions, both LCs and dDCs have the ability to induce a specific immune response against foreign pathogens, their selective capacity in initiating and driving cancer-specific immune response is largely debated (Table 2).

Early studies suggested that only LCs were capable of cross-presentation. LCs differentiated *in vitro* from human $CD34^{+}$ hematopoietic progenitors were shown to sample necrotic/apoptotic melanoma cells and efficiently prime $CD8^{+}$ T cells thereby generating melanoma-specific CTLs [20]. In another study, LCs that were induced to migrate from the epidermis in the presence

of external stimuli had the ability to cross-present both soluble and cell-bound protein antigens on their MHC class I molecules and to induce CTLs capable of killing antigen-loaded cells [13,21]. *In vivo*, it was reported that both intradermal and transcutaneous immunization resulted in $CD8^+$ T cell proliferation in draining lymph nodes [21,22].

On the other hand, LCs are believed to play an immunoregulatory role to promote tolerance and prevent excessive inflammation. For example, they were shown to constitutively promote local proliferation and activation of skin resident memory CD4+ regulatory T cells (Treg) and to migrate to skin-draining lymph nodes where they present self-antigens to T cells [23,24]. Moreover, the depletion of LCs in a mouse model of contact hypersensitivity resulted in a higher number of antigen-specific effector T cells, without affecting the Treg count [25,26].

Regarding dDCs, recent reports suggested that $langerin^+$ $CD103^+$ dDCs are particularly potent in terms of cross-presenting antigens to $CD8^+$ T cells [8,27]. Other dDC subpopulations, on the other hand, fails to cross-present endogenous and viral antigens [8,27] and seems to mediate mostly $CD4^+$ T cell priming [28]. I should be noted, however, that cross-presenting $langerin^+$ $CD103^+$ dDCs represent a very small population (2.6%) of dDCs [28,29].

Another level of complexity was revealed when it was found that targeting a given C-type lectin receptor does not invariably generate the same type of immune response in different DCs; similarly, within the same DC population, signaling via different C-type lectin receptors may lead to different outcomes. For example, early works suggested that targeting either DEC-205/CD205 or langerin/CD207 results in efficient cross-presentation and proliferation of $CD4^+$ and $CD8^+$ T cells [22]. However, newer results indicated that these two receptors might not be similarly involved in antigen presentation, depending on the DC subset that captures the targeting antibody. Indeed, LCs targeted through DEC-205/CD205 seem to perform cross-presentation and promote $CD8^+$ T cell proliferation [13,30], while those targeted through langerin rather tolerize CD8+ T cells for the antigen [13,29]. Conversely, antigen capture by langerin+ CD103+ dDCs via either langerin of DEC-205 consistently leads to potent CD8+ T-cell responses [13,29]. Further exploration of methods allowing selective targeting and stimulation of LCs and dDCs is needed to achieve the most appropriate cross-presentation of vaccine antigens.

Table 2: Role of skin DCs in immune activation and regulation.

Cell type	Phenotypic markers	Role in cellular immunity	Reference
Langerhans cells	$CD207^{high}$ $CD11b^{int}$ $CD103^{-}$	*In vitro* and *in vivo* induction of T cell proliferation	[14,22,23]
		Induction of antigen-loaded cells killing by CD8+ T cells	[22]
Dermal dendritic cells	$CD207^{+}$ $CD11b^{low}$ $CD103^{-}$	$CD207^{+}$ dDCs are particularly potent in inducing a CD8+ response	[15]
	$CD207^{+}$ $CD11b^{low}$ $CD103^{+}$		
	$CD207^{-}$ $CD11b^{+}$ $CD103^{-}$	Induction of a CD4+ T cell response	[15]
	$CD207^{-}$ $CD11b^{-}$ $CD103^{-}$		

Int: intermediate

Altogether, the current findings of skin immunobiology have so far proven the undeniable skin potential of mounting immune responses. The various, often controversial reports underline the skin DCs ability in driving the immune response either toward an immunostimulatory or an immunoregulatory state, depending on specific conditions (type and dose of antigen, danger signals, targeting receptor). This highlights not only the flexibility of the skin-induced immune responses, but also the high specialization and cooperation between different skin DC subsets. Further understanding of their activation conditions and their respective contribution in T cell priming and CTL induction is still needed for the development of improved skin-delivered vaccines.

4. Strategies of transcutaneous vaccination

4.1. Transcutaneous vaccination: making skin DCs the main vaccine recipients

Cutaneous vaccine delivery routes are distinguished as subcutaneous when the injection targets the hypodermis, intradermal when the vaccine is delivered within the dermis and transcutaneous (TC) when it is applied on the epidermis. Although subcutaneous injections

are widely used with acceptable results, this route ensures only suboptimal delivery of vaccines since the hypodermis is naturally devoid of skin-resident DCs (LCs and dDCs). Therefore, direct delivery of antigen to these cells appears as a potentially more efficient, alternative for cancer vaccination. Since intradermal vaccination does not favour uptake by LCs, the TC route is worth investigating. However, despite its promising potential, it is hampered by the *stratum corneum*, the impermeable outermost skin layer. The ultimate goal of TC vaccination is to ensure non-invasive antigen delivery through this barrier to the targeted DCs in the internal skin layers.

4.2. Barrier role of the stratum corneum

The *stratum corneum* exhibits highly selective permeability dictated by the size and lipophilicity of applied molecules. Depending on these parameters, TC passage of the vaccine molecules implies their uptake through multiple ports of entry that can be, either transepidermal, comprising the intercellular and the transcellular routes, or transfollicular (Figure 3 a).

For the transepidermal passage, only molecules smaller than 500 Daltons have a chance to cross the *stratum corneum* and reach internal skin layers [31]. When small molecules are uncharged (relatively lipophilic), they penetrate through the intercellular route, while when they are highly hydrophilic they are thought to prefer the transcellular route [32].

The *stratum corneum* is composed of keratin-rich dead corneocytes embedded in a lipid matrix [33]. In normal conditions, the fluid fraction is minimal and both lipids and keratin are solid, resulting in skin impermeability and elasticity. An increase in the fluid fraction can yield to mild transient permeabilization of the *stratum corneum* towards polar and apolar compounds. Achieving a hydration gradient can be performed by increasing the proportion of natural skin moisturizing factors (e.g. urea, glycerol) [34], by skin hydration prior to immunization or by applying occlusive bandage after vaccination. Once internalized, vaccine molecules diffuse toward the higher hydration gradient presented by the circulation under the epidermis.

The transfollicular route contributes largely and in different ways to TC crossing. The epithelium of the hair follicle *infundibulum* is immature, permitting the passage of soluble antigens [35] and selective entry of small particles [36]. Hair follicles have a reservoir function, however, they occupy less than 0.1% of the total skin surface, and their density varies

considerably according to body sites and individuals [35]. Besides, all hair follicles are not available for particles penetration: only “active” hair follicles are “open” for transfollicular passage, as hair growth and/or sebum production ensure removal of plugs formed by shed corneocytes and excess sebum [6].

4.3. Physical barrier disruption

The physical properties of conventional vaccines are usually not adapted for the TC route. In recent years, several barrier-disrupting and permeation-enhancing strategies have been developed, in addition to innovative vaccine formulations. Two types of strategies are currently used for *stratum corneum* barrier disruption: the first one relies on removal of one or more layers prior to vaccine application and the second relies on driving the vaccine components through the *stratum corneum* (Table 3).

Sandpapering, skin waxing and skin surface stripping are widely used to remove hair, excess sebum and a few layers of the *stratum corneum* [37,38] (Figure 3 (b)). Skin surface stripping was tested in preclinical trials and in humans to promote antigen penetration through the transepidermal or the transfollicular route. The technique resulted in an improvement in the immunogenicity of applied vaccines [4,39,40], but was uncomfortable to the patient. An alternative cyanoacrylate skin surface stripping procedure on human skin [41] proved to be more efficient and less uncomfortable.

Techniques using an external driving force include the use of jet injectors and micro-/nano-needles to deposit the vaccine directly inside the live skin layers, or sonoporation, electroporation and thermal poration to transiently and locally disrupt the *stratum corneum* (Figure 3 b).

All these techniques are minimally invasive compared to conventional injection routes, while they induce sufficient non-specific immunostimulation providing an adjuvant effect [42].

Table 3: Skin barrier disruption techniques.

Physical barrier disruption technique	Process	Properties	References
Abrasion, waxing, skin surface stripping	Removal of *stratum corneum*	Improve the immunogenicity of applied vaccines Uncomfortable when a high number of strikes is needed	[4,37–39]
Jet injectors	Skin piercing with compressed gas hitting the skin with high velocity	Delivery of liquid or powder vaccines May cause pain, bruising, and application-site burning	[42,43]
Microneedles	Hollow or vaccine-coated solid or dissolvable needles	Painless and self-administered	[44,45]
Thermal microporation or thermal ablation	*Stratum corneum* vaporization with highly focused thermal energy. Induction of micron-sized pores.	Delivery of hydrophilic molecules Induce activation and migration of LCs	[39,46]
Sonoporation/ electroporation	Transient molecular-scale disruption of the cellular plasma membrane	Expensive and needs a power supply	[39,42]
Permeation enhancers, addition of polar chains, conjugation to cell penetrating peptides	Molecular interactions with plasma membranes	Increase the permeability to macromolecules	[47,48]

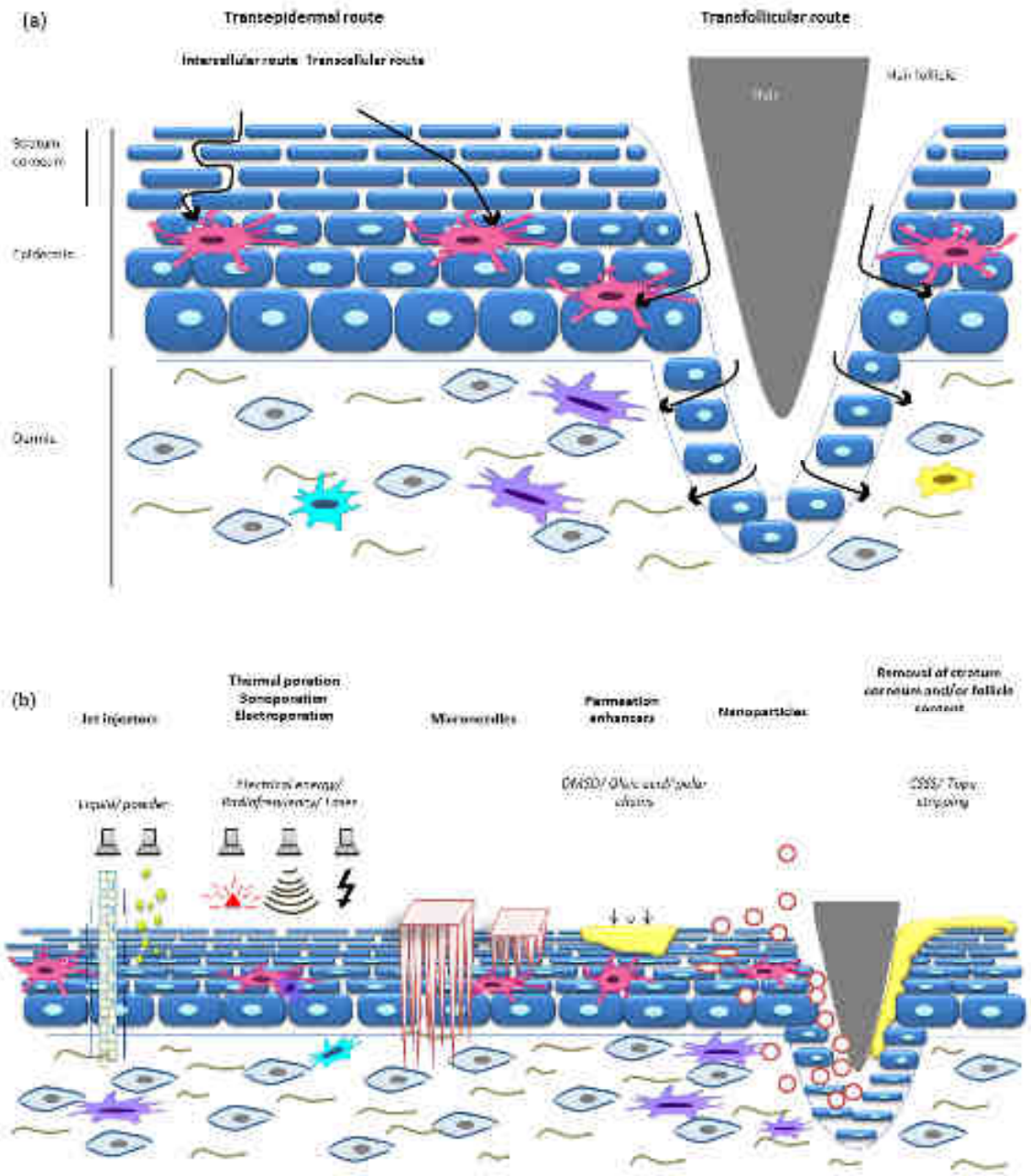

Figure 3. (a) Transcutaneous passage involves transepidermal and transfollicular ports. (b) Schematic representation of strategies of transcutaneous vaccination: jet injection of liquid and powder vaccines, thermal poration, sonoporation, electroporation, microneedles, permeation enhancers, nanoparticles and removal of stratum corneum and/or follicles content.

4.4. Innovative vaccine formulations for skin barrier crossing

To enable TC antigen delivery, another attractive strategy relies on the vaccine formulation itself.

4.4.1. Peptide-based vaccines combined to adjuvants

The current trend with vaccine design is to replace whole microorganism-based vaccines that are often toxic and reactogenic with proteins or even small synthetic peptides. Given their small size, these vaccines offer the additional advantage of being more suitable for the TC route. However, unlike whole microorganism-based vaccines, they are devoid of Microbe Associated Molecular Patterns (MAMPs) that provide danger signals to PRRs. Therefore, they are poorly immunogenic and require the co-administation of adjuvants to provide these signals and induce DC maturation. conventional adjuvants come in the form of emulsions (such as MF59 and Incomplete Freund's adjuvant, termed montanide when it is clinical grade) or colloids (such aluminum hydroxide called alum and aluminum phosphate). When they are injected, this physical form of results in a depot effect that ensures slow release of the vaccine components at the administration site, thereby increasing their uptake and presentation to DCs. Moreover, Alum induce danger signal by targeting the NALP3 PRR [49]. However, emulsions and colloids are not suitable for TC administration since they are unable to cross the cutaneous barrier [49]. Alternative modern adjuvants are pathogen-derived components, chosen to function as MAMPs, like lipopeptides, recombinant proteins and nucleic acid sequences.

4.4.2. Nanoparticles for transcutaneous immunization

Nanoparticles are well known for their capacity to permeate the skin and mediate delivery of compounds of different sizes and polarities, therefore, their use has become a popular strategy for TC vaccine delivery. Nanoparticles can effectively co-deliver the needed adjuvant along with the vaccine antigen(s), and they improve the stability of the vaccine by protecting it from the external environment and ensure its controlled slow release at the delivery site. Moreover, their formulation techniques are flexible allowing addition of the needed adjuvant and of various "ligand" molecules on their surface for targeted delivery. Their size and zeta potential can be modified for optimal transdermal passage, uptake by DCs and subsequent immune response.

"Nanoparticles" (NP) designate matricial as well as vesicular colloidal systems. Matricial systems are nanospheres made of a matrix of polymers or of solid lipid(s), where the active compounds are interspersed. Immunostimulating Complexes (ISCOMs) are one of the most successful examples. These are spherical cage-like particles, approximately 30-40 nm in diameter, made of cholesterol, phospholipids and glycosides (Quill A saponins), with a potent adjuvant property. Vesicular systems can also be made of polymers or lipids (the bi-layered lipide vesicles liposomes for example) but they have an inner liquid (aqueous or lipid) core. Active components can be incorporated either in their core or in the surrounding layer(s), or they can be attached or adsorbed on their surface (figure 4).

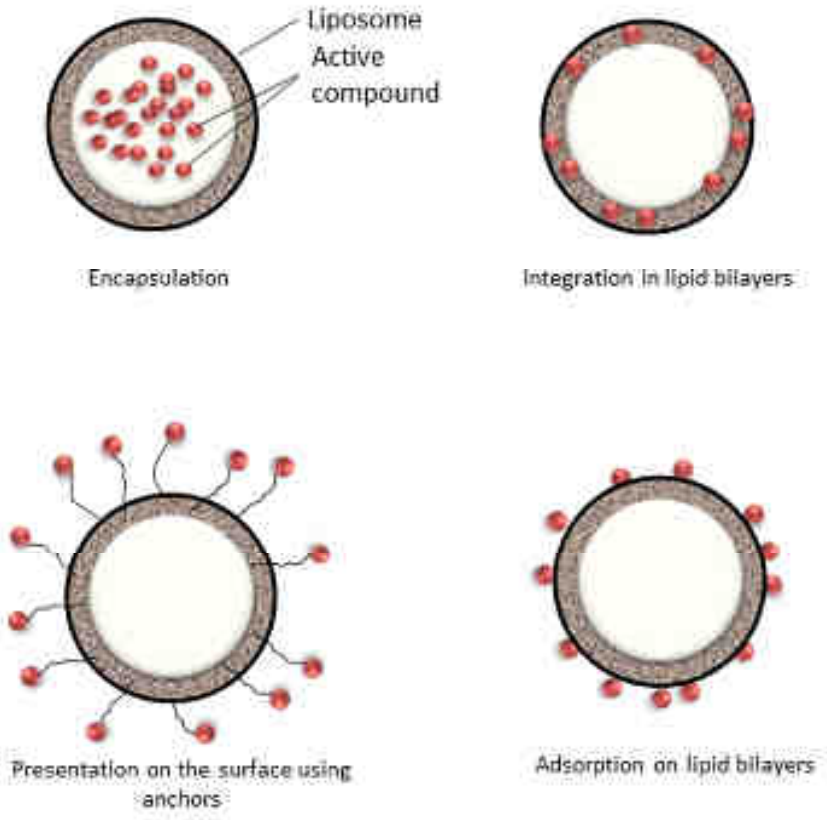

Figure 4: Interaction of active compounds with liposomes. Due to the physicochemical properties of liposomes, active compounds can either be incorporated in their core, or included in their surrounding layer(s), or be attached or adsorbed on their surface

4.4.3. Physicochemical properties influencing nanoparticles interaction with the skin immune system

Size. Nanoparticle size is a critical parameter that does not only largely predict TC passage, but also uptake by DCs and lymphatic draining efficiency. It was reported that the upper limit for intact skin absorption was 20 nm, while barrier-disrupted skin allows passage of nanoparticles up to 50 nm in diameter [50,51], and even 200 nm for ultradeformable ones [52]. Interestingly, the optimal particle size for transfollicullar passage was reported to be in the 600 nm range, allowing the highest penetration depth [53] (figure 5).

Nanoparticles ranging from 40 to 200 nm are optimal for fast and efficient uptake by DCs [54,55], including LCs [56]. Such virus-sized particles enter cells either by receptor-mediated endocytosis into clathrin-coated pits (<150nm), or through caveolae (50-80 nm). Particles 500-5000 nm, considered to be bacteria-like in size, are preferentially taken up by macrophages instead of DCs, through phagocytosis [54] (figure 5).

Regarding lymphatic draining, nanoparticles ranging from 10 to 100 nm seem to be most optimal. Indeed, they can efficiently drain to regional lymph nodes while being sufficiently retained in the vaccination site, thereby increasing the chance of antigen uptake and presentation by DCs. Larger particles (>500 nm diameter) are poorly drained to lymph nodes, whereas small ones (<10 nm) diffuse so rapidly that their chance to encounter DCs is minimized [57] (figure 5).

An *in vivo* study conducted by Fifis *et al* showed that among intradermally injected polystyrene nanoparticles ranging from 20 nm to 2000 nm, optimal immunogenicity was achieved by those in the viral size range of 40-50nm [58]. A closer assessment of the influence of minute differences in nanoparticle size showed that intradermal administration of 40-49 nm nanobeads activates IFN-γ secreting $CD8^+$ T cells, while that of 93-123 nm ones induces a $CD4^+$ T cell response and IL-4 [59] (figure 5). These findings underline the influence of the particle size on the cytokine profile and the type of elicited immune response, which may be of particular importance in the case of transcutaneous vaccination against cancer that requires $CD8^+$ T cell activation and IFN-γ secretion.

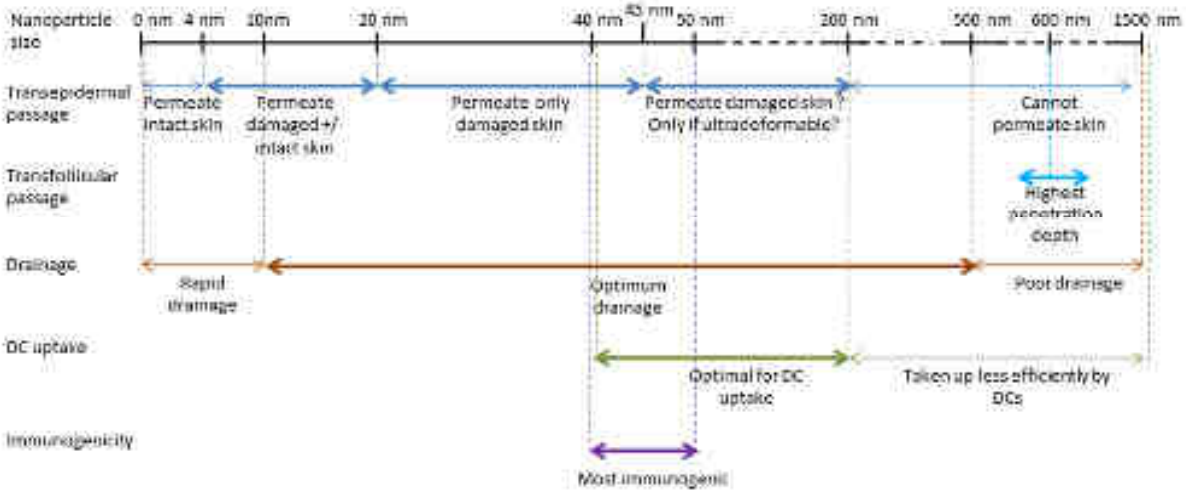

Figure 5: Influence of particle diameter on skin barrier crossing (transepidermal and transfollicular passage), drainage, uptake by dendritic cells (DCs) and immunogenicity. Other physicochemical parameters such as hydrophily and zeta potential can also affect particle behavior and properties.

Charge. The nanoparticle surface charge, reflected by its zeta potential, can largely affect its capacity to penetrate the skin. As the skin is negatively charged, it is expected to be more efficiently crossed by cationic or neutral nanoparticles. Indeed, such nanoparticles, like liposomes, were found to be more efficient in drug delivery into deep skin layers [60]. However, Kohli *et al* reported that only negatively charged latex particles could permeate through the skin. This unexpected finding was attributed to their passage *via* channels created by the repulsive forces between them and negatively charged skin lipids [51].

When it comes to internalization into DCs, it was demonstrated that charge is only important for larger particles: 1 µm polystyrene particles were more efficiently taken up when positively charged, while for those < 500 nm, the degree of internalization is independent of the surface charge [55,61].

How surface charge affects the induced immune response is still debatable. Nakanishi *et al* reported that protein antigens encapsulated into cationic liposomes are best delivered to APC cytosol and loaded on MHC class I, thus eliciting a cellular immune response [62]. Alternatively, Cui and Mumper showed that anionic chitosan-based nanoparticles induce higher antibody titers and cytokine production than cationic ones [63].

It is undeniable that these basic intrinsic properties can influence to a large extent nanoparticle penetration into the skin, their uptake by DCs and their immune activation properties. Nevertheless, current available data does not make it yet reasonable to draw final conclusions. Additionally, initial nanoparticle properties may not be sufficient to predict their behavior *in vivo* since they may be altered by their interaction with the skin lipids or with physiological fluids: nanoparticles can possibly aggregate to the micron-scale, their charge may change, etc.

Smart nanoparticle systems. These nanoparticles are designed to be applied through the transfollicular route and to release their active components only upon specific stimuli in order to increase follicular penetration of vaccine molecules. For example, bovine serum albumin nanoparticles encapsulating active compounds can be applied simultaneously with protease. Their subsequent enzymatic degradation ensures protease-triggered controlled release of their content [64].

4.4.4. Potential of liposomes for transcutaneous immunization

Liposomes were the first nanoparticles to be developed, about 40 years ago, and they remain the most investigated ones. They are the subject of a high number of patents and are available on the market as vectors of vaccines [65–67] and transcutaneously delivered drugs [68,69]. These are nanometric vesicles, composed of natural or synthetic biodegradable, cholesterol containing, phospholipid bilayers surrounding an aqueous core. Their zeta potential is defined by the nature of their phospholipids. Their structure allows them to transport both hydrophilic substances encapsulated in their core, and hydrophobic ones integrated in their lipid bilayers. Because their composition is closely related to that of biological membranes, they are highly tolerable and bear low intrinsic pro-inflammatory activity and are therefore among the most attractive nanoparticles for vaccination [70]. Liposomes provide passive targeting of skin DCs. Indeed, they are rapidly internalized by surrounding cells, ensuring a sufficient amount of vaccine is collected by the DCs, while “non-packaged” vaccines are less stable and risk rapid draining of their components.

Efficacy of liposomal carriers in TC crossing has been established since 1980, when they were used for the first time for topical drug delivery [71]. In this study, they were shown to achieve a four- to five-fold increase of the drug concentration in the epidermis and the dermis, as

compared to drug alone. Subsequent studies have however proposed their relatively rigid structure to be suboptimal for skin penetration. Liposomes were thus proposed to enhance drug deposition only in the upper layers of the *stratum corneum*, without reaching the internal living layers [72]. Because of their unique versatility in composition and size, liposomes are actively investigated as vehicles for TC vaccination. To increase skin penetration, many variants have been developed including transfersomes and ethosomes.

The term "transfersomes" was introduced for the first time by Cevc and Blume in 1992 [73] and is a trademark of IDEA AG, Munich, Germany. They are ultradeformable liposomes made with small unsaturated soybean lecithin and an edge activator, usually a surfactant. They are able to squeeze into pores much smaller than their size and to carry a remarkable amount of lipid into the skin. Therefore, they were expected to enhance cutaneous vaccine delivery through the *stratum corneum* with minimal barrier disruption [74]. However, results about their potential remain contradictory [32,75].

Ethosomes are obtained by adding a high percentage of ethanol (up to 45%) to conventional liposomes, thus significantly increasing their fluidity [75]. Rattanpack *et al* reported them to be the most efficient vesicular carriers. It is also possible to combine ethanol and surfactants in a single nanoparticle to maximize the fluidity enhancement effect. The resulting vesicles are called transethosomes [52,76].

4.4.5. Adapting nanoparticles for adequate skin DC targeting

In order to benefit from the skin potential in mounting protective immune responses following TC vaccination with nanoparticles, these particles should be able to reach, target and activate the most appropriate skin DCs (epidermal LCs and/or dDCs) that would induce Th1 and CTL responses. Targeting nanoparticles to receptors expressed on skin DCs improves the interaction between them. Espuelas *et al.* showed that adding a mannose residue to liposomes enhanced their uptake by human DCs through mannose receptor-mediated endocytosis [77]. When mannosylated liposomal cancer vaccines were injected SC in tumor-bearing mice, it was possible to decrease the adjuvant dose up to 100-fold without any loss in the anti-tumoral efficiency [78]. Translation of this strategy would be particularly interesting in TC vaccination where the minimal amount of vaccine that crosses the *stratum corneum* barrier would be compensated by a higher uptake by skin DCs.

5. Transcutaneous cancer vaccination using nanoparticles: where do we stand?

To date, the search by keywords “cancer vaccine” yields to more than 1800 returns in the National Institute of Health database for clinical trials (www.clinicaltrials.gov). Out of these, 185 studies (10%) are in phase 3 and only two therapeutic cancer vaccines, Sipuleucel-T (Provenge®)[79] and talimogene laherparepvec (IMLYGIC, Amgen, Inc.)[80], have been licensed for clinical use. These numbers underline not only the great efforts that are being dedicated to cancer vaccine research, but also the challenges faced in the development of such vaccines. Despite their extensive diversity (purified peptides, proteins, antigen-loaded autologous DCs, nanoparticle-based vaccines, etc), all of these vaccines aim to elicit protective CTL responses. Various administration routes have been applied in clinical trials. While most vaccines are delivered subcutaneously [81–88] a few are delivered intradermally, [89–91]. A combination of both routes has also been tested and is known as intracutaneous [92,93]. However, cancer TC vaccination in humans has rarely been tested [94] and never with nanoparticle-based vaccines (tables 4 and 5).

Table 4: Examples of the most promising cancer vaccines involving lipid-based carriers.

Vesicle type	Vaccine	Adjuvant	Administration route	Traget cancer type	Clinical trial	Study outcome	REF
Liposome	**Depovax (DPX)-0907:** 7 TAA-derived Tc peptides, and a tetanus toxoid-derived Th epitope	Polynucleotide based-adjuvant	SC	Breast, ovarian, prostate cancer	Phase I	- Specific $CD8^+$ T cell response (61% response rate) - Specific T cell memory	[82]
	BLP-25 , or Tecemotide, or Stimuvax : BP 25 peptide of the MUC1 protein	MPLA (TLR 4 agonist)	SC	Non-small-cell lung cancer	Phase III	- MUC1 proliferative T cell response - No survival benefits, unless with concurrent chemotheraoy	[83, 85, 100]
ISCOM	**NY-ESO-1 ISCOMATRIX** : Full length NY-ESO-1 protein	ISCOM vesicles	SC	Melanoma	Phase II	- High titer NY-ESO-1 antibodies - Circulating specific CD4+ and CD8+ cells - Response persistence for 252-1155 days - Absence of delayed hypersensitivity - No clinical benefits	[84, 86, 87]
	Full length NY-ESO-1 protein, and a recombinant fowlpox virus	Recombinant fowlpox virus	IM (prime boost protocol)	Melanoma	-	- CD8+ T cell response in 3/18 patients	[88]

Table 5: The most promising cancer vaccines involving the TC administration route.

Vaccine	Adjuvant	Administration route	Traget cancer type	Clinical trial	Study outcome	REF
CDX-1401 : full length NY-ESO-1-protein, fused to anti DEC-2015 mAb	Resiquimod (TLR 7/8 agonist) Or Poly ICLC (TLR 3 agonist)	Various combinations of TC and SC routes	melanoma, sarcoma, ovarian cancer and others	Phase I	- NY-ESO-1-specific cellular immunity of 56% of the patients - Disease stabilization in 13/56 patients, and occasional disease regression	[93]
Full length NY-ESO-1 protein	Imiquimod (TLR 7 agonist)	Protein (ID) and imiquimod (TC)	Melanoma	Phase I	- NY-ESO-1 specific antibodies - Absence of CD8+ response	[91]
	Resiquimod (TLR 7/8 agonist) and montanide	montanide-emulsfied protein (ID) and resiquimod (TC)	Melanoma	Phase II	- NY-ESO-1 specific antibodies in all subjects - Specific CD4+ T cells and CD8+ T cells (in 3/12 subjects)	[90]
Tumor-derived peptides/DMSO	Absence of adjuvant molecule- Tape tripping for skin-barrier disruption	TC	Melanoma	Phase I	- Extension of overall survival	[94]

5.1. Nanoparticle-based cancer vaccines in development

Various nanoparticles like nanoemulsions, polymeric and magnetite nanoparticles, ISCOMs and liposomes have shown efficacy in vaccine preclinical trials, but only the ISCOMs and liposomes have reached clinical studies.

5.1.1. ISCOM-based cancer vaccines

ISCOMs were first used to deliver viral and bacterial antigens. The promising results prompted the development of ISCOM-based antitumoral vaccines. The cage-like matrix of ISCOM nanoparticles is designated as ISCOMATRIX. An ISCOM-based cancer vaccine specific for NY-ESO-1 has been tested in clinical trials. NY-ESO-1 is a cancer-testis antigen expressed in normal testis but also in tumors of various tissues, including melanoma and ovarian cancer. In a phase I clinical trial involving patients with resected melanoma, it elicited NY-ESO-1-specific antibody responses, $CD4^+$ and $CD8^+$ T cells [84] and persistent memory [86]. In a phase II clinical trial conducted on advanced metastatic melanoma patients, antibody responses were confirmed, however patients failed to develop cellular immunity and had no clinical benefits [87]. In an attempt to increase the NY-ESO-1 specific $CD8^+$ T cell response, this vaccine was combined to a recombinant NY-ESO-1 fowlpox virus in a heterologous prime-boost strategy. In a phase I clinical trial, it gave positive results in 3/18 patients [88].

5.1.2. Liposome-based cancer vaccines

Cancer liposomal vaccines have been extensively used in preclinical studies where they have shown variable efficacy. A vaccine was developed against hepatocellular carcinoma. It bears a peptide derived from Glypican-3 (GPC3), a TAA overexpressed in this type of cancer. Although devoid of an adjuvant molecule, this vaccine resulted in an inhibition of tumor growth [95]. Another vaccine expressing murine ErbB2-derived peptide and incorporating the TLR4 agonist Monophosphoryl lipid A (MPLA) was evaluated in mice. Subcutaneous injection of this vaccine resulted in the induction of a CTL response, yet, showed only partial protection against ErbB2-expressing tumors [96]. We have designed a peptide-anchoring liposome-based vaccine expressing a human ErbB2-derived $CD8^+$ T cell epitope, a universal $CD4^+$ T cell epitope and, dipalmitoyil alanyl cysteine glycine (Pam_2CAG), a potent TLR2/6 ligand. This vaccine was evaluated in a mouse model bearing transgenic murine renal carcinoma cells expressing the human ErbB2 protein. It induced a specific immune response against the ErbB2 peptide and

exhibited an efficient antitumoral effect after subcutaneous injection [78] and needle-free airway administration [97,98].

One of the most promising liposome-based formulations that proved to be efficient in preclinical trials was developed by Immunovaccine (Halifax, Canada) under the name of VacciMax®. This vaccine-enhancement platform consists of a water-in-oil emulsion in which liposomes are emulsified in Incomplete Freund's adjuvant. A more stable, water-free, generation of VacciMax, called Depovax® (DPX) was developed for clinical trials. It consists of lyophilized liposomes re-suspended in montanide immediately prior to vaccination [99]. DPX-0907, one of the variants of Depovax®, contains a TLR ligand, a universal Th peptide derived from tetanus toxoid, as well as seven human leukocyte antigen (HLA)-A2 restricted peptides derived from various TAAs. These peptides are specifically presented by MHC class I on breast, ovarian, and prostate cancer cells [81]. A phase I clinical trial of DPX-0907 increased the frequency of $CD8^+$ T cells in advanced-stage breast, ovarian and prostate cancer patients, with 61% immunological response rate and induced antigen-specific T cell memory [82]. This clinical trial provides a rationale for further evaluation of the clinical benefits of DPX-0907, especially in breast and ovarian cancer subjects. It is to be noted however, that the integrity of the liposomes when suspended in a mineral oil such as montanide may have been affected and the clinical benefit of this formulation cannot therefore be attributed with certainty to the liposome formulation or to the adjuvant itself.

BLP25 or tecemotide, also known as Stimuvax®, is another promising liposomal anti-cancer vaccine that has reached late clinical stages. It consists of a multilamellar liposome incorporating a TLR4 ligand (MPLA), and BP25, a peptide derived of the mucin 1 (MUC1) protein. MUC1 is a TAA overexpressed in more than 90% of adenocarcinomas including breast and lung cancers. BP25 contains $CD4^+$ and $CD8^+$T cell epitopes. Phase I and II trials conducted on non-small-cell lung cancer patients showed tolerability, induction of MUC1-specific T-cell proliferation and cytokine production as well as extended median survival [83]. However, a randomized phase III trial designated START (Stimulating Targeted Antigenic Responses to NSCLC) did not exhibit any significant survival benefit [100]. Nonetheless, a prospective analysis of the START trial showed that BLP-25 can be beneficial for patients treated with concurrent chemotherapy, since it provides a 10.2 months extension of the median overall survival [85].

5.2. Transcutaenous cancer vaccines in clinical trials

In the vast majority of cancer vaccine trials involving the cutaneous route, antigen administration is performed either subcutaneously or intradermally, while the adjuvant is applied topically. The goal of this strategy is to deliver sufficient amounts of the antigen in the dermis and simultaneously activate LCs in the epidermis by the adjuvant.

This combination protocol was first used for a melanoma vaccine based on full length NY-ESO-1 protein. In a phase I clinical trial where non emulsified NY-ESO-1 was injected ID, and imiquimod, a TLR7 agonist was applied TC, a specific humoral response was elicited, however, this vaccine failed to induce a $CD8^+$ T cell response [91]. An improved vaccine in which NY-ESO-1 protein was emulsified in montanide and imiquimod was replaced with resiquimod, a related more potent TLR7/8 agonist, resulted in a potent humoral immune response and a $CD8^+$ T cell response was induced in 3/12 patients [90].

CDX-1401, another NY-ESO-1 based vaccine, is composed of the full length NY-ESO-1 protein fused to a human mAb targeting the DEC-205 receptor expressed on DCs. It was tested in a phase I clinical trial in 45 patients with diverse advanced malignancies, in combination with various TLR ligands (Resiquimod targeting TLR7/8 and poly-ICLC targeting TLR 3), both by the SC and the TC routes. Persistent cellular immunity and clinical benefits were observed in 56% and 29% of the patients respectively, distributed to all study cohorts, independently of the administration route [93].

As of today, only one clinical trial was conducted on a cancer vaccine administered exclusively through the TC route. This vaccine consisted of a mixture of melanoma-derived peptides dissolved in DMSO (Dimethylsulfoxide). Melanoma patients were vaccinated TC after skin barrier disruption by tape stripping. The vaccine provided an overall survival of 55.8 months for patients who responded to all vaccine peptides, compared to 20.3 months for partial responders [94].

These studies provide irrevocable evidence of the efficiency of skin DCs in inducing tumor-specific $CD8^+$ T cell responses when they are activated under the appropriate conditions and an additional rationale for the use of the TC route for cancer vaccination. Future studies will likely explore more closely the exact factors that drive optimal immune responses against

topically applied vaccines and uncover more potent adjuvant molecules that can better amplify tumor-specific CTL responses.

6. Conclusion

The key to successful cancer immunization resides in appropriate activation of DCs capable of reversing the tumor-induced immune tolerance. The skin has a unique and potent immune network, especially rich with DCs capable of inducing and tailoring immune responses. Skin DCs have shown a potential for driving tumor-specific immune responses in mouse models and in humans. Needle-free, nanoparticle-mediated, transcutaneous delivery of cancer vaccines is therefore intended to target skin DCs including LCs and dDCs, in order to improve tumor-specific immune response amplitude and quality. Several strategies acting on the level of the vaccine formulation and on the TC vaccination techniques have been developed to overcome the *stratum corneum* barrier. A careful choice of the vaccine carrier and adjuvant, in addition to the use of DC targeting molecules are expected to drive the development of next generation cancer vaccines.

7. Expert commentary

Cancer vaccination is far more challenging than microbial vaccination. While microbes express a large panel of MAMPs and antigens that are strong activators of innate and adaptive immunity, cancer cells express mostly self-antigens. TAAs are generally poorly immunogenic. Additionally, within a single tumor, cancer cells may have different TAA expression profiles and exhibit different escape mechanisms. Consequently, despite decades of efforts, cancer vaccination has not yet reached its golden age. The major challenge is therefore to induce a protective immune response against carefully selected TAA peptides properly presented by adequate DCs. Only two cancer vaccines are currently in clinical use.

On the other hand, recent efforts have focused on harnessing the antigen presentation potential of skin DCs. Several strategies have been developed to overcome the barrier of the *stratum corneum*, including the use of nanoparticles. Encouraging results reported in clinical trials of intradermal administration of a virosomal influenza vaccine are good proof of the feasibility of this approach.

Unfortunately, our **knowledge of skin immunobiology** is still incomplete. A large number of studies have been conducted, but their experimental settings varied extensively, thereby leading to contradictory results. For instance, the respective roles of LCs and dDC subpopulations in immune activation are far from being elucidated: the first studies suggested that LCs were the only players in antigen cross-presentation, but later, it was found that CD207/Lang$^+$ dDC are also involved. Therefore, this role remains to be attributed to one or the other or both populations.

Our knowledge about the ideal nanoparticles properties for TC vaccination is also limited. We need better investigation of **the influence of nanoparticles physicochemical characteristics** (size, charge, and composition) on skin barrier crossing, targeting and activation of adequate antigen presenting cells. Reported studies have used a large variety of models: nanoparticles were tested either *in vivo* or on human or porcine skin explants that may be frozen/thawed or fresh. These tests lead obviously to non-coherent, often contradictive results.

The high number of variables should therefore be counterbalanced by the establishment of common study design or, better, by organizing the laboratories into consortia. Centralization of generated data is expected to elucidate the networks of cellular cooperation that arise between these cells and identify optimal nanoparticle properties for specific targeting of the desired DC subpopulation.

Another challenge resides **in the choice of molecules to be incorporated in the nanoparticles. I**ndeed, single epitope vaccination approaches are MHC dependent and would be effective only in a subpopulation of cancer patients expressing the appropriate HLA genotype. Therefore, multi-epitope vaccines might be more adequate. Moreover, when the selected epitopes are derived from multiple TAAs, they decrease the risk of emergence of vaccine resistance following TAA dowregulation by tumor cells. This argues for the need for additional profiling of different malignancies, in order to identify the most relevant TAA peptides in each cancer type. Further studies are also needed to identify optimal targeting and adjuvant molecules to improve uptake and activation of DCs.

Finally, there is a need to improve the **reproducibility of transcutaneous vaccination techniques.** Current practices may lead to variable results between individuals depending on

the zone, size and hair follicle density of the application site. Development of transcutaneous vaccination devices that help standardizing the process deserves further attention.

For all these reasons, it will be many years before transcutaneous cancer vaccination unleashes its full potential. The half way target that currently seems the most reachable is the local treatment of melanomas, because lymphocytes primed by skin-derived DCs express skin homing receptors and are sufficiently recruited to the vaccination site. Additionally, combinatorial therapeutics, whether exclusively immunological, such as TC cancer vaccines and immune checkpoint inhibitors, or mixed immunological-chemical, such as TC cancer vaccines with concomitant chemotherapy, seem to be equally promising on the short-term.

8. Five-year view

This review of cancer vaccination strategies that are currently being investigated highlights the exponential growth of our understanding in the recent years. Important discoveries have been made in different converging fields, including tumor biology (TAA expression, escape mechanisms), cancer specific immunity (immune checkpoints, antigen presentation), vaccinology (adjuvantation, nanoparticles and cell targeting) as well as skin immune potential (LC, dDC). They are expected to progressively bridge the gap in knowledge regarding optimal TC cancer vaccine formulations and skin DC targeting strategies. As the respective roles of skin DC subpopulations will be better understood, appropriate ways of targeting them through nanoparticles and targeting molecules will be optimized. Identification of new adjuvants adapted to TC delivery will further improve vaccine efficacy.

Many clinical trials are already in the pipeline, with the most advanced being for melanoma treatment. Obviously, in the case of melanoma, topical application of the vaccine directly on the cancer lesion is expected to induce local protective immunity, but this is not the only intended effect. Indeed, similar to the currently used intratumoral melanoma vaccine (T-VEC), TC melanoma vaccines are expected to induce also systemic immunity, leading to the regression of metastatic lesions distant from the vaccination site. If such findings are confirmed, they will strongly encourage the application of TC vaccination to other kinds of tumors.

Finally, it will be interesting to evaluate in clinical trials the addition of TC vaccines to currently validated therapies based on immune checkpoint modulators and/chemotherapeutic drugs.

Their purpose would be to tip the balance from immune tolerance of tumors toward tumor rejection.

9. Key issues

- Cancers escape the immune system through many mechanisms, including immunoediting. Despite the potential immunogenicity of tumor cells, protective immune responses are rarely elicited and the balance is tipped towards tumor tolerance, thus favoring cancer aggressiveness and progression.
- To restore efficient immune rejection, cancer vaccines must re-educate the immune system to overcome tumor-induced tolerance.
- The skin harbors a complex network of dendritic cells. Langerhans cells and $CD207^{+}$/$Langerin^{+}$ dermal dendritic cells are thought to be potent inducers of CTL responses which are crucial for tumor specific immunity. Targeting these dendritic cells is possible through the transcutaneous route, if the vaccine can cross the impermeable *stratum corneum* barrier.
- Several strategies were recently developed to allow vaccine formulations to overcome the *stratum corneum* barrier. They include the incorporation of vaccine components into nanoparticles and the disruption of the skin barrier by microneedles and other means.
- Research is currently focusing on determining critical nanoparticle properties, such as size, charge and composition, for optimal delivery to skin dendritic cells and uptake. Because of their versatility and their similarity with biological membranes, liposomes are among the most promising nanoparticles adapted for transcutaneous immunization.
- In liposome-based vaccines, antigenic molecules can be incorporated into the liposome or expressed on its surface. Adapted adjuvants that are suitable for skin barrier crossing can be added. Additionally, it is possible to insert, in the liposome surface, DC targeting molecules whose receptors are differentially expressed on skin DC subsets. This strategy allows the delivery of a greater vaccine cargo to the desired cells. Sometimes, these receptors can also have an immunostimulatory role.

- Recently, several clinical trials have translated preclinical findings into human testing. Liposome-based vaccines are under current clinical investigation by conventional routes. On the other hand, peptide-based vaccines are being investigated by the TC route. A combination of the key elements of these success stories is expected to drive TC cancer vaccination using liposomal peptide vaccines into clinical development.

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Chapter 4: Evaluating cancer vaccines in humanized mouse models

Rodents have always been used for *in vivo* experimentation as surrogates to study human biology. In addition to their genetic resemblance to humans (Walsh *et al.*, 2017), their small size, their ease of maintenance and handling, their prolific reproduction and their short reproductive cycle are valuable qualities that prompted their wide use as model systems in the biomedical field and especially in immunology. However, it is frequent that biological products face partial or total failure in exerting their expected effects in clinical trials after being successful in these murine preclinical models (Mestas and Hughes, 2004). Besides, preclinical *in vivo* testing is of primordial importance, since candidate products cannot obviously be tested in humans for ethical, financial and logistic reasons. Therefore, the scientific community is in urgent need for a model that would better mimic the human biology and be more predictive of the human response to treatment.

Humanized mouse models represent a valuable tool to study human biology ex homine. They consist of mice with severe combined immunodeficiency (SCID) that are engrafted with human cells or tissues, or of genetically engineered mice that carry human genes. SCID mice are mostly engrafted with hematopoietic cells, such as the peripheral blood mononuclear cells (PBMCs), splenocytes (SPL), or hematopoietic stem cells (HSCs) to reconstitute a human immune system (figure 25). They can additionally be engrafted with fetal thymus or liver fragments. Following engraftment, they can reconstitute a network of functional immune cells capable of mounting innate and adaptive immune responses. The receptivity of SCID mice to xenografts is conditioned, not only by the presence or absence of a residual activity of their immune cells, such as B, T and NK lymphocytes, but also by the source of the transplanted cells.

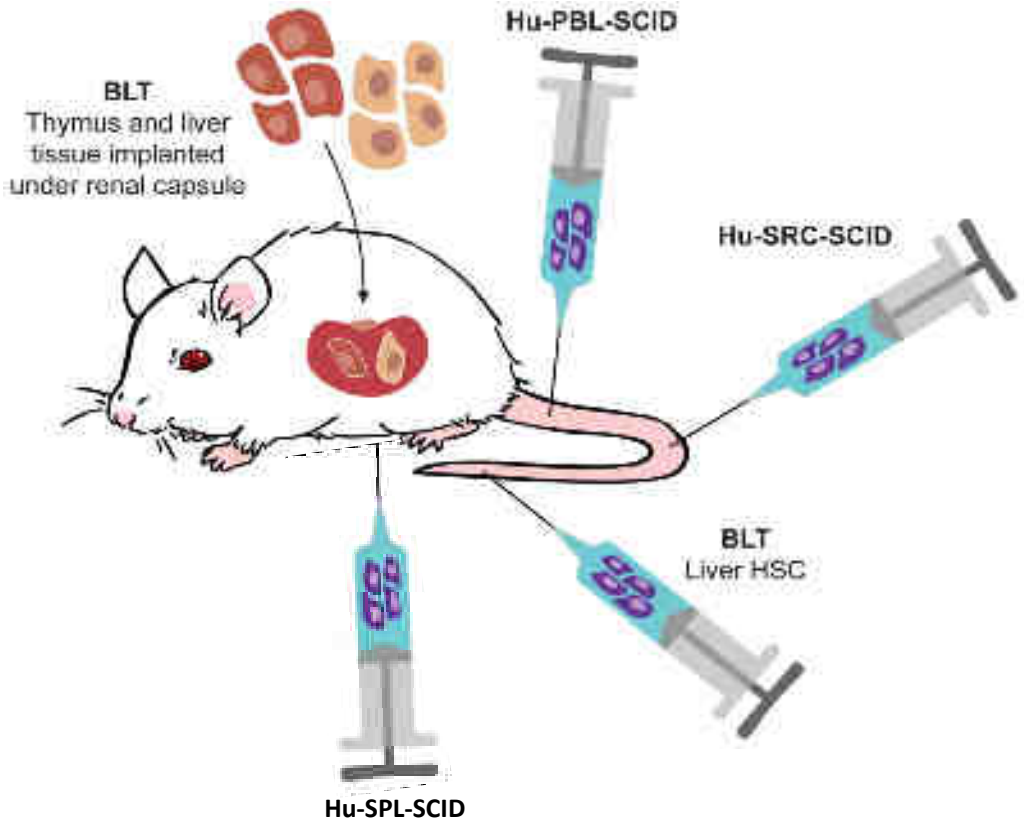

Figure 25: Models for engraftment of human immune systems into SCID mice *(Walsh et al.,2017)* ***(edited).*** *Immunodeficient mice support the engraftment and propagation of human immune cells of multiple sources. They can be reconstituted with human Peripheral Blood Mononuclear cells (PBMC), with splenocytes (SPL) or with Hematopoietic Stem Cells (HSC). The resulting humanized mice are respectively designated as Hu-PBL-SCID, Hu-SPL-SCID and Hu-SRC-SCID. When they are implanted with fetal liver and thymus fragments, they are termed BLT mice. Multiple injection routes have been reported, such as the intrahepatic, intracardiac, intravenous or intraperitoneal routes. The routes represented in this figure are indicative. BLT: Bone marrow Thymus Liver, Hu: Humanized, PBL: Peripheral Blood Lymphocytes, SRC: SCID Reconstituting Cells, HSC: Hematopoietic Stem Cells, SPL: Splenocytes*

1. Development and evolution of the concept: from mouse immunodeficiency to a humanized immune system

1.1. Evolution of immunodeficient mice: the first step towards a humanized model

The first key to a successful humanized mouse model is the receptivity of the host to the xenograft, which is largely dependent on the extent of immunodeficiency. Numerous trials of genetic modifications of normal mouse strains have resulted, according to Shultz *et al* (Shultz

et al., 2007), in three main breakthroughs that have revolutionized the field of humanized mouse models (table 4).

Table 4: Humanized immunodeficient mouse models (edited) *(Zhou et al., 2014)*

	Strain name	Mutated gene	Advantage	Disadvantage
The 1st immunodeficient mice	Nude	Foxn1^{nu}	No T cells	NK activity high, very low engraftment of human cells
1st breakthrough	SCID	Prkdc^{scid}	No functional T and B cells	NK activity high, low engraftment of human cells
2nd breakthrough	NOD/SCID	Prkdc^{scid}	No functional T and B cells, lowered NK level, promoted engraftment of human cells and tissues	Short lifespan, NK activity still present
3rd breakthrough	NSG	Prkdc^{scid} Il2rg^{tm1Wjl}	No functional T and B cells, no NK cells, long lifespan, high engraftment of human cells and tissues	No human MHC, no human cytokines
	NOG	Prkdc^{scid} Il2rg^{tm1Sug}		
	NRG	Rag2^{tm1Fwa} Il2rg^{tm1Sug}		

SCID: Severe Combined Immunodeficiency, NOD: Non-Obese Diabetic, NSG: NOD-SCID-Gamma null, NRG: NOD-RAG null- Gamma null, NK: Natural Killer, MHC: Major Histocompatibility Complex.

After the nude athymic mouse that lacked functional T cells (Dwyer *et al.*, 1971; Wortis, 1971; Wortis *et al.*, 1971), the first achievement in this field was the identification of an immunodeficient mouse strain that carries a mutation in the protein kinase, DNA activated, catalytic polypeptide (Prkdc) (Bosma *et al.*, 1983) gene. The enzyme is involved in the V(D)J recombination of the BCR and TCR genes. The mutated gene induces an arrest in B and T lymphocyte development, leading to a severe combined immunodeficiency (SCID) phenotype that results in acceptance of xenografts of human cells (Mosier *et al.*, 1988). Later, mutations were induced in the Recombination-Activating Genes 1 and 2 (RAG-1 and RAG-2) that have a similar role in V(D)J recombination and result in a comparable phenotype (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). SCID and RAG^{-} mice have however high levels of NK cells and exhibit poor human cell engraftment rates.

The second breakthrough came along in 1995 when Non Obese Diabetic (NOD) mice were backcrossed with the SCID mice, resulting in improved engraftment rates. This effect was

partially due to the decreased NK cell activity exhibited by NOD-SCID mice, but also to additional defects in complement, DCs and macrophages (Shultz *et al.*, 1995). However, the engraftment rate was still less than optimal.

The third breakthrough was the targeted mutation of the gene encoding the IL-2 receptor γ chain (IL-2rγ-/-). This hallmark greatly improved the humanized mouse model, driving a very rapid development of this field. Indeed, since the IL-2rγ chain is shared with the receptors for IL- 4, 7, 9, 15 and 21 (Nakajima *et al.*, 1997) (figure 26), the mutated chain results in a severe deficiency in T, B (figure 27) and NK cells, in addition to DCs and neutrophils. As one would expect, these mice showed improved engraftment and functionality of human cells, especially of T cells, as compared to the previous immunodeficient models (Ito *et al.*, 2012; Lepus *et al.*, 2009).

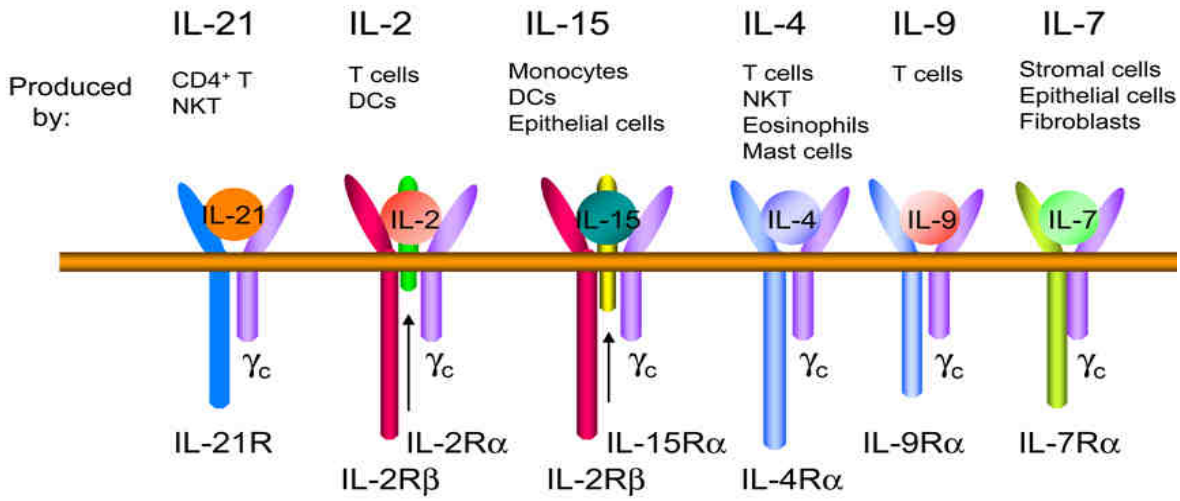

Figure 26: Members of the cytokine-receptor family bearing the common γchain. *There are three classes of IL-2 receptors, binding IL-2 with low affinity (IL-2Rα alone), intermediate affinity (IL-2Rβ + γc), and high affinity (IL-2Rα + IL-2Rβ + γc); only the high affinity IL-2 receptor is shown in the figure (edited) (Rochman et al., 2009).*

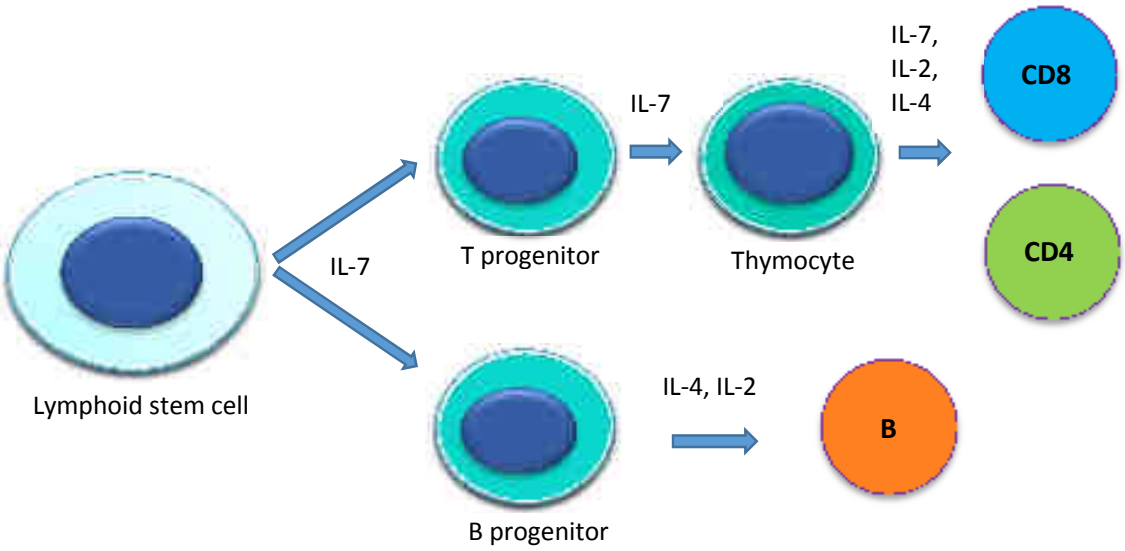

Figure 27: Role of IL-7, IL-2 and IL-4 in the generation of T and B cells. *IL-7 deficiency impairs the early phases of T and B cell development, by inhibiting B and T cell progenitor differentiation. In addition to IL-7, IL-2 and IL-4 are crucial for the generation of circulating B and T cells.*

1.2. Engraftment of a functional immune system: from immunodeficiency to humanization

The second key to the generation of a successfully humanized mouse, designated as Hu-SCID, is the ability of the implanted immune cells to engraft in homing sites, to develop and to remain functional in their exotic microenvironment. Therefore, the choice of the human immune cell source has a great impact.

1.2.1. The Hu-PBL-SCID model

In this model, the implanted immune cells are the mononuclear cell fraction of the peripheral blood (PBMC). Because of its numerous practical advantages, the Hu-PBL-SCID model is the most widely used model for the assessment of human immune function and testing of vaccine efficacy in humanized mice. Indeed, this source of human cells is relatively easy to secure from blood donation byproducts, and abides to fewer ethical or legal restrictions than other sources. However, the Hu-PBL-SCID model results in a poor engraftment of B cells as compared to that of T cells, (Bazin *et al.*, 1996; Wagar *et al.*, 2000). Moreover, one of the important pitfalls of this model is that the engrafted human $CD4^+$ T cells are mostly reactive against MHC molecules expressed on murine cells. Therefore, the engrafted lymphocytes attack the host

cells, causing a strong Graft Versus Host Disease (GVHD) (King *et al.*, 2009; Tary-Lehmann *et al.*, 1994). Using this model, we have noticed premature death of about 40 % of the engrafted mice (personal non-published data).

1.2.2. The Hu-SRC-SCID model

In the Hu-SRC-SCID model, immunodeficient mice are injected with hematopoietic stem cells (HSC). Therefore, hematopoiesis occurs in the mouse, repopulating it for a long term with multiple cell lineages, including B cells, T cells, T regs and DCs (Ishikawa *et al.*, 2005; Shultz *et al.*, 2007).

Hu-HSC-SCID mice respond to antigen challenge by IgM and IgG pecific antibody production. However, their T cell responses are low. Indeed, during their development, T cells undergo a positive selection step in the thymus, where only T cells recognizing MHC molecules expressed on thymic cells continue their development. In the Hu-SRC-SCID mouse model, the thymic selection of human T cells occurs in the mouse thymus, resulting in T lymphocytes that recognize the murine, but not the human MHC. This defect strongly impairs the activation of these murine MHC-restricted T cells by human DCs expressing human MHC molecules (Ishikawa *et al.*, 2005; Shultz *et al.*, 2007; Traggiai *et al.*, 2004).

To improve the T cell thymic selection and generate T cells that are restricted to human MHC, a human thymus can be engrafted along with the HSC (Lockridge *et al.*, 2013). Another alternative consists in genetic modifications of the NSG mice models to express human MHC (Danner *et al.*, 2011; Marron *et al.*, 2002; Suzuki *et al.*, 2012).

Typically, HSCs can be derived from umbilical cord blood, bone marrow or fetal liver. Granulocyte-Colony Stimulating Factor (G-CSF) can also mobilize them into peripheral adult blood. A comparative study of the engraftment rate of HSCs of several origins showed that, while rates obtained with cells originating from fetal liver and umbilical cord blood are acceptable, those with stem cells mobilized into adult blood are poor (Lepus *et al.*, 2009). Therefore, the Hu-HSC-SCID model is difficult to use for testing vaccine candidates, since the sources of human cells repopulating the mice are not readily accessible.

1.2.3. The BLT mouse model

The NOD/SCID-hu BLT mouse model, abbreviated as BLT (Bone marrow, Liver, Thymus), is generated by the engraftment of human fetal liver and thymus tissues under the renal capsule of NOD-SCID mice, followed by the injection of $CD34^+$ HSCs originating from the same fetal liver. This model shows a good reconstitution of B cells, T cells, DCs and macrophages. Additionally, T cell selection occurs exclusively in the implanted thymus (reviewed by (Cachat *et al.*, 2012; Lan *et al.*, 2006; Shultz *et al.*, 2007)). Therefore, the responses elicited in these mice are restricted to human MHC. BLT mice secrete high IgG titers against T-cell dependent antigens (Melkus *et al.*, 2006). However, this model is laborious and, therefore, less frequently used. It is not suitable for screening large numbers of vaccine candidates.

1.2.4. The Hu-SPL-SCID model

In this model, immunodeficient mice are transplanted by intraperitoneal injection of a suspension of human splenocytes. Similarly to the Hu-PBL-SCID model, the mice are engrafted with phenotypically differentiated cells. The Hu-SPL-SCID model seems to resolve the low engraftment problem previously observed with the Hu-PBL-SCID model. Indeed, in comparison to PBL, SPL showed better homing to the secondary lymphoid tissues, that resulted in an improved engraftment, especially of T cells (Thirdborough *et al.*, 1993). Additionally, implanted splenocytes remained highly functional and were shown to mediate rejection of skin allografts (Alegre *et al.*, 1994). Hu-SPL-SCID mice were capable of mounting an IgG mediated immune response specific to immunizing proteins, such as the F protein of the Respiratory Syncitial Virus (Chamat *et al.*, 1999), the horse ferritin (Brams *et al.*, 1998) and the Merozoite-surface-protein-3 of *Plasmodium falciparum* (Bouharoun-Tayoun *et al.*, 2004). Finally, this model does not imply remarkable GVHD reactions.

Reconstituting immunodeficient mice with spleen cells presents several advantages over the use of the other sources of human cells cited above. Human spleen fragments may be obtained following clinically indicated splenectomy or from deceased tissue donors. A high number of splenocytes can be isolated from a single spleen specimen. For the purpose of evaluating vaccine candidates, it is therefore possible to test several vaccines with the splenocytes of the same donor as well as the response of several donors to the same vaccine. From the experimental point of view, the model is easy to establish by simple injection of cell

suspensions in the peritoneal cavity and does not require surgical interventions. Finally, from the regulatory point of view, it does not imply ethical and legal restrictions as stringent as those imposed by the use of fetal liver and thymus tissue.

The different humanized immunodeficient mouse models are compared in Table 5.

Table 5: Comparison of humanized immunodeficient mouse models.

Humanized mouse model	Human immune cells	Advantage	Disadvantage
Hu-PBL-SCID	Peripheral blood mononuclear cells (PBMCs)	- Accessible from blood donation by-products - Few ethical restrictions - Engraftment of T cells	- Poor B cell engraftment - CD4+ T cells are activated against murine cells - Strong Graft Versus Host Disease
Hu- SRC-SCID	Hematopoietic stem cells (HSCs)	- Engraftment of multiple immune cell lineages, including T and B cells - Antibody responses	- Less accessible than PBMCs - Ethical restrictions - T cells recognized antigen presented on mouse MHC
SCID-hu or -BLT	Fetal liver and thymus tissues	- Similar to Hu-SRC-SCID - T cells are restricted to human MHC	- Less accessible than PBMCs and HSCs - Ethical restrictions - Laborious
Hu-SPL-SCID	Splenocytes	- Accessible from surgical waste tissue - Few ethical restrictions - B and T cells engraftment - Antibody responses	- Used by very few laboratories - Les well characterized than the remaining models

Hu: Humanized, PBL: Peripheral Blood Lymphocytes, SCID: Severe Combined Immunodeficiency, PBL: Peripheral Blood Lymphocytes, SRC: SCID Repopulating Cells, BLT: Bone marrow Liver Thymus, SPL: Splenocytes.

In my host laboratory at the Lebanese University, we have optimized the Hu-SPL-SCID model by replacing SCID mice with NSG mice. The Hu-SPL-NSG model was used in this work.

2. Humanized mice in cancer vaccine research

In *vivo* evaluation of cancer vaccine candidates implies the investigation of their potential to elicit immune responses of the desired profile, capable of inhibiting tumor growth. Because of the intrinsic genetic differences between conventional animal models used in preclinical trials and humans, immune responses observed in animals following vaccine administration may be poorly predictive of those developed by the patients. Mice with a humanized immune system

are expected to be more valuable models in assessing the validity of vaccine candidates before initiating costly clinical trials.

In cancer vaccine research, the humanized mouse model should fill three requirements. Firstly, it should mimic the human humoral and cellular systemic and local immune responses to vaccine administration. Secondly, the mice should accept adoptive transfer of human tumor cells or tissues and exhibit a microenvironment that resembles the one observed naturally in human patients. Thirdly, the model should permit the evaluation of the capacity to the immune response elicited by the vaccine to fight tumor establishment or reduce its growth.

2.1. Humanized mice for preclinical testing of vaccines immunogenicity

Humanized mice have been extensively tested as models for different human infectious diseases such a HIV (Tager *et al.*, 2013) and Influenza virus (Yu *et al.*, 2008). For example, when NOD-SCID-Jak3-/- mice, exhibiting a phenotype similar to NSG mice, received the H3N2 inactivated influenza virus vaccine, they were found to produce specific antibodies of similar affinity to those produced by vaccinated human volunteers. Moreover, the predictive potential of these humanized mice was verified when they failed to mount an immune response to an experimental H7N9 vaccine, thus reproducing results previously observed in clinical trials. Interestingly, the H7N9 vaccine had been immunogenic in BALB/c mice (Wada *et al.*, 2017). In another example, humanized BLT mice inoculated with the HIV virus were shown to mount HIV-specific T $CD8^+$ cellular immune responses that accurately resemble those of human patients during acute infection (Dudek and Allen, 2013).

Fewer attempts have been made to evaluate the immunogenicity of cancer vaccines in humanized mouse models. In a recent report, Kametani *et al.* used PBMC-NOG-hIL-4-Tg mice. These are NOG mice transgenic for human IL-4 and reconstituted with human PBMC. Animals received a tumor vaccine consisting of a peptide derived from the ErbB2/Her2/Neu tumor antigen adjuvanted with Freund's adjuvant. They developed a humoral response characterized by high titers of human IgG antibodies (Kametani *et al.*, 2017). Potent cellular and cytotoxic responses against cancer were also induced in humanized mouse models. For example, in a study conducted by Spranger *et al.* in 2012, DC cells differentiated from peripheral blood monocytes and transfected to encode the melanoma antigen MART-1 were shown to induce an *ex vivo* cytotoxic activity against MART-expressing cells (Spranger *et al.*, 2012).

2.2. Humanized mice in cancer vaccine research: evaluation of protection

2.2.1. Humanized SCID mice can accept adoptive transfer of tumor xenografts and reconstitute their natural microenvironment

The profound immunodeficiency of the SCID mice and their derivatives was found to facilitate the engraftment of a large panel of human primary tumors and cell lines and to reconstitute the tumor microenvironment (reviewed by (Ito *et al.*, 2012; Zhou *et al.*, 2014)).

Hu-PBL-SCID mice carrying human bladder cancer xenografts were shown to undergo a successful reconstitution with human lymphocytes, to secrete human immunoglobulins and to support tumor infiltration with T cells (Gong *et al.*, 2015). Comparable results were obtained in a model of cervical cancer carcinoma where the slowly growing tumors were found to be infiltrated with lymphocytes (Ye *et al.*, 2006). In a Hu-SRC-SCID model of breast cancer, concurrent transplantation of human HSCs and breast cancer cells resulted both in the engraftment of a functional immune system and in tumor development and dissemination. The tumor was infiltrated with activated specific T-cells and NK cells (Wege *et al.*, 2011).

2.2.2. Humanized SCID mice as platforms for cancer vaccine evaluation

The established capacity of humanized mouse models to mimic the human immune response and the tumor microenvironment prompted their use for the development of cancer immunotherapies (Kozlowska *et al.*, 2016; Siegler *et al.*, 2005; Trieu *et al.*, 2004) and especially cancer vaccines.

Among the multiple examples that illustrate their potential in this field, we cite a model of colorectal carcinoma model in SCID/Beige nude mice, completely lacking NK cells that were engrafted with HSCs. These mice were vaccinated with autologous *in vitro*-generated DCs, transfected to express a co-stimulatory molecule, the CD137 ligand (CD137L), and pulsed with tumor-cell antigens (Fu *et al.*, 2017). CD137 is expressed on activated T cells, and the inducible expression of its ligands is currently one of the targets of cancer immunotherapies (Vinay and Kwon, 2014). This vaccination protocol was found to protect Hu-SRC-SCID mice from tumor development. Indeed, mice showed a delayed tumor growth and a decreased tumor volume and weight, as compared to non-vaccinated Hu-SRC-SCID mice, indicating that tumor rejection was exclusively due to the vaccine, and not to HLA-mismatch between tumor cells and cancer cells (Fu *et al.*, 2017).

In another cancer vaccine trial, humanized mice were injected with MHC-matched melanoma cells. They subsequently received an engineered oncolytic adenovirus encoding for GM-CSF, on which MHC class I peptides of the MAGE-A1 melanoma antigen were adsorbed. The vaccine was shown to eradicate the established tumors and to induce tumor-specific $CD8^+$ T cells (Capasso *et al.*, 2016).

Humanized mouse models also served as platforms for a limited number of protein/peptide based vaccine evaluation studies. In one of these studies, Hu-PBL-SCID mice were vaccinated with a recombinant protein consisting of the two melanoma antigens MAGE-A1 and MAGE-A2, in addition to the recombinant Mycobacterium tuberculosis heat shock protein 70 (TBHSP70) that served as a danger molecules. This protocol was shown to induce vaccine specific lymphocytes, among which CTLs mediate the lysis of MAGE-expressing cancer cells *in vitro*. In a prophylactic vaccination setting, Hu-PBL-SCID mice immunized with the MAGE-A1/MAGE-A2/TBHSP70 showed slower tumor growth and higher survival rates as compared to control mice (Junwei *et al.*, 2016).

Altogether, the growing evidence of the capacity of the humanized mouse models to mimic the human adaptive immune response argues for their use in vaccine development. In particular, their capacity to induce $CD8^+$ T cell responses against tumor antigens on one hand, and to engraft and reconstitute tumor microenvironment on the other hand, strongly suggests them as platforms for the evaluation of cancer vaccine immunogenicity and efficacy.

OBJECTIVES

Despite their improved efficacy in recent years, the majority of currently used cancer treatments remain poorly specific for tumor cells and induce major side effects. For decades, data has accumulated providing proof that tumors are immunogenic and capable of triggering immune responses. These responses may, under certain circumstances, be able to control them, but tumors usually escape destruction by undergoing genetic and epigenetic modifications, inducing tolerization and immune response inhibition. Therefore, the idea of developing therapeutic cancer vaccines that would reverse this context and initiate/amplify the immune response against tumors is gaining increased attention. Translation of this concept into a reality is rapidly evolving thanks to the recent discoveries in the field of cancer immunobiology. It is today established that tumors display TAAs that may be presented by DCs and recognized by $CD4^+$ and $CD8^+$ T cells. However, they usually do not activate an efficient tumor-specific immune response because the tumor microenvironment is generally immunosuppressive. A cancer vaccine should therefore include all the elements needed to target and activate the DCs and generate tumor antigen presentation in an immunostimulatory context. Ultimately, it should lead to the production of specific Th1 cells to induce differentiation of CTLs that are capable of infiltrating the tumor and killing cancer cells.

Currently, various clinical trials employing multiple strategies in the design and delivery of cancer vaccines are ongoing and showing promising results. Extensive optimization efforts are made to replace conventional approaches relying on the use of whole TAA proteins and conventional adjuvants by innovative ones using minimal peptide sequences and small, less reactogenic, stimulatory molecules derived from bacteria. These elements are generally combined altogether in various vectors such as nanoparticles. Liposomes emerged as interesting vectors for such vaccines. Indeed, they are biocompatible and their composition, size, charge and surface functionalization can be easily manipulated, thereby offering a wide range of potential strategies to optimize cancer vaccine delivery.

Regarding the vaccine delivery route, special attention has been recently devoted to the skin. Being an immunological barrier that normally protects the organism from external aggressions, the skin is rich in multiple DC subpopulations. The conventional subcutaneous route, which has proven its efficacy in microbial vaccination, is currently being explored in cancer vaccination. However, this route does not optimally deliver the vaccine to skin DCs

since the hypodermis is devoid of DCs, and leads to rapid vaccine draining to the lymph nodes. A more efficient approach to target skin DCs may be to apply the vaccine on the skin surface to reach the epidermis and the dermis. However, the skin is also a mechanical barrier with an impermeable uppermost layer, the *stratum corneum*. Therefore, innovative solutions to cross this barrier are needed. Fortunately, one of the advantages of liposomes is that they are adapted to multiple delivery routes, including the transcutaneous one. Moreover, their penetration in the skin can be further increased through modification of their physicochemical properties.

In my host laboratory at the University of Strasbourg, liposomal constructs incorporating the necessary elements for the induction of an efficient antitumor immune response were previously developed. They incorporated a TLR 2/6 agonist (Pam_2CAG) as immunostimulatory molecule, a $CD8^+$ T cell epitope peptide derived from a TAA expressed of cancer cells, as well as a universal $CD4^+$ T cell epitope peptide derived from the influenza virus hemagglutinin protein. The immunogenicity and tumor-specific efficiency of these liposomes were established following administration by the subcutaneous and the intranasal routes in tumor-bearing conventional mice (Kakhi *et al.*, 2015, 2016; Roth *et al.*, 2005; Thomann *et al.*, 2011).

In parallel, in the vaccine development field, it is not infrequent that vaccine candidates that are originally selected and optimized based on preclinical experimentation in rodents yield disappointing results in clinical trials. Severely immunodeficient mice reconstituted with human immune cells offer the possibility to explore the human immune response to various antigens. They may therefore be promising models to solve this issue, by allowing the *in vivo* evaluation of vaccine candidates destined for humans.

In my host laboratory in the Lebanese University, a humanized mouse model was previously developed, in which immunodeficient mice are reconstituted with human splenocytes (Hu-SPL-SCID) (Bouharoun-Tayoun *et al.*, 2004; Chamat *et al.*, 1999). We have since adapted this model to the NSG mouse. The Hu-SPL-NSG model proved to be superior to more conventional models relying on the use of peripheral blood cells, yielding more potent primary human immune responses against a variety of antigens, including the hepatitis B vaccine and experimental vaccine candidates against *Plasmodium falciparum*. However, these antigens were adjuvanted with conventional potent adjuvants (Ghosn, 2015).

In this context, the objective of my thesis project consisted in developing liposome-based vaccines for transcutaneous cancer vaccination in humans. Therefore, we aimed at optimizing a liposome vaccine that would induce a potent immune response after TC administration to a conventional mouse model. Additionally, we aimed to verify that the liposome constructs can induce an immune response by human immune cells in vivo, using a humanized mouse model. To this end, the experimental work was divided into two parts, the results of which will will be presented in two chapters, each corresponding to a scientific article in preparation that answers one of these two general objectives.

The specific objectives are presented in detail below.

Objective 1: Development of liposome constructs adapted for TC cancer vaccination and evaluation of their immune potential

The first specific objective of my thesis project consisted in developing and validating liposome-based vaccines suitable for transcutaneous cancer vaccination. Specifically, we aimed to refine the liposome-based vaccine composition previously validated in the BALB/c model and to identify a formulation that induces a potent $CD4^+$ and $CD8^+$ T cell immune response after TC administration in mouse models. The results of this part are presented in the **scientific article #1, in preparation.**

1.1 ***Optimization of the composition of the liposomal constructs for TC immunization:*** Based on the original liposomal vaccine developed in our team (Thomann *et al.*, 2011), we formulated new vaccine candidates for the TC route by modifying three elements:

- **The immunostimulatory molecule:** In order to choose the most optimal adjuvant for the induction of vaccine-specific responses by the TC route, we replaced the original immunostimulatory molecule, which was a TLR 2/6 agonist (Pam_2CAG) by a TLR4 agonist (MPLA).
- **The construct targeting to DCs:** a bivalent mannose residue was previously developed in our laboratory (Espuelas *et al.*, 2003), and liposomal vaccines incorporating this residue were shown to exhibit improved uptake by DCs (Espuelas *et al.*, 2008), permitting a reduction in the minimal dose of immunostimulatory molecule required

to induce an immune response (Thomann *et al.*, 2011). Therefore, mannose residues addition was tested in the constructs designated for TC vaccination.

- **The flexibility/deformability of the lipid vesicle:** The above mentioned elements were incorporated in two types of vesicles, either conventional liposomes, or ultradeformable liposomes or transfersomes^{TM} which are assumed to provide better penetration through the skin, to test whether the latter would induce improved immune responses.

We also formulated fluorescent liposomes incorporating a hydrophobic fluorochrome in their lipid bilayer. This strategy offers the opportunity to track the cells that internalize the liposomes, including skin DCs, and assess their migration to draining lymph nodes following TC application.

The formulated constructs had to fulfill the following requirements:

1- **Lipid vesicle size:**
   a. Average diameter: we aimed to obtain lipid vesicles with an average diameter of 100 nm, because in this size range various nanoparticles were reported to be suitable for skin barrier crossing (Lilia Romero and Morilla, 2011; Rattanapak *et al.*, 2012)
   b. Size distribution/homogeneity: we aimed to formulate liposomes with a narrow size distribution and with a polydispersity index (PDI) < 0.3.

2- **Peptide coupling rate:** the peptide coupling rate to the preformed liposomes incorporating a functionalized anchor had to be highly efficient in order to minimize peptide loss.

3- **Formulation technique:** the reproducibility of the formulation technique was essential to obtain homogenous formulations during the whole process. This allows us to avoid the variations of the *in vivo* results that may be caused by a variation of the vaccine formulation itself.

1.2 Evaluation of the local and systemic immune response induced by the constructs

In a first step, the original construct was evaluated by the SC and the TC routes to provide a proof of concept of their immunogenicity after TC administration on one hand, and to

compare the immune response induced by the two administration routes on the other hand.

We next compared the local and systemic immune responses induced by liposomes incorporating the two different TLR agonists.

We finally investigated the influence of the lipid vesicle fluidity on the immune response induced by the TC route.

1.3 Assessment of the migration of skin DCs after TC immunization

Because the liposomal constructs were shown to induce an immune response after TC administration, we assessed their capability to induce the migration of skin DCs to lymph nodes draining the application zone.

Objective 2: Evaluation of liposomes immunogenicity in the Hu-SPL-NSG model

The second specific objective of my thesis was therefore to determine whether liposome constructs that were previously validated in murine models can induce detectable human immune responses in the Hu-SPL-NSG model. The results of this part are presented in the scientific **article #2, in preparation.**

To our knowledge, there are very few reports in the literature regarding administration of liposome-based formulations to humanized mice and evaluation of their immunogenicity in this model. Therefore, before attempting to test the capacity of TAA-expressing liposomes to induce a tumor-specific CTL response in the Hu-SPL-NSG mouse model, several preliminary steps were needed to establish the proof of concept of this approach. To this end we used several alternative liposome-based vaccine formulations.

2.1. Assessment of the safety of the TLR agonists towards human splenocytes

In a first step, it was primordial to assess the safety of liposomes incorporating different TLR agonists towards human cells. While their immunostimulatory effect is essential for activating immune responses, excessive stimulation may lead to cell death or alter the capacity of human cells to engraft in the NSG mouse.

Therefore, we first performed in vitro evaluation assays of liposomes incorporating different TLR agonists. Then we completed the selected liposome by adding the vaccine epitopes and tested it in vivo. We evaluated its effect on mice survival and on the homing of the injected splenocytes to the mouse spleen and their capacity to secrete total human immunoglobulins.

2.1. Evaluation of the liposomes capacity to induce an immune response in the Hu-SPL-NSG mouse model

We next aimed to determine whether the liposomal constructs can induce a detectable human immune response in the Hu-SPL-NSG model. This approach faces several challenges. For instance, the ability of the Hu-SPL-NSG mouse to mount cellular responses is far less well established than that to mount humoral responses (Bouharoun-Tayoun *et al.*, 2004; Brams *et al.*, 1998; Chamat *et al.*, 1999). Moreover, cellular immune responses can be evaluated in secondary lymphoid organs only after animal euthanasia, while humoral responses can be assessed regularly in mice sera in the ongoing experiment. For all these reasons, we formulated liposomal constructs incorporating, in addition to the HA peptide and the selected TLR agonist, a B cell epitope rather than a T cell epitope. The B epitope is derived from *Pseudomonas aeruginosa*, and has been previously evaluated in liposomal constructs administered to immunocompetent mice by the intraperitoneal and the intranasal routes (Heurtault *et al.*, 2009). The humoral immune response was assessed by searching for anti-PAK antibodies at several time points after immunization. The $CD4^+$ T cell response was evaluated *in vitro* in the mice spleen pools after sacrifice by detecting the production of IFN-γ, IL-4 and IL-10 by ELISPOT.

EXPERIMENTAL RESULTS

Chapter 1: Transcutaneous immunization with liposome-based cancer vaccines induce CD4+ and CD8+ T cell responses in BALB/c mice

My host team in the University of Strasbourg has developed innovative liposome-based vaccines that co-deliver the crucial elements for a tumor-specific immune response. The chosen liposomes are conventional ones, composed of phosphatidylcholine, phosphatydilglycerol and cholesterol, the main constituents of cell membranes, and, therefore, are tolerated by the immune system. The elements delivered by these liposomes are namely a $CD8^+$ T cell peptide epitope derived from the human TAA ErbB2, a universal $CD4^+$ T cell peptide epitope derived from the hemagglutinin of the influenza virus, in addition to an immunostimulatory molecule, a TLR2/6 agonist (Pam_2CAG). In conventional mouse models, these constructs were shown to induce efficient anti-tumor immune responses after subcutaneous (Roth et al., 2005; Thomann et al., 2011) and intranasal (Kakhi et al., 2015, 2016) administration in normal mice.

When I first joined the team, one previous attempt had been performed to deliver the liposome-based constructs by the TC route and seemed to give promising results. In this experiment, the mice were shaved on their back and were allowed to rest for 24h before applying the formulation by massage. Therefore, we decided to adopt this same vaccination strategy. As a preliminary step, we optimized the vaccine penetration through the skin by dividing the vaccine dose into three subsequent sub-doses, and by massaging every sub-dose for one minute. Then, to improve the immunogenicity of the liposomal formulations by the TC route, we decided to benefit from their versatility by modifying their composition and their physicochemical properties.

Because the immunostimulatory molecule plays a central role in the induction of an immune response, we replaced the TLR2/6 agonist that was incorporated in the original formulation, with a TLR 4 agonist (MPLA), and compared the resulting constructs. Additionally, in order to allow a more efficient delivery of the vaccine components to the DC, we attempted to target the liposome constructs to the skin DCs by incorporating a DC-targeting molecule, di-mannose. Because the ability of the liposomes to cross the cutaneous barrier depends on the fluidity of

their lipid bilayer, we replaced the conventional liposome vesicle with an ultradeformable one, called transfersome, in order to optimize the skin crossing ability of our formulations. Finally, to evaluate the local and systemic immune response induced by these constructs variants, we tested them in a conventional mouse model. The corresponding results are presented in the following **scientific article # 1** (in preparation).

Scientific article #1:

Transcutaneous immunization with liposome-based cancer vaccines induce $CD4^+$ and $CD8^+$ T cell responses in BALB/c mice

Hanadi Saliba, Vincent Flacher, Célia Jacoberger, Yohan Gerber, Maroua Messous, Hasnaa Bouharoun-Tayoun, Benoît Frisch, Soulaima Chamat, Béatrice Heurtault, Sylvie Fournel

In preparation

Introduction

The recent years have witnessed major research advances in therapeutic anti-tumor vaccination, which is intended to induce and amplify the physiological immune responses against cancer (reviewed by (Butterfield, 2015; Guo *et al.*, 2015)), (Adams *et al.*, 2008; Berinstein *et al.*, 2015; Chen *et al.*, 2015; Dhodapkar *et al.*, 2014; Fujiyama *et al.*, 2014). These advances were made possible by the identification of tumor-associated antigens (TAA) against which an immune response may be induced, leading to the destruction of TAA-expressing tumor cells (Baldwin, 1971; van der Bruggen *et al.*, 1991; Van den Eynde and van der Bruggen, 1997; Vigneron, 2015). They have also highlighted the pivotal role played by dendritic cells (DCs) in eliciting such protective immune responses (Palucka and Banchereau, 2013).

Tumor-specific responses are induced when dying cancer cells release exosomes and/or apoptotic bodies containing TAAs and molecules that indicate cell damage and therefore play the role of danger signals (Dhodapkar *et al.*, 2007; Pathak *et al.*, 2012; Tang *et al.*, 2012). Following TAA internalization by DCs, their processing leads to cross-presentation of derived peptides on MHC class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively (Alloatti *et al.*, 2016; Joffre *et al.*, 2012). Following recognition of TAA-derived epitope peptides by their specific antigen receptors, $CD4^+$ and $CD8^+$ T cells differentiate into tumor specific helper (Th) and cytotoxic (CTL) effector cells, respectively. The outcome of this differentiation depends on the maturation state of the DC that is induced by danger signals provided by Microbe-Associated Molecular Patterns (MAMPs) in infectious context or by Damage-Associated Molecular Patterns (DAMPs) in non-infectious context such as cancer. These molecules engage specific receptors on the DC surface, named PRR for Pattern Recognition Receptors (PRRs), such as Toll-Like-Receptors (TLRs (Dudek *et al.*, 2013; Pathak *et al.*, 2012). Beside danger signals, optimal differentiation of CTLs needs complementary signals provided by Th cells, especially IFNγ producing Th1 cells, after recognition of their specific antigen presented by the same DC that is presenting the antigen to the CTL. Following recognition of TAA-derived peptides on the cancer cells surface, CTLs mediate their apoptosis. Therefore, to induce a cancer-specific response, a cancer vaccine should incorporate three elements, namely a i) $CD4^+$ T cell epitope, a ii) $CD8^+$ T cell epitope derived from the TAA, both necessary for the activation of cancer-specific T cells, and iii) a danger signal that induces DC maturation.

One of the most extensively used vaccination sites is the skin, and more specifically through the subcutaneous route. Recently, the transcutaneous (TC) route has emerged as a tempting alternative (DeMuth *et al.*, 2013; Glenn *et al.*, 2000; Kim and Prausnitz, 2011). Indeed, while the hypodermis is virtually devoid of DCs, the epidermis and the dermis harbor several DC populations. The epidermal Langerhans cells (LCs), characterized by their high expression of langerin/CD207, and the dermal dendritic cells (dDCs), comprised of different subpopulations that can express or not langerin/CD207, normally ensure skin immunosurveillance (Malissen *et al.*, 2014; Merad *et al.*, 2002, 2008). After sampling the constituents of their environment, activated skin DCs that sense danger signals migrate to the skin draining lymph nodes where they activate lymphocytes to mount an adaptive immune response (Worbs *et al.*, 2017). Upon activated T cell recirculation, an adaptive immune response is elicited locally and systemically (Hopkins and McConnell, 1984; Masopust and Schenkel, 2013; Thomas *et al.*, 2012). However, for a vaccine to be efficient by transcutaneous immunization (TCI), it must efficiently cross the *stratum corneum,* which is the impermeable uppermost layer of the epidermis (Marks, 2004).

One of the advances in vaccine technology resulted in the emergence of innovative liposome-based formulations (Bangham and Horne, 1964; Bangham *et al.*, 1974) that are suitable for skin barrier crossing (Ashtikar *et al.*, 2016; Hansen and Lehr, 2012) and can deliver all the minimum vaccine key elements to the skin DCs (Chikh and Schutze-Redelmeier, 2002; Iwama *et al.*, 2016; Roth *et al.*, 2005; Shariat *et al.*, 2014; Thomann *et al.*, 2011). Liposomes are versatile phospholipid vesicles. It is possible to modulate each of their physicochemical characteristics to adapt their use for different applications. In particular, it is possible to increase their fluidity to optimize them for TC delivery. Liposomes with ultradeformable bilayers, called transfersomes^{TM}, are reported to exhibit increased passage capacities through mildly disrupted skin barriers, by squeezing into pores smaller than their size (Ascenso *et al.*, 2015; Benson, 2006; Cevc *et al.*, 1998)

Our team has previously developed several liposomal formulations that co-deliver a $CD4^+$ T cell epitope derived from the hemagglutinin of the influenza virus (HA 307–319, PKYVKQNTLKLAT-C) (O'Sullivan *et al.*, 1991) and a $CD8^+$ T cell epitope derived from the human TAA ErbB2 (p63–71, CG-TYLPTNASL) (Nagata *et al.*, 1997), in addition to TLR agonists that functions as danger signal (Roth *et al.*, 2005; Thomann *et al.*, 2011). Different TLR agonists have been tested, including S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteinyl-alanyl-glycine

(Pam_2CAG), a diacetylated lipopeptide derived from the N-terminal moiety of *E. coli* lipoprotein that interacts with TLR 2/6 heterodimers (Oliveira-Nascimento *et al.*, 2012; Omueti *et al.*, 2005). When incorporated into liposomal constructs, this TLR agonist was found to induce the maturation of human monocyte-derived DCs *in vitro*, as indicated by the expression of CD80, CD83, CD86 and HLA-DR molecules (Espuelas *et al.*, 2005a). Additionally, the above-mentioned liposomes incorporating the T $CD4^+$ and T CD8+ epitopes trigger protective tumor-specific responses *in vivo* (Heurtault *et al.*, 2009; Thomann *et al.*, 2011). GlaxoSmithKline has recently designed adjuvant systems consisting of liposomes incorporating monophosphoryl lipid A (MPLA), a TLR4 agonist (Alving and Rao, 2008; Alving *et al.*, 2012a). MPLA is a chemically detoxified form of the lipid A, the anchor moiety of lipopolysaccharides (LPS) (a highly immunostimulatory structure found on the outer cell surface of Gram negative bacteria). These adjuvant systems are currently used in multiple vaccines, especially those where CTL responses are needed (Alving *et al.*, 2012b).

Addition of targeting molecules such as mannose residues to the liposomes can facilitate their uptake into the DC through interaction with mannose-specific receptors (East and Isacke, 2002; Espuelas *et al.*, 2008; Markov *et al.*, 2015). Our team has reported that, when adding di-mannose residues to liposome surface, it is possible to reduce the amount of incorporated TLR agonist without losing their immune-activating potential (Thomann *et al.*, 2011).

In the present work, our aim was to optimize liposomal constructs previously developed in our laboratory for TC cancer vaccination. We formulated several variants that differ for the TLR agonist, the presence of a DC targeting molecule and the lipid vesicle composition. We evaluated their immune potential after TC immunization of BALB/c mice by testing their capacity to elicit an HA-specific $CD4^+$ and ErbB2-specific $CD8^+$ T cell response. Moreover, we tested the ability of the liposomal constructs to induce skin DC migration to draining lymph nodes.

Material and methods

1. Formulation and characterization of liposomal constructs

1.1. Lipids and adjuvants

Egg yolk L-α-phosphatidylcholine (PC), soy PC (SPC) and cholesterol (Chol, recristallized in methanol) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France) and their purities exceeded 99%. L-α-phosphatidyl-DL-glycerol transesterified from egg yolk PC (PG) was purchased from Avanti Polar lipids (Alabama, USA). The lipopeptide S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteinyl-alanyl-glycine (Pam_2CAG), the thiol-functionalized lipid anchor dipalmitoylglycerol maleimide (DPGMal) and the mannosylated lipid anchor dioleylglycerol di-mannose (DOG-Man_2) were synthesized at the laboratory as previously described (Espuelas *et al.*, 2003, 2008; Heurtault *et al.*, 2009; Roth *et al.*, 2004). The lipopolysaccharide derivative Monophosphoryl lipid A (MPLA) was purchased from Invivogen (San Diego, CA). All reagents were conserved under argon at -20 °C.

1.2. Synthetic peptides

The peptides ErbB2 p63–71 (CG-TYLPTNASL, MW = 1139 g/mol) (CTL eptitope) (Nagata *et al.*, 1997), and influenza virus haemagglutinin-derived HA 307–319 (PKYVKQNTLKLAT-C, MW = 1606 g/mol) ($TCD4^+$ epitope) (O'Sullivan *et al.*, 1991) were obtained from Genosphere Biotechnologies (Paris, France). Their purity, as assessed by HPLC, was > 85%.

1.3. Formulation of lipid vesicles

1.3.1. Formulation of fluorescent liposomal SUVs (DiI-liposomes)

Multilamellar fluorescent vesicles (DiI-MLV) were prepared by lipid film hydration technique. Briefly, a chloroform/methanol (9/1 v/v) solution containing PC, PG, Chol, adjuvant, DOG-Man_2 and DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) were mixed in a round-bottom Pyrex tube, and slowly evaporated under a continuous flow of argon. The molar proportions of each of the constituents is shown in table 1. The resulting lipid film was completely dried under high vacuum for 1 hour. It was then hydrated in 10 mM Hepes buffer (pH 7.4) containing 5% w/v sorbitol by rigorous vortex mixing, to yield a phospholipid concentration of 15 mM. The resulting DiI-MLV suspension was sonicated (1s cycle every 3 s) for 1 hour at room temperature under a continuous flow of argon, using a Vibra Cell 75041

ultrasonicator (750 W, 20 kHz, Fisher Bioblock Scientific, Illkirch, France) equipped with a 3 mm-diameter tip probe (40% amplitude). The resulting DiI-SUV preparations were centrifuged twice at 10000 g to remove the titanium dust originating from the probes. They were then concentrated to a 30 mM PC concentration. To this end, DiI-SUV were centrifuged at 5000 *g* and 4 °C, in a concentrator equipped with a semi-permeable membrane of 10000 KDa cut-off (Sartorius Stedim Biotech, Aubagne, France). Formulations were snap frozen in liquid nitrogen after addition of a 5% glucose as a cryoprotectant and stored at -80°C until use.

Table 1: Composition of formulated liposomes, fluorescent liposomes and transfersomes

	Formulations	Composition	Molar Proportion
Floures cent	Lp*	PC/PG/Chol/DiI	80/20/50/1
	Lp*Man/MPLA	PC/PG/Chol/DiI/MPLA/DOG-Man_2	77/20/50/1/0.001/3
Liposomal contructs	Lp	PC/PG/Chol	80/20/50
	Lp Pam_2CAG/HA/ErbB2	PC/PG/Chol/DPG-Mal/Pam_2CAG/HA/ErbB2	75/20/50/5/0,2/1.25/1.25
	Lp Pam_2CAG/Man/HA/ErbB2	PC/PG/Chol/DPG-Mal/Pam_2CAG/DOG-Man_2/HA/ErbB2	72/20/50/5/0,2/3/1.25/1.25
	Lp MPLA/HA/ErbB2	PC/PG/Chol/DPG-Mal/MPLA/HA/ErbB2	75/20/50/5/0.001/1.25/1.25
	Lp MPLA/Man/HA/ErbB2	PC/PG/Chol/DPG-Mal/MPLA/DOG-Man_2/HA/ErbB2	75/20/50/5/0.001/3/1.25/1.25
Transfersomal constructs	Tf	SPC/SDC	73/23
	Tf Pam_2CAG/HA/ErbB2	SPC/SDC/DPG-Mal/Pam_2CAG/HA/ErbB2	68/27/5/0,2/1.25/1.25
	Tf Pam_2CAG/Man/HA/ErbB2	SPC/SDC/DPG-Mal/Pam_2CAG/ DOG-Man_2/HA/ErbB2	65/27/5/0.2/3/1.25/1.25
	Tf MPLA/HA/ErbB2	SPC/SDC/DPG-Mal/MPLA/HA/ErbB2	68/27/5/0.001/1.25/1.25
	Tf MPLA/Man/HA/ErbB2	SPC/SDC/DPG-Mal/MPLA/ DOG-Man_2/HA/ErbB2	65/27/5/0.001/3/1.25/1.25

PC: phosphatidylcholine, PG: phosphatidylglycerol, Chol: cholesterol, DiI: 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate, DPG-Mal: dipalmitoyl glycerol-maleimide, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, MPLA: monophosphoryl lipid A, DOG-Man_2: dioleyglycerol-dimannose, SPC: soy phosphatidylcholine, SDC: sodium deoxycholate,

1.3.2. Formulation of liposomal SUV

Liposomes were prepared by mixing in a round-bottom Pyrex tube a chloroform/methanol (9/1 v/v) solution containing PC, PG, Chol, adjuvant, DPG-mal and Man (DOG-Man_2). The next steps are similar to previously described formulation of fluorescent liposomes.

1.3.3. Formulation of ultradeformable liposomes or transfersomes™

Uf-SUVs were prepared as described before by Cevc *et al* (Cevc and Blume, 1992; Cevc *et al.*, 1998). Briefly, appropriate proportions of SPC, DPG-Mal, adjuvant and DOG-Man_2 were dissolved in chloroform/methanol (9/1 v/v). Solvent was evaporated under high vacuum in a Pyrex tube for 1 h. The resulting lipid film was dispersed in ethanol (8.5% (v/v) at a final phospholipid concentration of 15 mM) and mixed vigorously for 2 min. A 10 mM Hepes buffer (pH 6.5) containing 5% w/v sorbitol and 5 mM SDC was then added to the suspension to obtain a final concentration of 4 mM of the surfactant. The mixture was stirred vigorously for 2 min. The resulting MLV suspension was manually extruded (LiposoFast-Basic, Avestin, Canada) for 20 cycles through a 100-nm pore size polycarbonate mesh, in order to obtain Tf-SUV. Formulations were snap frozen in liquid nitrogen after addition of 10% glucose as a cryoprotectant and stored at -80°C until use.

1.4. Peptide conjugation to Lp-SUV or Tf-SUV

Potential disulfide bonds of cysteine residues between peptides that may result in peptide dimerisation, were reduced with 0.7 M eq. tris(2-carboxyethyl) phosphine (Interchim, Montluçon, France), for 15 minutes under argon. Equimolar quantities of $CD4^+$ and $CD8^+$ T cell epitope peptides were then coupled to freshly prepared SUVs by Michael addition in 10 mM Hepes buffer (pH 6.5) containing 5% (w/v) sorbitol (0.5 molar eq of each peptide vs surface accessible thiol-reactive maleimide functions). After an incubation of 3 hr under argon at room temperature, a 10-fold excess of β-mercaptoethanol was added for 30 minutes to inactivate all unreacted maleimide groups on internal or external surface of SUVs. Then, the formulation was extensively dialysed (Spectra/Por, exclusion limit of 12–14 kDa, Spectrum laboratories, DG Breda, Netherlands) against a 10 mM Hepes buffer (pH 7.4) containing 5% (w/v) sorbitol to eliminate unreacted reagents and peptides.

1.5. Physicochemical characterization of the constructs

1.5.1. Nanoparticle size measurement by dynamic light scattering

The average size of formulated Lp-SUVs and Tf-SUVs was measured by dynamic light scattering using a Zetasizer Nano-ZS (Malvern instruments, Orsay, France) with the following

specifications: sampling time, 30 s; viscosity, 1.014 cP; refractive index, 1.34; scattering angle, 90°; temperature, 25°C. SUVs were diluted at 1/100 in 10 mM Hepes buffer (pH 7.4) containing 5% (w/v) sorbitol, and the results were the average of three consecutive measurements. Data were analyzed using the multimodal number distribution software included with the instrument. Particle size is expressed in intensity. Sample are considered monodispersed when the polydispersity index (PDI) is < 0.3.

1.5.2. Phosphatidylcholine content

The PC content of formulated Lp-SUVs and Tf-SUVs was determined using an enzymatic assay with the LabAssay™ Phospholipid kit (Wako Pure Chemical industries Ltd, Richmond, VA). Briefly, 1-2 µL of SUV or Tf preparation were incubated in triplicates in a 96-well plate with 200 µL of the enzymatic reagent. The reagent contains a phospholipase C (0.47 U/mL) that releases the choline, which, by its turn, is oxidized by the choline oxidase. The reaction produces hydrogen peroxide needed by the peroxidase (2.16 U/mL) to convert a chromogen into a blue product. After 10 min at 37 °C, absorbance was measured at 590 nm using a microplate reader (Safas SP2000, Xenius 5801, Monaco). A standard curve of choline chloride served to establish a calibration curve.

1.5.3. Quantification of conjugated peptides

The quantification of conjugated peptides in each formulation was performed after acid hydrolysis using a fluorometric assay with fluorescamine (4-phenyl-spiro [furan-2(3H), 1'-phthalan] -3,3' –dione, Sigma-Aldrich) (Boeckler *et al.*, 1999; Böhlen *et al.*, 1973). Briefly, amino acids were generated after formulation hydrolysis at 110 °C for 12 h in a heating module (Pierce Reacti Therm III™). After neutralization by the addition of sodium hydroxide, 40 µL of the hydrolysis solution was added to 1.5 mL of 50 mM sodium borate buffer (pH 9), followed by the addition of 500 µL of fluorescamine solution in dioxane (300 mg/mL). Fluorescence was measured immediately at $\lambda_{excitation}$ = 400 nm and $\lambda_{emission}$ = 480 nm. A physical mixture of plain liposomes and peptides served to establish a calibration curve. Coupling yields were calculated relative to the quantity of surface-exposed maleimide functions.

2. Animals

For the evaluation of the immunogenicity of $CD8^+$ T cell epitope-bearing liposomes, specific-pathogen-free 6-8 week old female BALB/c mice (Charles River, Laboratories Saint-Germain-sur-l'Arbresle, France) were used. *In vivo* experiments were performed in full compliance with the CEE directive 2010/63 of September 22nd, 2010 relating to the protection of animals used for experimental purposes and in compliance with the French law (décret n° 2013–118 of February 1st, 2013). Moreover the experiments were performed in accordance with the guidelines of Animal Care and Use Committee of Alsace (authorization numbers: AL/106/113/02/13).

3. Immunization of mice

Mice were shaved on the dorsum and allowed to rest for 20 h. Then the shaved skin was swabbed with ethanol, and 100 µl of constructs were applied in three steps (three doses: 50 µl, 30 µl and 20 µl approximately) by massage for 1 min for each dose (total 3min). Mice were housed individually for the following 24 hours in order to avoid grooming.

4. Immunogenicity of transcutaneously administered liposomal constructs

4.1. Splenocyte and lymph node cell suspension

Mice were euthanized at day 30 after immunization by cervical dislocation. Spleen and lymph nodes draining the application zone (axillary, inguinal and brachial) were harvested in ice-cold RPMI 1640 culture medium, supplemented with 100 UI/mL penicillin, 100 mg/mL streptomycin, 10% heat-inactivated Fetal Bovine Serum (FBS), and 10 µM β-mercaptoéthanol. All culture medium reagents were purchased from Sigma Aldrich, Saint-Louis, USA. Spleens and lymph nodes of mice of the same group were pooled. Organs were dissociated onto a 70 µm nylon mesh cell strainer (BD FalconTM, Le pont de Claix, France). Resulting cell suspensions were centrifuged at 220 g for 5min. The lymph node cell pellet was resuspended in RPMI medium, where FBS is replaced with 2 % normal mouse serum (Dominique Dutscher, Brumath, France). To lyse red blood cells, the spleen cell pellet was resuspended in ammonium-chloride-potassium (ACK) lysing buffer (0.15 M NH_4Cl, 1 M K_2CO_3, and 0.1 M EDTA,

pH 7.4) and incubated at room temperature for 30 sec. To stop the lysis, ACK was diluted 10 times in RPMI, and the cells were centrifuged. The spleen cell pellet was resuspended in RPMI 2% normal mouse serum. Cells were counted and incubated in enzyme-linked lmmunospot (ELISpot) plates at a 4.10^6 cells/mL.

4.2. Direct/ Standard IFNγ ELISPOT assay

PVDF-bottomed plates (Multiscreen® HTS filter plates, Merck Millipore, Molsheim, France) were activated with 35% ethanol for 1 min, followed by three washes with sterile PBS. All plates were coated with 100 µL/well of purified anti-mouse IFNγ antibody (BD Pharmingen™, Le pont de Claix, France), clone AN-18, at 15 µg/mL in PBS overnight at 4 °C. After three washes, membranes were blocked with RPMI 10 % FBS for at least 2h at 37 °C, and washed again with RPMI without FBS. Splenocytes or lymph node cell suspension were cultured in triplicates (4.10^5 cells per well), in presence of recombinant human IL-2 (30 UI/well), and HA or ErbB2 peptides (10 µg/mL). Cells cultured with medium, and cells cultured with 5 µg/mL of concanavalin-A (Con A, Sigma Aldrich, St. Quentin Fallavier, France) served as negative and positive controls, respectively. After 20-24 h at 37°C, 5% CO2, cells were removed by 6 washes with PBS 0.01% Tween 20 (washing buffer). A volume of 100 µl of biotinylated anti-IFNγ antibody (BD Pharmingen™, Le pont de Claix, France), clone R4-6A2, was diluted at 1 µg/mL in PBS 0.01% tween 20 and applied for 2h at 37 °C. Plates were then washed 6 times with washing buffer and alkaline phosphatase-conjugated extravidin (Sigma-Aldrich, 1/5000, St. Quentin Fallavier, France) was added. After 45 min incubation at 37 °C, three washes in washing buffer, and three washes in PBS alone, spots were revealed by the addition of 100 µL of BCIP/NBT substrate (Sigma Aldrich). Coloration was allowed to develop for 30-60 minutes. To stop the reaction, plates were extensively washed with water and dried overnight before analysis (Bioreader 4000 PRO-S, Biosys, Karben, Germany). The number of spots/well was normalized per 10^6 cells and averaged for each replicate.

4.3. Indirect IFNγ ELISPOT assay (cultured ELISpot, double stimulation)

To allow the proliferation of peptide specific T cells, spleen cells and lymph nodes cells were cultured in a 6-well flat-bottom plate ($4\text{-}5.10^6$ cells/mL) in RPMI 10% FBS, in the presence of HA or ErbB2 peptide (10 µg/mL) at 37°C in a humidified 5% CO2 atmosphere for 3 days. On day 2,

half of the supernatant was replaced with fresh culture medium containing 20 UI/mL recombinant human IL-2. Cells were harvested on day 3, centrifuged at 220 g for 5min. The cell pellet was re-suspended in RPMI medium, where FBS is replaced with 2 % normal mouse serum. A standard ELISpot assay was then performed.

5. Tracking of DC skin migration to draining lymph nodes after TC application

This part of the work was performed in collaboration with Drs Christopher Mueller and Vincent Flacher (CNRS UPR 3572, IBMC).

5.1. Preparation of lymph node cell suspensions

After immunization by TC route (#2.3), mice were euthanized by cervical dislocation 48h after immunization. Lymph nodes draining the immunization sites, namely brachial lymph nodes, were harvested in ice-cold RPMI 1640 medium supplemented with 10% FBS. They were then dissociated using thin forceps in RPMI 1640 medium supplemented with 2% FBS and containing the following enzymes: 160 µg/mL Collagenase D (Roche, Roche Applied Science, Hamburg, Germany) and 120µg/mL DNAse I (Roche, bovine pancreas grade II). After digestion for 1 h at 37°C with stirring, the lymph node cell suspensions was filtered on 100 µm nylon mesh cell strainer and washed with PBS. Finally, cells were counted before being re-suspended in PBS with 2% FBS and 5mM EDTA.

5.2. Flow cytometry analysis

Cells were stained with a Fixable Viability Dye (eBioscience, San Diego, USA) then incubated with FcR Blocking Reagent (Miltenyi-Biotec, Auburn, USA). Antibody panels used for cell-surface staining are detailed in table 2. Intracytoplasmic staining with anti-langerin mAb was carried out using the Cytofix/Cytoperm™ kit (Becton-Dickinson, Le Pont-de-Clays, France) according to the manufacturer's instructions. Briefly, cells were washed with PBS before being resuspended in the BD Cytofix/Cytoperm™ solution. After an incubation step of 20 min at 4°C, cells were washed with the BD Perm/Wash™ buffer stained with anti-Langerin. All incubations with mAb were performed in a volume of 100 µL at 4°C in the dark. Experimental data were acquired on a Gallios flow cytometer (Beckman-Coulter, Brea, CA) and analyzed with the FlowJo software (Version 7.6.5 Treestar, Ashland, OR).

Table 2: Dyes and antibodies used for flow cytometry staining.

Staining		Fluorochrome	Clone	Final concentration / Dilution	Provider	Incubation Time
Viability	Fixable Viability Dye	eFluor780	-	1/1000	eBioscience	15 minutes
Fc-receptor Blocking	FcR Blocking Reagent	None	Not indicated	1/100	Miltenyi-biotec	15 minutes
Extra-cellular	I-A/I-E (MHC II)	Alexa Fluor 700	M5/114.15.2	1µg/mL	Biolegend	15 minutes
	Anti-CD11c	PE-Cy7	HL3	2 µg/mL	BD Biosciences	15 minutes
	Anti-CD103	PE	M290	2 µg/mL	BD Biosciences	15 minutes
Intra-cytoplasmic	Anti-Langerin	Alexa Fluor 488	929F3	2 µg/mL	Dendritics	20 minutes

Results

1. Formulation and physicochemical characterization of different variants of liposomal constructs

1.1. Formulation and characterization of conventional liposome-based constructs

In a previous work, our team designed liposome-based constructs that co-deliver ErbB2 (T $CD8^+$ epitope) and HA (T $CD4^+$ epitope) peptides and incorporate a TLR2/6 agonist (Pam_2CAG) as a danger signal. It was demonstrated that subcutaneous delivery (SC) of these constructs induces a potent protective antitumor response against ErbB2-overexpressing tumors in mice (Espuelas *et al.*, 2005b; Thomann *et al.*, 2011). To evaluate whether similar formulations can induce a tumor specific immune response by the TC route, multiple variants of the original liposome-based vaccine (Lp $Pam_2CAG/HA/ErbB_2$) were prepared. They incorporated in addition to the two epitope peptides a TLR4 agonist (MPLA) or a TLR2/6 agonist (Pam_2CAG) as danger signal. They also incorporated or not di-mannose (Man) as a DC targeting molecule.

Liposome-based SUVs, resulting from the sonication of MLVs, had a mean diameter of 60-70 nm, with a narrow distribution (low CV) and a polydispersity index always lower than 0.3 reflecting the monodispersity of the liposome diameter distribution. Their size was independent of the coupling. In all cases, the peptide coupling yield to the surface-exposed maleimide function exceeded 88% (table 3). Size and coupling were affected neither by the replacement of Pam_2CAG in the original formulation by MPLA nor by the addition of mannose. The reproducibility of our formulation technique ensures a constant amount of peptides was delivered per vaccine administration dose, equivalent to 15 µg of ErbB2 and 20 µg of HA/100 µL of liposome suspension.

Table 3: Physicochemical characteristics of the liposome-based constructs.

Composition	Average diameter ± width (nm) (% of the population)	CV (%)	PDI	Peptide coupling rate (%)
PC/PG/Chol	69 ± 12 (96%)	8%	0.185	-
PC/PG/Chol/DPG-Mal/Pam$_2$CAG	69 ± 10 (93%)	8%	0.216	91
PC/PG/Chol/DPG-Mal/Pam$_2$CAG/DOG-Man$_2$	62 ± 11 (91%)	17%	0.218	94
PC/PG/Chol/DPG-Mal/MPLA	66 ± 8 (92%)	13%	0.222	88
PC/PG/Chol/DPG-Mal/MPLA/DOG-Man$_2$	68 ± 12 (92%)	14%	0.214	95

The liposome average size was measured by the dynamic light scattering method, and the peptide coupling rate was determined after acid hydrolysis using a fluorometric assay with fluorescamine (n=4 preparations, with 3 measurements on each preparation). CV: Coefficient of variation, PDI: polydispersity index, PC: phosphatidyl choline, PG: phosphatidyl glycerol, Chol: cholesterol, DPG-Mal: dipalmitoyl glycerol-maleimide, Pam$_2$CAG: dipalmitoyl-cysteine-alanyl-glycine, MPLA: monophosphoryl lipid A, DOG-Man$_2$: dioleyglycerol-dimannose

1.2. Formulation and physicochemical characterization of different variants of transfersome-based vaccines

Transfersomes^{TM} (Tf) are flexible deformable vesicles known for their capacity to squeeze into pores smaller than their own diameter (Cevc and Blume, 1992, 2001; Cevc *et al.*, 1998). Therefore, they are described as highly efficient for skin barrier crossing. In this context, we formulated and evaluated formulations incorporating the same elements as the conventional liposomes, but replaced the liposomal vesicle by an ultradeformable vesicle (transfersome^{TM,} Tf). SUV-Tf resulting from the extrusion of MLV-Tf had a mean diameter of 80-90 nm and a narrow size distribution (PDI<0.3). Similarly to conventional liposomes, Tf that incorporated a TLR2/6 agonist (Pam$_2$CAG) or a TLR4 agonist (MPLA), as well as those that bore the DC targeting molecule, mannose, did not exhibit any remarkable variation in their physicochemical properties. Their mean diameter remained <100 nm (80-90 nm), with a narrow distribution (low CV) and a polydispersity index always lower than 0.3 reflecting the monodispersity of the transfersome diameter distribution. The peptide coupling rates were slightly decreased compared to those previously obtained with the liposomes in this work, but always exceeded 78 % (table 4). Thus, we have repeatedly formulated transfersome-based peptide-expressing constructs, incorporating a danger signal and a DC targeting molecule.

***Table 4*: Physicochemical characteristics of the transfersome-based vaccines.**

Composition	Average diameter ± width (nm) (% of the population)	% CV	PDI	Peptide coupling rate (%)
SPC/SDC	91 ± 11 (97%)	7%	0.136	-
SPC/SDC/DPG-Mal/Pam_2CAG	92 ± 9 (99%)	6%	0.150	83
SPC/SDC/DPG-Mal/Pam_2CAG/ DOG-Man_2	91 ± 10 (99%)	4%	0.238	83
SPC/SDC/DPG-Mal/MPLA	88 ± 11 (95%)	15%	0.237	78
SPC/SDC/DPG-Mal/MPLA/ DOG-Man_2	80 ± 11 (100%)	6%	0.124	87

Average size was measured by the dynamic light scattering method, and the peptide coupling rate was determined after acid hydrolysis using a fluorometric assay with fluorescamine. (n=3 preparations, with 3 measurements on each preparation). CV: Coefficient of variation, PDI: polydispersity index, SPC: soy phosphatidyl choline, SDC: sodium deoxycholate, DPG-Mal: dipalmitoyl glycerol-maleimide, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, MPLA: monophosphoryl lipid A, DOG-Man_2: dioleyglycerol-di-mannose

1.3. Formulation of fluorescent liposomes

To follow the development of the immune response by tracking the liposomes in the lymph nodes of immunized mice, we formulated fluorescent liposomes by incorporating a fluorescent molecule, DiI, in their lipid bilayer. The formulations also comprised i) the danger molecule (MPLA) to allow DC maturation and migration to the draining lymph nodes, and ii) mannose residues for optimal DC targeting. Fluorescent liposomes had a mean diameter of 60 nm. DiI incorporation did not alter neither their narrow distribution (low CV) nor their polydispersity index (<0.3) thus reflecting the monodispersity of the fluorescent liposome diameter distribution (table 5).

Table 5: Physicochemical characteristics of the fluorescent liposome-based constructs.

Composition	Average diameter ± width (nm) (% of the population)	CV (%)	PDI
PC/PG/Chol/DiI	58 ± 10 (94%)	8.9%	0.204
PC/PG/Chol/DiI /Pam_2CAG/DOG-Man_2	57 ± 4 (96%)	3%	0.210

The liposome average size was measured by the dynamic light scattering method (n=3 preparations, with 3 measurements on each preparation). CV: Coefficient of variation, PDI: polydispersity index, PC: phosphatidyl choline, PG: phosphatidyl glycerol, Chol: cholesterol, DiI: 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate ,MPLA: monophosphoryl lipid A, DOG-Man_2: dioleyglycerol-di-mannose

2. Systemic immune response induced by a physical mixture of ErbB2 and cholera toxin: ErbB2 is immunogenic by the TC route.

Before evaluating the immunogenicity of liposome formulations administered by the TC route, we investigated whether the potential $CD8^+$ T cell peptide epitope ErbB2 would be capable of eliciting a cellular immune response when administered in a physical mixture with cholera toxin, according to a previously validated robust transcutaneous (TC) immunization protocol. Cholera toxin has been described, along with other ADB-ribosilating toxins, as a potent adjuvant for inducing $CD8^+$ T cell responses in TC immunization (Glenn *et al.*, 1999; Olvera-Gomez *et al.*, 2012; Partidos *et al.*, 2004). Following TC application of a physical mixture of cholera toxin and the target peptide, cholera toxin ensures a strong DC activation and sufficient skin permeation to elicit potent responses (Olvera-Gomez *et al.*, 2012). We therefore immunized mice by applying a physical mixture of ErbB2 peptide and cholera toxin on ethanol-wet, previously shaved dorsum. After sacrifice, spleen and lymph node cells were cultured in the presence of the ErbB2 peptide; the number of ErbB2-specific IFNγ secreting lymphocytes was measured by ELISpot. As shown in figure 1, an ErbB2-specific cellular immune response was induced in spleen and lymph node cell cultures of immunized mice after re-stimulation with ErbB2 peptide, but not with the HA peptide used as negative control. These results indicate that TC delivery of ErbB2 on ethanol-treated skin induces a local and systemic specific cellular immune response.

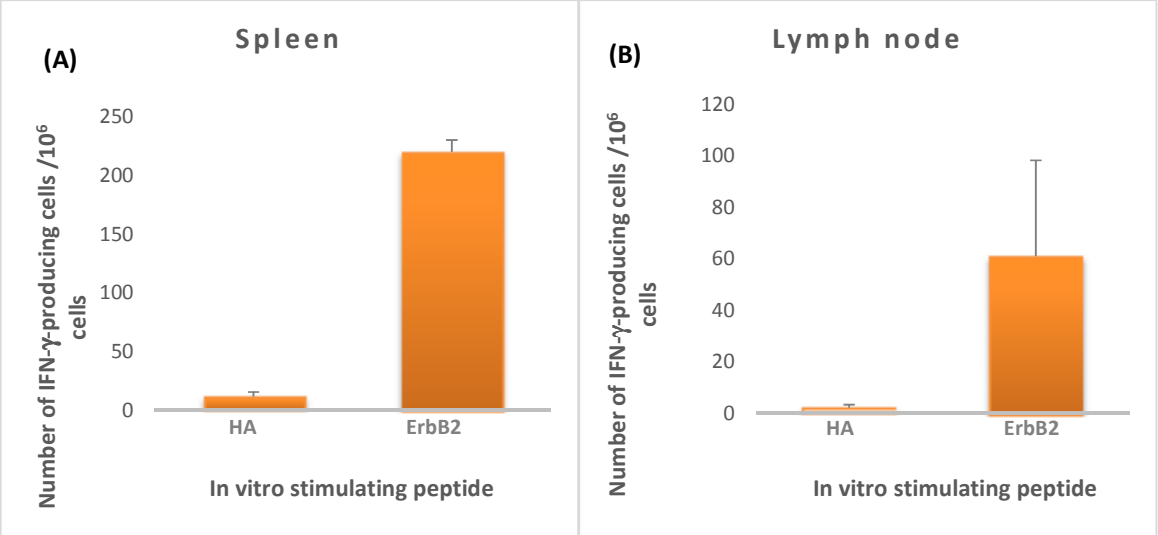

Figure 1: ErbB2-specific IFNγ-response to transcutaneous immunization with a physical mixture of cholera toxin and ErbB2 peptide. *Immunizations were carried out at days 0, 4 and 8, where mice received a TC application of cholera toxin (20 µg) mixed with ErbB2 peptide (15 µg). Mice were sacrificed on day 28. Pools of spleen or lymph node cells were prepared for each experimental group (n=5), and cultured either alone (Background, B) or in the presence of ErbB2 or with HA (10 µg/mL) as a negative control peptide (peptide, P). The number of IFNγ-secreting cells/10^6 cells was measured by ELISpot assay. Data from one representative experiment among 5 is shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *display the number of spots specific to each peptide antigen (P-B) in the spleen and lymph nodes respectively.*

3. Evaluation of the immune response induced by different ErbB2-bearing liposomal constructs by TCI: proof of immunogenicity

3.1. Liposomes adjuvanted with a TLR2/6 agonist induce an ErbB2-specific response after TCI

To evaluate if liposomal constructs are suitable for TCI, we compared the immunogenicity of the original formulation (Lp Pam_2CAG/HA/ErbB2) after TCI and after SC injection.

For this, mice received 3 doses of Lp Pam_2CAG/HA/ErbB2 formulation, either through the TC route or by SC injection and the number of T cells producing IFNγ in response to the peptide presented by the liposome was evaluated by ELISPOT in the spleen (systemic immune response) and the draining lymph nodes (local immune response).

As expected, SC administration of liposome constructs containing peptides and danger molecules induces a systemic and local specific cellular immune response as shown by the

increased number of IFNγ-producing cells in comparison with administration of plain liposomes (figure 2). It is worth noting that the observed response is weak against ErbB2 in the lymph nodes. Interestingly, similar results were obtained after TC administration with an increase of the specific immune response as compared to plain liposomes. These results show that Lp Pam_2CAG/HA/ErbB2 formulations delivered by the TC route, as well as by the SC route, induce an immune activation in the skin that elicits ErbB2 and HA-specific T cell proliferation and differentiation. Furthermore, they indicate that the TC immunization route is comparably efficient to the SC route.

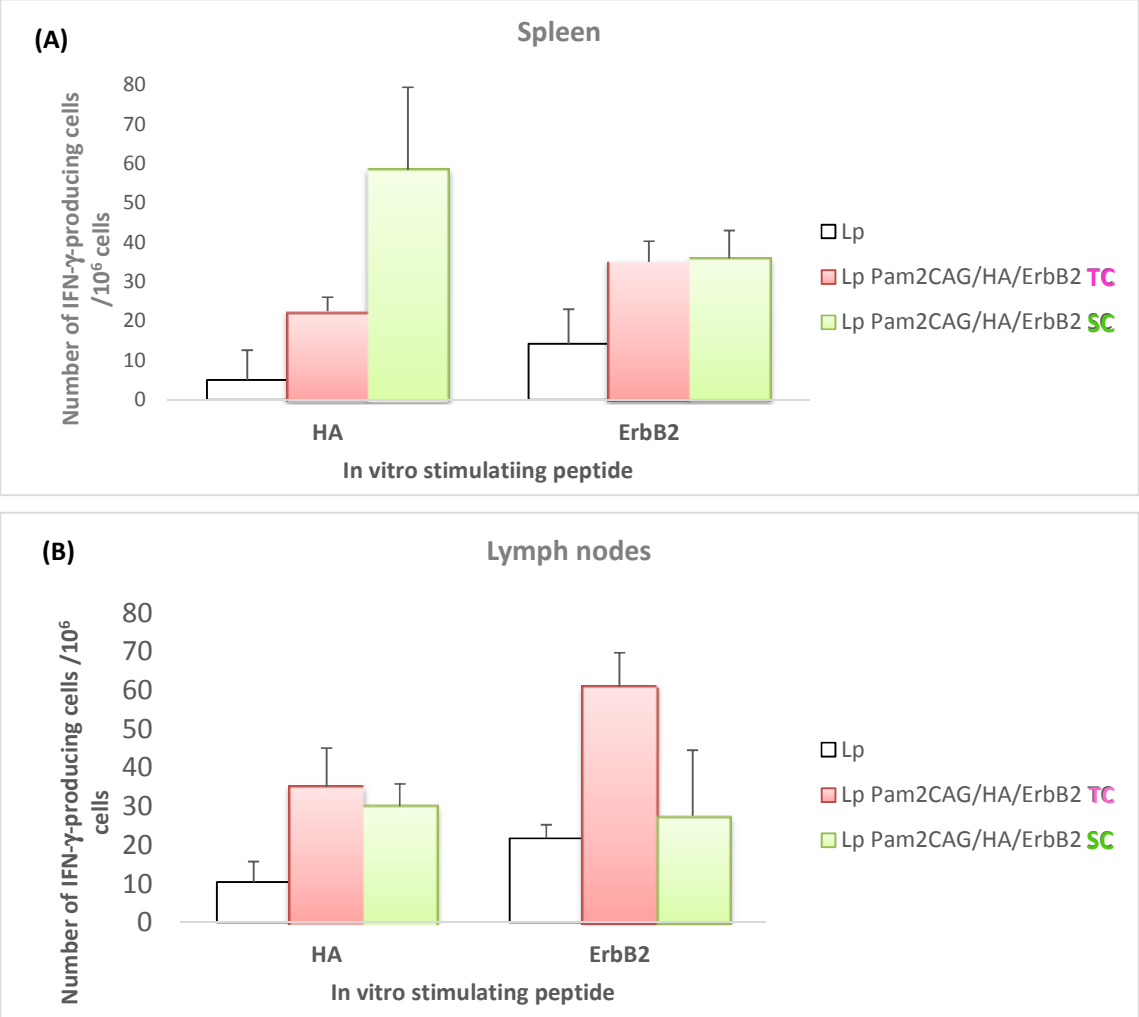

Figure 2: ErbB2 and HA-specific IFNγ-production induced by transcutaneous (TC) or subcutaneous (SC) immunization with the original (Lp Pam$_2$CAG/HA/ErbB2) formulation. *Mice received on days 0, 4 and 8 a TC application of plain liposomes (lp) (white) or Lp Pam$_2$CAG/HA/ErbB2 (pink) or a SC injection of Lp Pam$_2$CAG/HA/ErbB2 (green). Spleen and lymph node cells were collected on day 28 and cultured either alone (Background, B) or in the presence of ErbB2 or HA (peptide, P, 10 µg/mL). The number of IFNγ-secreting cells/10^6 cells was measured by direct ELISpot assay. Data from one representative experiment of five independent ones, using 5 mice per group each, is shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *display the number of spots specific to each peptide antigen (P-B) in the spleen and lymph nodes of immunized mice, respectively. Lp: liposome, Pam$_2$CAG: dipalmitoyl-cysteine-alanyl-glycine.*

3.2. The TLR4 agonist is less suitable than the TLR2/6 agonist for T cell activation after TCI with liposomal constructs

In an attempt to optimize the vaccine for TCI, the TLR2/6 agonist Pam_2CAG was replaced in the liposome construct by a TLR4 agonist, MPLA. Liposome-incorporated MPLA is known for its ability to favor the induction of CTL responses (Alving *et al.*, 2012a), suitable for anti-tumoral immune response. Mice were immunized with liposomes-ErbB2-HA-MPLA by the TC route and the immune response was evaluated by IFNγ ELISpot as described above.

Whereas the immune response induced by both constructs was similar in the spleen, the TLR4 agonist bearing construct did not induce any specific immune response in the lymph nodes (figure 3). These results show that DC activation by TLR4 elicits a systemic immune response but is less effective in inducing a local one.

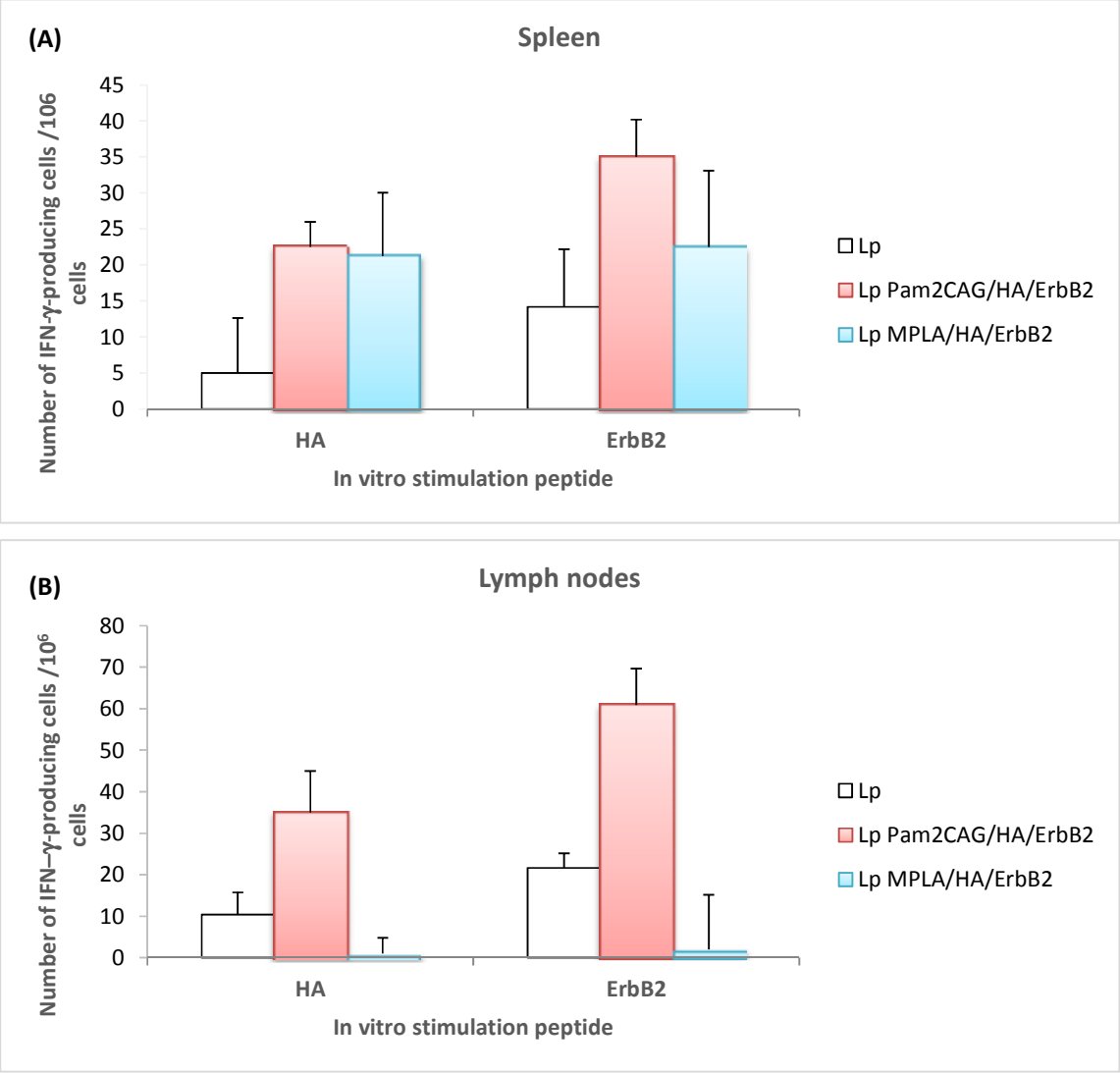

Figure 3: ErbB2 and HA-specific IFNγ response induced by TC immunization of mice with Pam₂CAG (TLR2/6 agonist) or MPLA (TLR4 agonist) adjuvanted liposomes. *Plain liposomes (lp) (white),* Lp Pam_2CAG/HA/ErbB2 *(pink), or* Lp MPLA/HA/ErbB2 *(blue) were applied to the skin of 3 mouse groups (5 mice/group). Immunizations were carried out at days 0, 4 and 8. Spleen and lymph node cells were collected on day 28 and cultured either alone (Background, B) or in the presence of ErbB2 or HA (peptide, P,* 10 µg/mL*). The number of IFNγ-secreting cells/10^6 cells was measured by direct ELISpot assay. Data from one representative experiment of two independent ones are shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *show the number of spots specific to each peptide (P-B) in the spleens and lymph nodes respectively. Lp: liposomes, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, MPLA: monophosphoryl lipid A.*

3.3. The DC targeting molecule has only a minor effect on ErbB2-specific responses induced by TCI

Another approach to optimize the liposomal constructs for TCI consisted in targeting them to receptors expressed on the surface of DCs via the mannose receptor. We have previously observed that when mannose was incorporated in the lipid bilayers of the Lp, it dramatically increased their uptake by DCs *in vitro* (Espuelas *et al.*, 2008). *In vivo*, targeting of SC injected Lp-based vaccines allowed us to decrease 100-fold the danger molecule, without affecting the amplitude of the immune response. Similarly, it allowed 100-fold dilution of the injected formulation without affecting its protective effect in tumor-bearing mice (Thomann *et al.*, 2011).

To assess the efficacy of DC targeting in TC vaccination, mice were immunized with the same liposomes described in paragraph 3.2, to which we added or not mannose as a DC targeting molecule. The immune response was evaluated by IFNγ ELISpot as described above.

As expected, the amplitude of ErbB2-specific responses induce by TCI was slightly increased in the spleen by the addition of the DC targeting molecule on constructs bearing TLR 2/6 as well as TLR4 agonists, indicating a slight potentiating effect of DC targeting on the systemic immune response (figure 4).

In contrast, in the lymph nodes, ErbB2 specific responses induced by TLR2/6 agonist bearing formulations were decreased and those induced by TLR4 agonist formulations remained unchanged, suggesting that the addition of a DC targeting molecule did not improve the local specific immune response (figure 4).

Neither the local nor the systemic immune response to HA was affected by mannose addition to the formulations (figure 4).

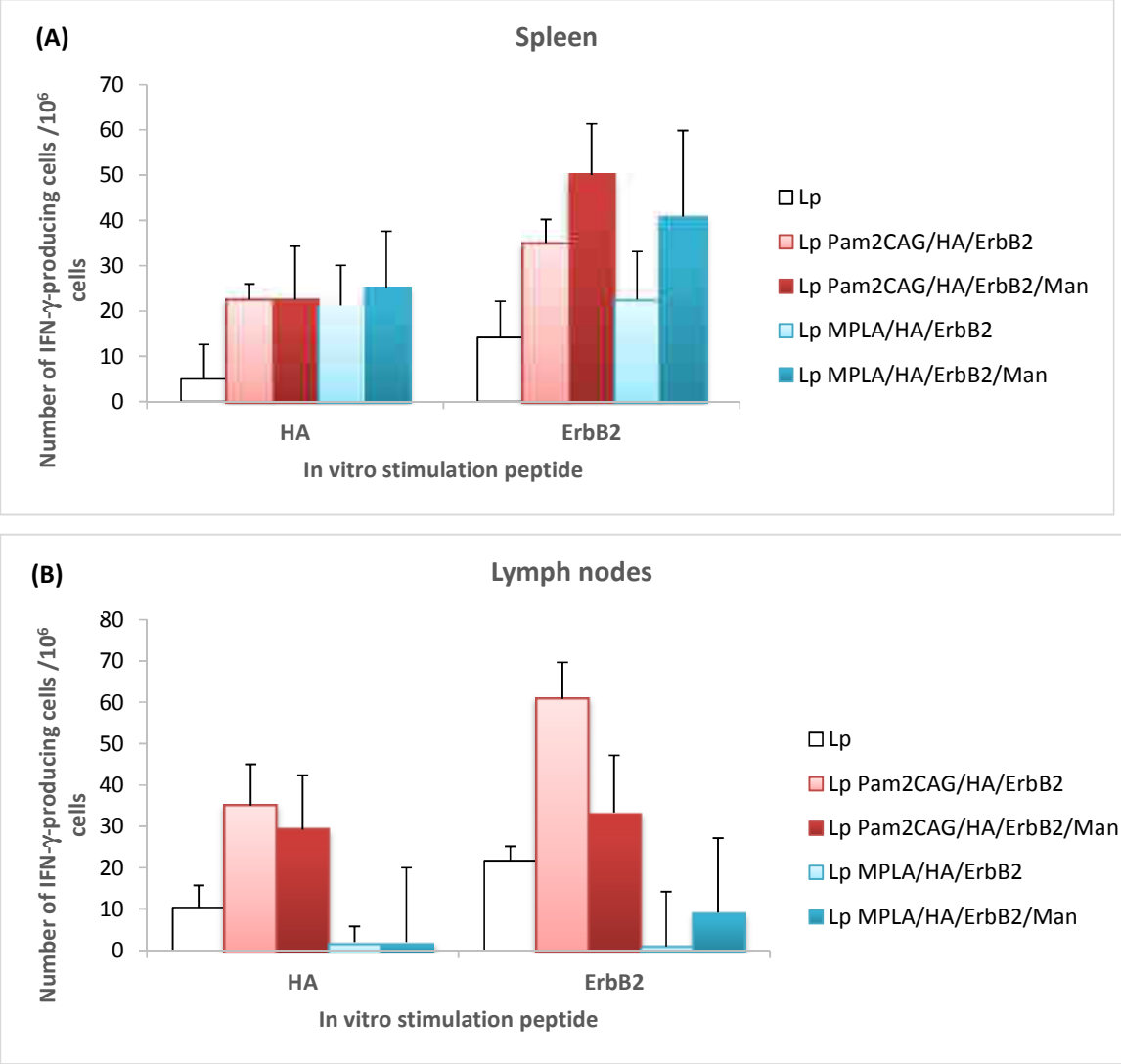

Figure 4: ErbB2 and HA-specific IFNγ response induced by TC immunization of mice with DC targeted liposomal vaccines. *Plain liposomes (lp) (white), adjuvanted liposomes Lp Pam₂CAG/HA/ErbB2* (pink), or *Lp MPLA/HA/ErbB2* (light blue)*, and targeted adjuvanted liposomes, Lp Pam₂CAG/HA/ErbB2/Man (red), or Lp MPLA/HA/ErbB2/Man (dark blue), were applied to the skin of 5 mouse groups (5 mice/group). Immunizations were carried out at days 0, 4 and 8. Spleen and lymph node cells were collected on day 28 and cultured either alone (Background, B) or in the presence of ErbB2 or HA (peptide, P, 10 µg/mL). The number of IFNγ-secreting cells/10⁶ cells was measured by direct ELISpot assay. Data from one representative experiment of two independent ones are shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *show the number of spots specific to each peptide (P-B) in the spleens and lymph nodes respectively. Lp: liposome, Pam₂CAG: dipalmitoyl-cysteine-alanyl-glycine, MPLA: monophosphoryl lipid A, Man: mannose.*

3.4. Validation of the power of the ELISpot assay

Once the immunogenicity of liposomal vaccines by the TC route was established, it became important to address the reproducibility of the T-cell responses in our model. However, in all the above results, the total number of peptide-specific IFNγ producing cells remained generally low with all the tested formulations, resulting in timid margins in mice immunized with peptide and adjuvant-bearing liposomes as compared to mice immunized with plain liposomes. Therefore, a robust method of analysis was needed to ensure high sensitivity while maintaining a minimal risk of false positive responses. Indeed, even if IFNγ ELISpot has emerged as a powerful tool to detect rare antigen-specific T cells, the interpretation of ELISpot data becomes problematic when the signal is low. Several empirical methods were developed and employed for analyzing ELISpot responses in clinical trials for infectious diseases and cancer. Empirical methods are dependent on the reagents, the settings of the test and on populations that are being tested, and therefore, are internally set up. A rational step-by-step empirical method was illustrated by Dubey *et al.*, to set and validate positivity criteria for the ELISpot assay (Dubey *et al.*, 2007). This method was adopted herein, and was used to analyze the results of the direct as well as the indirect ELISpot assay.

3.4.1. Dubey's method: how to define the positivity cut-off?

The power of Dubey's method is to establish a cut-off to compare results obtained in different *ex vivo* experiments while avoiding the variations inherent to animal experimentation. This cut-off determines the number of spots above which a complete liposome construct (containing peptides + adjuvant) is considered to induce a specific response in comparison with the the plain liposome taken as a negative control. To establish this positivity cut-off, we retrospectively analyzed IFNγ ELISpot data of mice immunized with the plain construct in all our experiments. A two-dimensional distribution was generated by the natural log (ln) of spot numbers in unstimulated cultures (b = ln B) against that of the peptide-stimulated culture (p = ln P). Since in each immunization experiment, the immune response had been analyzed in two types of ELISpot assays, known as direct and indirect ELISpot, two different two-dimensional distributions were generated, one for each ELISpot type.

Positivity regions were then defined in such a way that they did not include any data point from control mice, by setting a minimum spot number for peptide-stimulated cultures and a

threshold ratio for peptide/background spot number. For the direct ELISpot, the Dubey's method defines 2 positivity regions according to the number of spots in peptide-stimulated cultures. When this number was low ($5 \leq P < 20$ spots/10^6 cells) the ratio threshold was set at $R_1 = P/B \geq 1.6$; when it was high ($P_2 \geq 20$ spots/10^6 cells), we selected a lower threshold ($R_2 \geq 1.4$) (Figure 5 (A). For the indirect ELISpot assay, positive results were defined as a combination of a minimum peptide-stimulated spot count $P \geq 20$ spots/10^6 cells and a $R \geq 1.3$

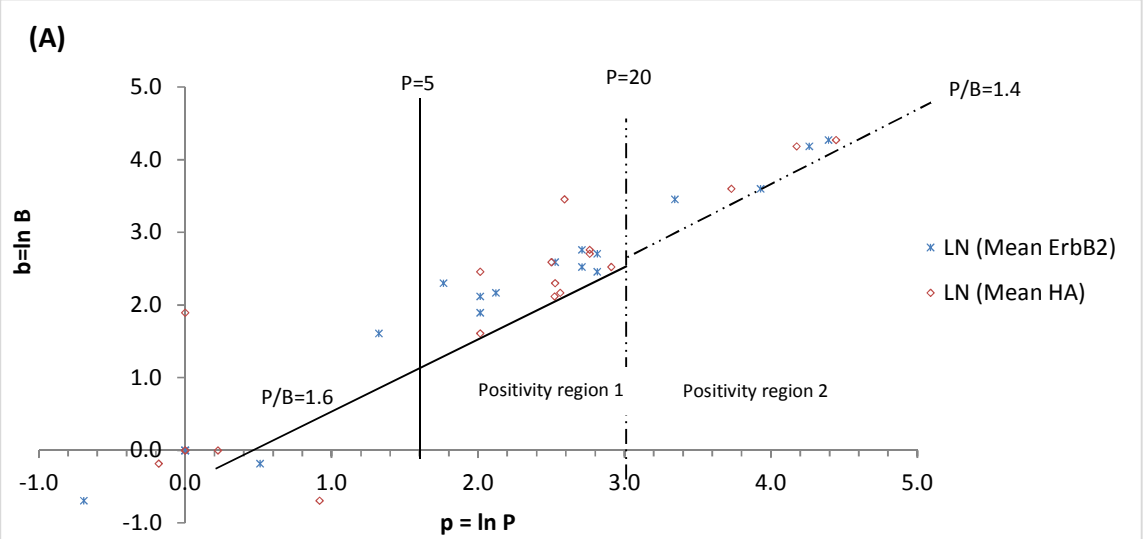

Figure 5: ELISpot positivity criteria are defined by the responses of control mice vaccinated with plain formulations. *IFNγ spots of spleen and lymph node cell pools from mice immunized with the plain formulations are plotted in a 2-dimensional distribution. The ln of spot numbers in peptide-stimulated cultures (p = ln P) is plotted against the ln of spot numbers in unstimulated cultures (b = ln B). The region of positivity is obtained by defining a minimal spot count ($P \geq P_0$) and a peptide/background ratio r.* ***(A)*** *For direct ELISpot, 2 positivity regions were defined ($5 \leq P_1 < 20$ and $R_1 \geq 1.6$; $P_2 \geq 20$ and $R_2 \geq 1.4$.)* ***(B)*** *For indirect ELISpot, the positivity region was defined by $P_0 \geq 20$, and $R_0 \geq 1.3$.*

3.4.2. Dubey's method: how to analyze ELISpot responses

To evaluate whether the peptide-induced spot counts observed in mice immunized with the various liposome constructs reflect a significantly positive response, we retrospectively analyzed the peptide/background spot number ratios (R=P/B) of the vaccine constructs according to the positivity criteria set above. Table 6 summarizes Dubey's analysis of the

experiments represented in the figures 2, 3 and 4, where the responses were observed in a direct ELISpot assay and the number of peptide-stimulated spots exceeded 20 spots/10^6 cells ($P_2 \geq 20/10^6$ cells). Therefore, the positivity threshold as $R_2 \geq 1.4$.

Responses that were apparently positive in figures 2, 3 and 4 were all significant according to Dubey's positivity criteria, with the exception of HA-stimulated spleen-cultures of mice immunized with Lp MPLA/HA/ErbB2/Man. Dubey's method proved to be concordant with the "visual" interpretation of ELISpot results. Therefore, Dubey's method is valid to be used in the rest of this work.

Table 6: Analysis of ELISpot responses to (A) HA and (B) ErbB2 peptides in mice immunized with mannose-targeted and untargeted Lp Pam_2CAG/HA/ErbB2 (TLR2/6 agonist) or Lp MPLA/HA/ErbB2 (TLR4 agonist), according to Dubey's method.

(A)	**HA-specific response**	**Lp Pam_2CAG/HA/ErbB2 (SC)**	**Lp Pam_2CAG/HA/ErbB2 (TC)**		**Lp MPLA/HA/ErbB2 (TC)**	
		No mannose	**No mannose**	**+ mannose**	**No mannose**	**+ mannose**
Spleen	Peptide/background ratio	2.4	1.5	1.4	1.5	1.3
	Interpretation	***Positive***	***Positive***	***Positive***	***Positive***	***Negative***
Lymph nodes	Peptide/background ratio	1.4	1.6	1.5	1.0	0.9
	Interpretation	***Positive***	***Positive***	***Positive***	***Negative***	***Negative***

(B)	**ErbB2-specific response**	**Lp Pam_2CAG/HA/ErbB2 (SC)**	**Lp Pam_2CAG/HA/ErbB2 (TC)**		**Lp MPLA/HA/ErbB2 (TC)**	
		No mannose	**No mannose**	**+ mannose**	**No mannose**	**+ mannose**
Spleen	Peptide/background ratio	1.8	1.7	1.8	1.5	1.5
	Interpretation	***Positive***	***Positive***	***Positive***	***Positive***	***Positive***
Lymph nodes	Peptide/background ratio	1.4	2.0	1.5	0.9	1.2
	Interpretation	***Positive***	***Positive***	***Positive***	***Negative***	***Negative***

Spleen and lymph node cells of immunized animals were cultured either alone (background) or in the presence of ErbB2 or HA peptide. IFNγ production was assayed by direct ELISPOT. Since the number of peptide-stimulated spots P consistently exceeded 20 spots/10^6 cells, the peptide/background ratio R ≥ 1.4 was adopted. Lp: liposome, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, SC: subcutaneous, TC: transcutaneous, MPLA: monophosphoryl lipid A.

3.4.3. Dubey's method: our results are reproducible and show that the liposome constructs induce a specific immune response by the TC route

We then retrospectively analyzed the results of all the *ex vivo* experiments using the Dubey's method. A response was considered positive if it met the positivity criteria defined by Dubey's

method either in the direct or indirect ELISpot. The response rate against each of the formulations was defined as the number of experiments yielding a positive response *100 /the total number of experiments. This analysis is illustrated in Table 7.

Table 7: Response rate of spleen and lymph node cell pools of mice vaccinated with liposomal vaccines to (A) HA and (B) ErbB2 peptide antigens.

(A) HA-specific response	Lp Pam_2CAG/HA/ErbB2 (SC)	Lp Pam_2CAG/HA/ErbB2 (TC)		Lp MPLA/HA/ErbB2 (TC)	
	No mannose	No mannose	+ mannose	No mannose	No mannose
Spleen	100 % (4/4)	75 % (3/4)	67 % (2/3)	100 % (3/3)	100 % (2/2)
Lymph nodes	75 % (3/4)	100 % (4/4)	50 % (1/2)	0 % (0/3)	0 % (0/2)

(B) ErbB2-specific response	Lp Pam_2CAG/HA/ErbB2 (SC)	Lp Pam_2CAG/HA/ErbB2 (TC)		Lp MPLA/HA/ErbB2 (TC)	
	No mannose	No mannose	+ mannose	No mannose	+ mannose
Spleen	75 % (3/4)	75 % (3/4)	100 % (3/3)	67 % (2/3)	67 % (2/3)
Lymph nodes	75 % (3/4)	75 % (3/4)	50 % (1/2)	0 % (0/3)	0 % (0/2)

Spleen and lymph node cells of immunized animals were cultured either alone (background) or in the presence of ErbB2 or HA peptide. IFNγ production was assayed by direct ELISPOT. A result is considered positive if detected either in the direct or indirect ELISpot assay, according to the positivity criteria defined by Dubey's method.

Based on all the results compiled in table 7 we can conclude that:

1- Liposome administration by the TC route induces a cellular immune response against ErbB2 (Table 7A) and HA (Table 7B).

2- After TC administration, TLR2/6 (Pam_2CAG) bearing liposome constructs induce a local (in lymph nodes) and systemic (in the spleen) immune response whereas addition of TLR4 (MPLA) bearing liposomes induce only a systemic immune response.

3- DC targeting by mannose does not increase the immune response after TC immunization with the liposome construct containing MPLA. A slight increase of the systemic immune response against ErbB2 is observed with constructs containing TLR2/6 agonist.

These results indicated a high reproducibility of the immune response in our TC vaccination model, despite the technical challenges of TC immunization. Thus, liposome-based formulations are promising TC vaccines that deserve further attention in tumor immunotherapy.

4. Influence of the lipid vesicle fluidity on the systemic immune response

4.1. Transfersome-based formulations induce a T-cell response comparable but not equal to that induced by their liposomal counterparts.

To assess the immunogenicity of the Tf-based constructs after TCI, and to address the influence of the danger signal in these constructs, the same immunization protocol that was adopted for conventional liposomes was followed, using TLR2/6 agonist-containing transfersomes (Tf Pam_2CAG/HA/ErbB2) or TLR4 agonist-containing transfersomes (Tf MPLA/HA/ErbB2).

In contrast to their liposomal counterparts, TLR2/6- and TLR4-containing transfersomes induced poorly reproducible HA-specific immune responses (not shown). Therefore, we compared their immunostimulatory potential according to ErbB2-induced responses.

As shown in figure 6, whereas Tf incorporating a TLR2/6 agonist induced an ErbB2-specific immune response in the spleen and lymph nodes, the TLR4 agonist-bearing Tf induced an immmune response only in the lymph nodes. Interestingly, these results came in contrast with those obtained with TLR4 agonist-incorporating conventional Lp induced an immune response only in the spleen, not in the lymph nodes.

These results suggest that similarly to their liposomal counterparts, transfersome-based formulations trigger a specific T cell response. Interestingly, the vesicle type (Lp or Tf) seems to affect the localization of the immune response induced by certain adjuvants like the TLR4 agonist (MPLA).

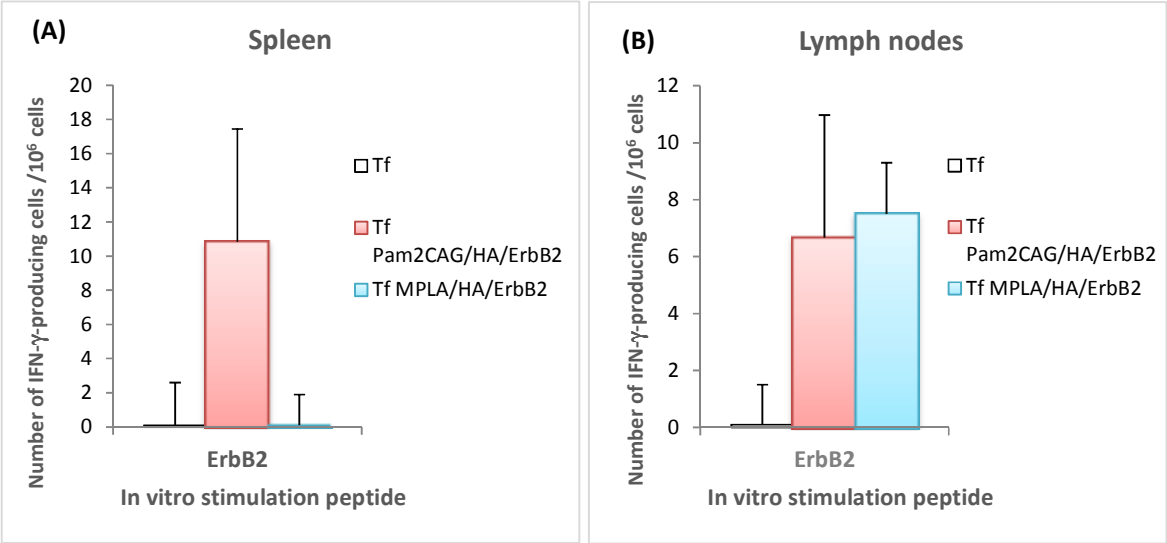

Figure 6: ErbB2-specific IFNγ response in mice immunized with Pam_2CAG (TLR2/6 agonist) or MPLA (TLR4 agonist) adjuvanted transfersomes. *Plain transfersomes (Tf) (white), Tf Pam_2CAG/HA/ErbB2 (pink), or Tf MPLA/HA/ErbB2 (blue) were applied to the skin of 3 mouse groups (5 mice/group). Immunizations were carried out at days 0, 4 and 8. Spleen and lymph node cells were collected on day 28 and cultured either alone (Background, B) or in the presence of ErbB2 (Peptide, P). The number of IFNγ-secreting cells/10^6 cells was measured by direct ELISpot assay. Data from one representative experiment of two independent ones are shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *show the number of $ErbB_2$-specific spots (P-B) in the spleens and lymph nodes respectively. Lp: liposomes, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, MPLA: monophosphoryl lipid A.*

4.2. DC targeting molecules modulate the immunogenicity of transfersome-based vaccines

Targeting the conventional Lp to DCs did not seem to influence their immunogenicity by TCI. To check if this is the case with Tf too, mice received a TC application of Tf incorporating or not the DC targeting molecule, di-mannose.

As seen in figure 7, addition of a DC targeting molecule to TLR2/6 agonist-bearing Tf has a deleterious effect on the systemic immune response in spleen but a favorable one on the local immune response in lymph nodes. These results came in contrast to those obtained with targeted liposomal vaccines where mannose addition induced only a slight effect and in the opposite way.

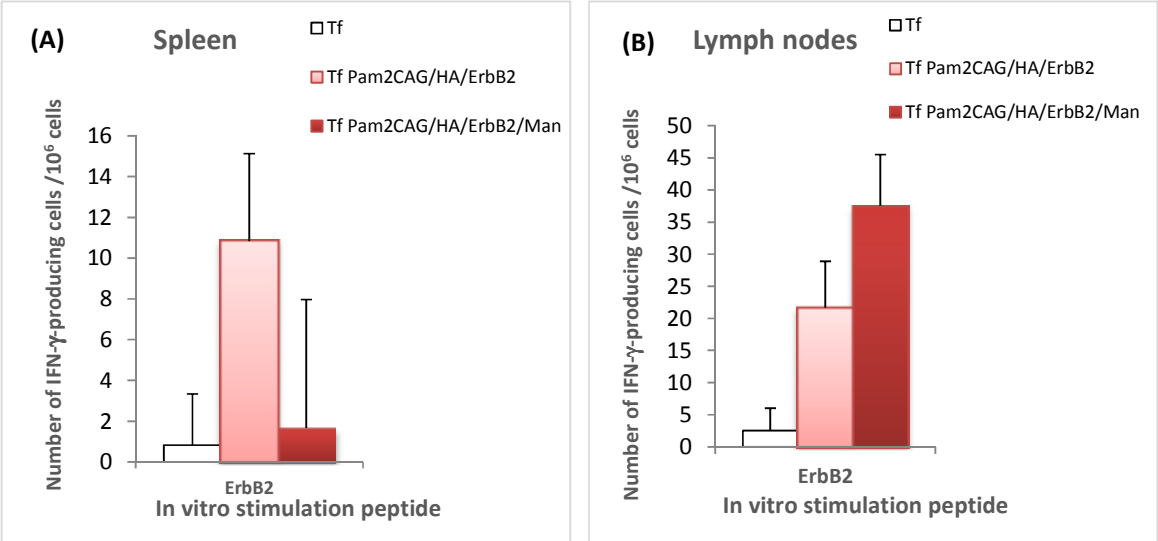

Figure 7: ErbB2-specific IFNγ response in mice immunized with non-targeted or DC targeted transfersomal vaccines adjuvanted with Pam_2CAG. *Plain transfersomes (Tf) (white), adjuvanted transfersomes, Tf Pam_2CAG/HA/ErbB2 (pink), and targeted adjuvanted transfersomes, Tf Pam_2CAG/HA/ErbB2/Man (red), were applied to the skin of 5 mouse groups (5 mice/group). Immunizations were carried out at days 0, 4 and 8. Spleen and lymph node cells were collected on day 28 and cultured either alone (Background, B) or in the presence of ErbB2 (Peptide, P). The number of IFNγ-secreting cells/10^6 cells was measured by direct ELISpot assay. Data from one representative experiment of two independent ones are shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *show the number of spots specific to each peptide (P-B) in the spleens and lymph nodes respectively. Tf: transfersome, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, Man: dioleyil-glycerol-di-mannose (DOG-Man_2).*

In contrast, addition of a DC targeting molecule to TLR4 agonist-bearing transfersomes allows them to induce a systemic immune response and seems to slightly increase the amplitude of the local immune response. (figure 8).

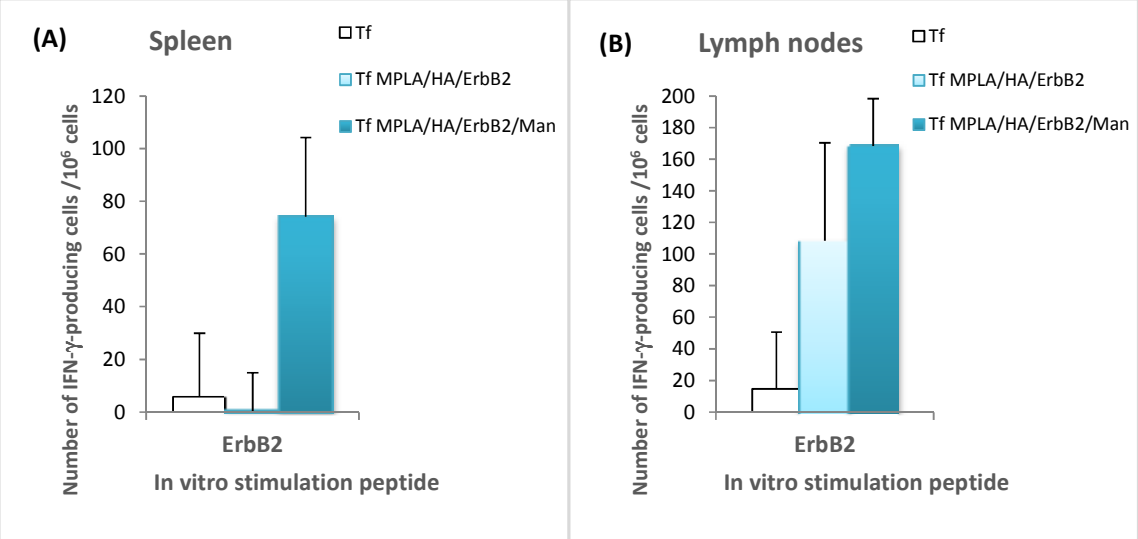

Figure 8: ErbB2-specific IFNγ response in mice immunized with DC targeted liposomal vaccines adjuvanted with MPLA. *Plain transfersomes (Tf) (white), adjuvanted transfersomes, Tf MPLA/HA/ErbB2 (light blue), and targeted adjuvanted transfersomes, Tf MPLA/HA/ErbB2/Man ((dark blue) were applied to the skin of 5 mouse groups (5 mice/group). Immunizations were carried out on days 0, 4 and 8. Spleen and lymph node cells were collected on day 28 and cultured either alone (Background, B) or in the presence of ErbB2 (Peptide, P). The number of IFNγ-secreting cells/10^6 cells was measured by an indirect ELISpot assay. Data from one representative experiment of two independent ones are shown. Results are expressed as mean+/- SEM of ELISpot triplicates.* ***(A)*** *and* ***(B)*** *show the number of spots specific to each peptide (P-B) in the spleens and lymph nodes respectively. Tf: transfersome, MPLA: monophosphoryl lipid A, Man: dioleyl-glycerol-di-mannose (DOG-Man_2).*

Altogether, these results suggest that DC targeting has a more significant effect in transfersomes than in liposomes. Moreover, its role seems to depend on the danger molecule type and varies according to the localization of the immune response. Indeed, when the systemic ErbB2-specific response is suppressed by mannose association with a TLR2/6 agonist (Pam_2CAG), and increased by its association with a TLR2/4 agonist (MPLA). The local immune response is increased by mannose addition to both TLR agonists.

4.3. Transfersome-based vaccines induce a high ErbB2-specific, but a low HA-specific response rate

To evaluate the reproducibility of the experiments performed with transfersomes, we retrospectively evaluated the peptide/background spot number ratios (R=P/B), according to the positivity criteria set above, in 4 different experiments. The results were analyzed by the

method used in paragraph 3.4.2 for the conventional liposome (see table 6). All the results are compiled in table 8.

Table 8: Response rate of spleens and lymph node cell pools of mice vaccinated with transfersome-based vaccines to (A) HA and (B) ErbB2 peptide antigens.

(A) ErbB2-specific response	Tf Pam_2CAG/HA/ErbB2 (TC)		Tf MPLA/HA/ErbB2 (TC)	
	No mannose	+ mannose	No mannose	+ mannose
Spleen	50 % (2/4)	25 % (1/4)	25 % (1/4)	75 % (3/4)
Lymph nodes	100 % (4/4)	100 % (4/4)	50 % (2/4)	75 % (3/4)

(B) HA-specific response	Tf Pam_2CAG/HA/ErbB2 (TC)		Tf MPLA/HA/ErbB2 (TC)	
	No mannose	+ mannose	No mannose	+ mannose
Spleen	50 % (2/4)	25 % (1/4)	25 % (1/4)	50 % (2/4)
Lymph nodes	0 % (0/4)	75 % (3/4)	25 % (1/4)	25 % (1/4)

Spleen and lymph node cell pools of immunized animals were cultured either alone (background) or in the presence of ErbB2 or HA peptide. IFNγ production was assayed by direct ELISPOT. A result is judged positive if it was detected either in the direct or indirect ELISpot assay, according to the positivity criteria defined by Dubey's empirical method.

By analyzing these results, we first observed that the response rates induced by transfersomes were generally lower and more variable than those observed with liposomes. However, we could draw some preliminary conclusions on the cellular immune response induced by TC immunization with transfersomes.

1- Transfersome administration by the TC route induces a cellular immune response against ErbB2 (Table 8A), but almost none against HA (Table 8B)

2- Incorporation of a TLR2/6 agonist (Pam_2CAG) in transfersome-based constructs administered by the TC route induces a systemic immune response (in the spleen) against both peptides and a local response (in lymph nodes) only against ErbB2. In contrast, the TLR4 agonist (MPLA) induces only infrequent immune responses (systemic and local) against both peptides.

3- DC targeting by mannose increased the systemic as well as the local immune responses, mainly the local HA-specific response by the TLR2/6 agonist-bearing Tf.

This study shows that TCI with transfersome-based vaccines incorporating a TLR agonist and expressing a $CD8^+$ ($ErbB_2$) and a $CD4^+$ (HA) T cell epitope induce an immune response which seems less consistent than that of similar liposome-based formulations. Additionally, mannose targeting seems to improve the reproducibility of these responses.

5. Liposomes induce skin DC migration to draining lymph nodes after TC immunization

To elicit an immune response, a vaccine should be able to induce the migration of DCs towards lymph nodes that drain the vaccination site, where an adaptive immune response is initiated. We therefore investigated the capacity of the liposome-based constructs to induce DC migration to the brachial lymph nodes that drain the immunization site, after TC application. We started the analysis with the constructs incorporating MPLA. This TLR agonist was selected before the experiments comparing MPLA and Pam_2CAG efficacy were completed. To this end, BALB/c mice received liposomes incorporating MPLA and mannose, or plain liposomes, both labeled with the DiI fluorochrome, or the buffer in which the liposomes are dispersed. Brachial lymph nodes were harvested 48 hours later, and different DC subpopulations, including $Lang^+$ dDCs, $Lang^-$ dDCs and Langerhans cells, were analyzed by flow cytometry.

We first attempted to determine in each of these skin DC subpopulations the percentage of DiI^+ cells, as this would indicate that they have internalized the fluorescent liposomes. The results are shown in figure 9. They indicate that the percentage of DiI^+ cells in all 3 skin DC subpopulations present in the lymph node is almost nil.

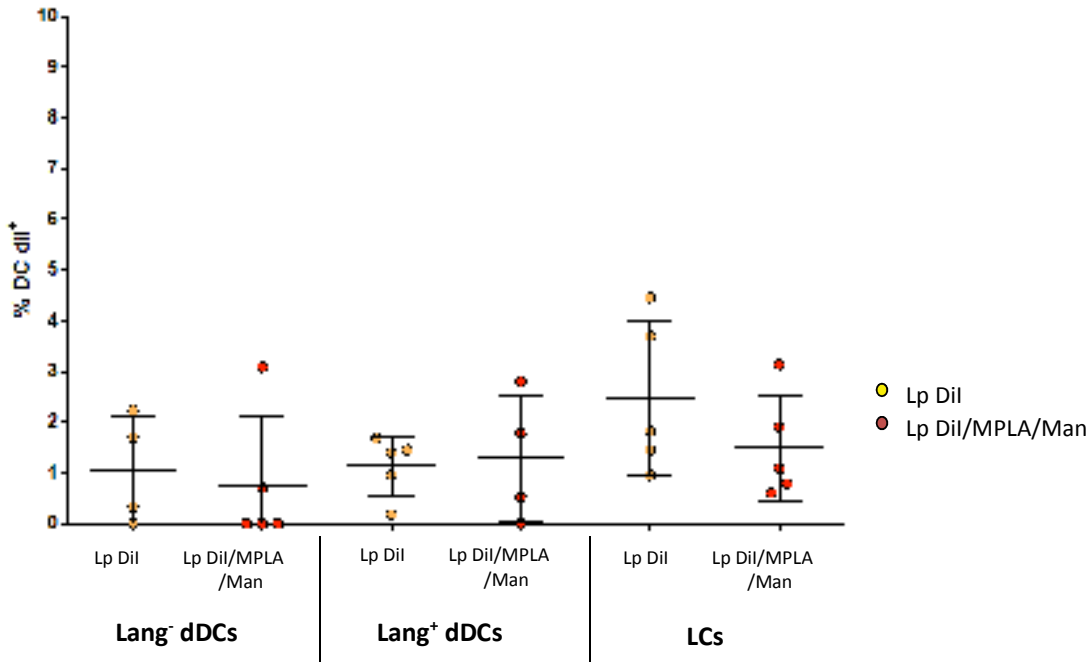

Figure 9: Skin-DCs found in the lymph nodes 48h after immunization with fluorescent liposomes do not show any fluorescence. Liposome dispersion buffer, plain fluoresecent liposomes (Lp DiI) *(yellow) and fluorescent liposomes incorporating MPLA and mannose (Lp DiI/MPLA/Man) (brown) were applied to the skin of 5 BALB/c mice. After 48 h, brachial lymph nodes were harvested and different DC subpopulations were analyzed by flow cytometry. Every spot represents the % of DiI⁺ DCs in one mouse, after the mean percentage of DiI+ DCs obtained in mice immunized with the dispersion buffer was substracted. The mean and standard deviation were also added to the figure. Lp: liposome, DiI: 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate , MPLA: monophosphoryl lipid A, Man: mannose*

To determine whether the absence of fluorescence is caused by a lack of skin DC migration or because DiI fluorescence fades due to liposome internalization and processing in the endosome for 48 hours, we compared the total number of cells of the different skin DC subpopulation in the lymph nodes of mice that received the different liposomal constructs or the negative control. Figure 10 shows that in control mice that received buffer, a low number or skin DCs can be detected in lymph node. In contrast, both liposomal constructs application induces migration of skin DCs, independently of whether they incorporate or not MPLA and mannose. However, all the skin DC subpopulations do not seem to migrate similarly, since LCs and langerin ⁻ dDCs seem to migrate in higher numbers, as compared to langerin⁺ dDCs.

These results show that our liposomal constructs induce the migration of skin DCs after TC immunization. However, it is quite surprising that the plain liposomes, which are devoid of danger signal, induce skin DC migration in the same way as those incorporating MPLA and mannose.

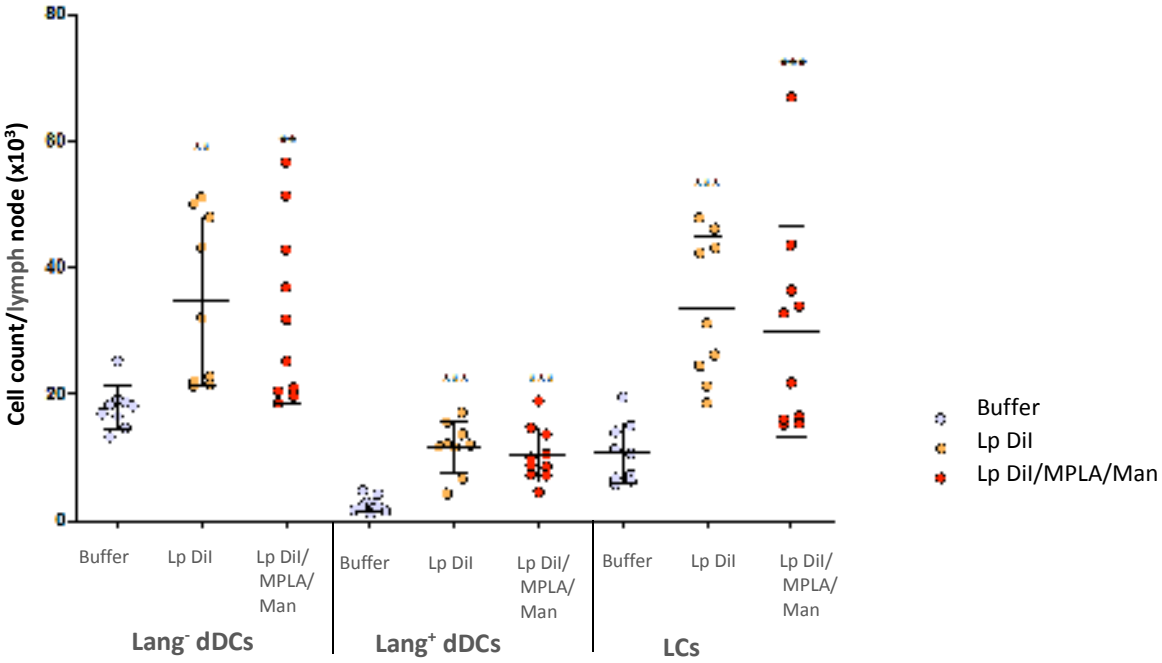

Figure 10: Liposomal constructs induce skin DC migration towards lymph nodes. Liposome dispersion buffer, plain fluorescent liposomes (Lp DiI*) (yellow) and fluorescent liposomes incorporating MPLA and mannose (Lp DiI/MPLA/Man) (brown) were applied to the skin of 10 BALB/c mice. After 48 h, brachial lymph nodes were harvested and different DC subpopulations were analyzed by flow cytometry. After 48 h, brachial lymph nodes were harvested and different DC subpopulations were analyzed by flow cytometry. Every spot represents the % of DCs in one mouse. The mean and standard deviation were also added to each condition. n= 10 mice, in 2 different experiments. Test ANOVA, **=p<0.005 and ***= p<0.0005. Lp: liposome, DiI: 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate , MPLA: monophosphoryl lipid A, Man: mannose*

Discussion

The variable efficacy of therapeutic cancer vaccines that aim to fight established cancers highlights the need to improve tumor-directed T cell responses in the patient.

A liposome-based cancer vaccine has been previously developed by our team. It allows the delivery of all vaccine components needed by the immune system to induce a cancer specific response. To induce DC maturation, the liposomal constructs incorporate a danger signal provided by a TLR 2/6 agonist (Pam_2CAG), and to activate T lymphocytes they incorporate a $CD4^+$ (HA 307-319) and a $CD8^+$ (ErbB2 63-71) epitope peptides. These constructs proved to be immunogenic when delivered by the subcutaneous (Roth *et al.*, 2005; Thomann *et al.*, 2011) and intranasal routes (Kakhi *et al.*, 2015, 2016), and, most importantly, they protected mice against tumor challenge in therapeutic vaccination settings.

The skin is an immunological barrier rich in multiple DC subpopulations that can mount adaptive immune responses and protect the organism from external aggressions. Given the central role of DCs in the antitumor immune response, the skin appears as a highly suitable administration site of cancer vaccines. Application of these vaccines on skin surface, termed transcutaneous (TC) vaccination, is expected to target skin DCs in the best way to harness their immunostimulatory potential. The TC immunization route has recently made its first steps towards clinical use by the success of a phase I clinical trial of an influenza vaccine delivered to the skin using a microneedle array. The vaccine has proven to be safe and as immunogenic as the intramuscularly delivered one, providing a proof of concept and a strong rationale for the investment of further efforts in the development of vaccines for the TC route (Rouphael *et al.*, 2017).

Because the physicochemical properties of liposomes are reported to favor their TC passage through the skin, we aimed in the present work to deliver our liposomal cancer vaccines by the TC route and to assess their capability to induce potent cellular adaptive immune responses. We also attempted to increase the potency of the immune response by optimizing their composition and physicochemical characteristics.

In a first step, we chose to use an easy-to-use vaccination technique that consists in applying the vaccine dose by massage on previously shaved, ethanol-wiped and dry skin. Ethanol

application on the skin (Hirschberg *et al.*, 2012) is intended to solubilize skin lipids and induce disorder in the lipid structure of the *stratum corneum*, thus mildly disrupting the skin barrier and increasing its fluidity and permeability. Another strategy for skin barrier disruption is the use of tape stripping, a more aggressive technique that removes few layers of the *stratum corneum*, thus favoring the penetration of applied vaccine (Inoue *et al.*, 2005; Loan Honeywell-Nguyen *et al.*, 2002). However, tape stripping induces cellular damage. Upon epidermis disruption, danger signals and subsequent signaling through PRR induce the secretion of proinflammatory cytokines such as IL-1α, IL-1β, tumor necrosis factor (TNF)-α and granulocyte/macrophage-colony-stimulating factor (GM-CSF) (Clausen and Stoitzner, 2015; Kaurav *et al.*, 2016; Li *et al.*, 2011; Partidos and Muller, 2005). By contrast, our immunization technique involving a very mild *stratum corneum* disruption using ethanol avoids these uncontrolled inflammatory effects.

To assess the development of a systemic and local peptide-specific cellular immune response, we investigated the presence of specific T cells producing IFNγ, the surrogate of T lymphocyte responses of Th1 and CTL profiles, using an ELISpot assay. In addition to the spleen, we tested the lymph nodes that drain the skin application zone, namely the inguinal, axillary and brachial lymph nodes. The ELISpot technique is a sensitive and powerful tool that allows the detection of rare antigen-specific lymphocytes to the single-cell level, as rare as 3 cells per well (Karulin *et al.*, 2015). Moreover, the sensitivity of the standard ELISpot assay can be increased by culturing the cells *in vitro* during a longer incubation time that can reach 12 days, in presence of antigen and IL-2, to induce the expansion of antigen-specific lymphocytes. This increased-sensitivity ELISpot variant is known as indirect ELISpot assay, or cultured ELISpot assay (Calarota and Baldanti, 2013). In the present work, because the induced immune responses were low, we assessed their presence using both a standard and an indirect ELISpot assay.

The interpretation of ELISpot data becomes problematic when the signal is low, and is accompanied by a variable background spot count. In our case, the spot count in antigen-stimulated wells was frequently increased as compared to control wells. However, the amplitude of the detected response remained low, indicating that a statistical analysis of the significance of these results is not suitable. Alternatively, several empirical methods, or

empirical rules ("ER"), usually based on observations from a specific study were developed and employed for detecting positive ELISpot signals in clinical trials for infectious diseases and cancer (Dubey *et al.*, 2007; Lai *et al.*, 2011; Lewis *et al.*, 2000; Moodie *et al.*, 2010). A step-by-step empirical method was illustrated by Dubey *et al.,* to set and validate positivity criteria for an ELISpot assay for the detection of responses against a set of HIV peptides (Dubey *et al.*, 2007). Since empirical methods are dependent on the reagents, the test settings and the populations that are being tested, they are internally set up. Therefore, we followed Dubey's steps to generate positivity criteria that can be used in our conditions to analyze the direct and indirect ELISpot assay.

Before evaluating the immunogenicity of liposome formulations administered by the TC route, we investigated whether the potential $CD8^+$ T cell peptide epitope ErbB2 can elicit a cellular immune response following a previously validated robust TCI protocol. As described in the literature, cholera toxin is a powerful adjuvant (Kawamura *et al.*, 2003; Schnitzler *et al.*, 2007) that induces potent humoral as well as cellular responses by the TC route, including CTL responses (Glenn *et al.*, 1998, 1999; Kahlon *et al.*, 2003). However, due to its toxicity, cholera toxin cannot be used in humans and exerts multiple secondary effect and, therefore, is used in the present study only as a positive control for TC immunization. The mechanism by which cholera exerts its adjuvant effect is debated. While some studies reported that its immunostimulatory potential is unconventional and independent of TLR signaling (Kahlon and Dutz, 2003; Olvera-Gomez *et al.*, 2012), others suggest it to strongly depend on the activation of TLR4 (Liu *et al.*, 2016; Phongsisay *et al.*, 2015). Indeed, our results showed that administration of a physical mixture of cholera toxin and ErbB2 by massage on ethanol-wiped and dried skin induces a potent local and systemic immune response, thus indicating that ErbB2 is suitable to be administered by the TC route. Therefore, we prepared liposomal constructs incorporating this peptide associated to a T $CD4^+$ epitope and a danger signal.

We formulated conventional liposomes (Lp-SUV) as well as ultradeformable ones, or transfersomes (Tf-SUV). Tf were chosen because the presence of ethanol and a surfactant in their composition increases their fluidity and makes them ultradeformable. The surfactant decreases their resistance at tension points and allows them to deform, thus squeezing into pores smaller than their size. Therefore, they were reported to cross the *stratum corneum*

barrier more efficiently than conventional liposomes (Cevc and Gebauer, 2003; Cevc *et al.*, 1998).

Lp-SUV were obtained by sonication of Lp-MLV prepared from PC, PG and Chol, in proportions similar to those previously described by our team (Espuelas *et al.*, 2008; Kakhi *et al.*, 2015, 2016; Thomann *et al.*, 2011). Tf-SUV were obtained by manual extrusion of Tf-MLV prepared with SPC, SDC and ethanol (Cevc and Blume, 1992; Kakhi *et al.*, 2016). These formulation techniques allowed us to reproducibly obtain monodispersed liposomal constructs, with a size < 100 nm, which is suitable for skin barrier crossing and uptake by DCs.

In order to display the peptides on the surface of Lp-SUVs and Tf-SUVs, an amphiphilic thiol reactive anchor, DPGmal, was added to the vaccine composition and was inserted in the lipid bilayer. Cysteine-containing peptides were subsequently covalently linked to the maleimide group of the anchor by the Michael addition (Schelté *et al.*, 2000). This soft coupling step in aqueous medium preserves the narrow size distribution of the preformed liposomes resulting in reproducible liposome-based constructs.

Beside epitope peptides, immunostimulatory molecules, namely a TLR 2/6 agonist and a TLR 4 agonist (Pam_2CAG and MPLA), in addition to a DC-targeting molecule (DOG-$Mann_2$, or mannose) were incorporated in the lipid bilayer of SUVs. In order to maintain the overall charge of formulations, additions of amphiphilic molecules in a proportion > 1% was compensated by an equivalent decrease in PC proportion. Through its diacylglycerol moiety, Pam_2CAG a diacetylated lipopeptide derived from the N-terminal moiety of E-coli lipoprotein), interacts with TLR 2/6 heterodimers (Oliveira-Nascimento *et al.*, 2012; Omueti *et al.*, 2005). MPLA, a chemically detoxified form of the lipid A, the anchor moiety of lipopolysaccharides (LPS) is currently one of the leading innovative vaccine adjuvants (Alving and Rao, 2008). Because the adjuvant molecule is a decisive factor of the immune response induced by a vaccine, we compared both adjuvants in order to choose the most appropriate one for this route of administration.

Mannose was chosen to be a di-antennary molecule, because previous work in our team comparing the efficacy of mono-, di-, and tetramannosylated ligands showed that both di- and tetramannosylated onces drastically improve liposome uptake by DCs with no significant

difference among them (Espuelas *et al.*, 2008). Because dimannnose is easier to synthetize, it was chosen to be used in the present work,

In a proof of concept, we first compared the immune response induced by administration of the original liposomal construct incorporating the TLR2/6 agonist by the TC route and the SC route. Our results show that TC application of this construct triggers local (in the lymph nodes) and systemic (in the spleen) immune responses against the $CD4^+$ and the $CD8^+$ T-cell peptides that are similar to those induced by subcutaneous injection. Efficacy of liposomal carriers in TC barrier crossing was established in 1980 when they were used for the first time for topical drug delivery and were found to achieve a four- to five-fold increase of the drug concentration in the epidermis and the dermis, as compared to drug alone (Mezei and Gulasekharam, 1980). However, several studies report that liposomes fuse on the *stratum corneum* surface instead of penetrating it, as shown by the observation of the behavior of bilayer-forming surfactant L-595 (sucrose laurate ester) rigid vesicles applied on the skin under an electron microscope and their absence in the viable layers (Loan Honeywell-Nguyen *et al.*, 2002). Interestingly, Trauer *et al* found that massage may improve the delivery of particulate substances, including conventional liposomes, into hair follicles, which constitute a port of entry to the internal skin layers (Trauer *et al.*, 2014). Therefore, the induction of an immune response after TC application of our liposome-based constructs indicates that liposomes were indeed capable of reaching the internal skin layers and to be taken up by skin DCs in order to induce T cell responses. We believe that this may be facilitated by our massage vaccination techniques. To our knowledge, TC immunization with conventional liposomes delivering peptides/proteins has been limited to encapsulated model-antigens such as ovalbumin or tetanus toxoid, where the humoral response, but rarely the cellular response was assessed (Gupta *et al.*, 2014; Zhang *et al.*, 2017). In this report, we show that liposomes displaying simple epitopes on their surface are able to induce a primary immune response via the TC route.

For the development of efficient vaccines, the determination of the most optimal immunostimulatory molecule is essential and may influence the type of the induced immune response. Therefore, the first attempt to improve our liposome-based formulations was by replacing the TL2/6 agonist (Pam_2CAG) with a TLR4 agonist (MPLA). Pam_2CAG induces the maturation of human monocyte-derived DCs *in vitro*, as indicated by the expression of CD80, CD83, CD86 and HLA-DR molecules (Espuelas *et al.*, 2005b), and triggers protective

tumor-specific responses *in vivo* (Heurtault *et al.*, 2009; Thomann *et al.*, 2011), whereas liposome- incorporated MPLA triggers T cell responses (Alving *et al.*, 2012b). The comparison of the immune response induced after TCI with the liposomal constructs incorporating Pam_2CAG or MPLA showed that both constructs can induce a systemic immune response, however, only Pam_2CAG-bearing construct could induce a detectable local immune response. While TLR2, TLR4 and TLR6 are expressed on LCs (Fehres *et al.*, 2015; de Koning *et al.*, 2010), their functionality seems to be debated. Indeed, Flacher *et al.* reported that while TLR2 is functional and its engagement induces LC maturation, TLR4 signaling is impaired (Flacher *et al.*, 2006). Accordingly, intradermal administration of several TLR agonists revealed that signaling through TLR2, but not through TLR4, induces a local inflammatory response by LCs (Oosterhoff *et al.*, 2013). On the other hand, dDCs express both TLR2 and TLR4 (Aar *et al.*, 2007; Rozis *et al.*, 2008). The ability of our TLR4 agonist-bearing liposomes to induce systemic responses suggests that either TLR4 is functional on LCs, in contrast to other findings in the literature, or that dDCs alone are responsible for the induction of the observed immune response.

Targeting of vaccines to C-lectin type receptors, such as mannose receptors, has been shown to increase uptake by DCs (Espuelas *et al.*, 2008; Jeong *et al.*, 2014; Markov *et al.*, 2015; Thomann *et al.*, 2011; Vyas *et al.*, 2010), thus resulting in an improved DC activation, and vaccine-specific T cell priming and proliferation. The most widely targeted receptor is the mannose receptor. When the liposome-based constructs developed by our team were targeted to DCs by mannose addition, they were found to conserve their immunostimulatory potential despite a 100-fold reduction of the adjuvant amount, and to conserve their antitumor efficacy despite a 10-fold reduction of the vaccine dose (Thomann *et al.*, 2011). Therefore, in an attempt to increase the immunogenicity of our liposome-based constructs in the present work, we targeted them to DCs by adding a DC targeting molecule to their composition, namely, di-mannose. Liposome targeting to DCs had variable effects. It slightly increased the amplitude of the cellular response against ErbB2 peptide in the spleen, while it was at most ineffective for local immune responses in the lymph nodes. The mannose receptor is expressed on dDCs, however its expression on LCs seems controversial. Some studies report that it is expressed on LCs in normal skin (Condaminet *et al.*, 1998; de Koning *et al.*, 2010), while others report it to be only induced under inflammatory conditions (Fehres *et al.*, 2015;

Plzáková *et al.*, 2004; Polak *et al.*, 2014; Wollenberg *et al.*, 2002). Therefore, improved immunogenicity following mannose addition to our liposomal constructs does not provide enough information regarding the targeted DC subpopulation in the light of current evidence.

For improved targeting of LCs and/or dDCs, further studies should be pursued to investigate the expression of mannose receptor on LCs. Choosing another receptor to target, such DEC-205, is also an interesting alternative. DEC-205 is expressed on both LCs and dDCs, even if its expression is low on LCs, and its targeting has resulted in potent immune response induction (Stoitzner *et al.*, 2014).

Because the flexibility of the vesicular carrier affects its passage through the skin barrier, it also modulates its activity. Despite their use for TC drug delivery (Jain *et al.*, 2017), liposomes were reported by some authors to be suboptimal for TC delivery and to enhance deposition in the upper layers of the skin rather than transdermal penetration, thus mediating topical and not TC delivery (Ashtikar *et al.*, 2016). Therefore, modified liposomes were formulated to improve TC passage such transfersomes (Cevc and Blume, 1992; Cevc *et al.*, 1998). In the present work, we aimed to optimize the liposomal constructs for the TC route by replacing the conventional liposomal vesicle with a transfersome. Surprisingly, transfersome-based constructs did not improve the induced cellular immune response. On the contrary, their almost failed to induce reproducible HA-specific responses.

Comparative studies of transfersomes and liposomes were mostly conducted in the context of drug delivery. A few studies assessed their immunostimulatory capacity, in which the results were sometimes discordant. Cationic ultradeformable liposomes delivering HBs antigen DNA were found to induce potent cellular and humoral immune responses that were superior to those induced by conventional liposomes, and comparable to intramuscular injection of naked DNA (Wang *et al.*, 2007). Gupta *et al* found that transfersomes induce higher anti-TT titers as compared to conventional liposomes (Gupta *et al.*, 2014). Similarly, transdermal immunization with ultradeformable liposomes was found to induce stronger cellular and humoral immunity against merozoite surface protein-1 (PfMSP-1_{19}) of *Plasmodium falciparum,* as compared to conventional liposomes (Tyagi *et al.*, 2016). By contrast, other studies showed that transfersomes exert the same effect as conventional liposomes. A comparative *in vitro* skin permeation study, conducted by Rattanpack *et al.* on transfersomes and liposomes, showed

that transfersomes do not significantly increase penetration as compared to conventional liposomes (Rattanapak *et al.*, 2012). Accordingly, transfersomes were found to induce the same antibody titers as those induced by liposomes entrapping the model antigens Ova (Zhang *et al.*, 2017).

A possible explanation of the decreased reproducibility of responses with the transfersomes is that their slightly bigger size could have negatively influenced their TC passage. When insufficient vaccine amount is delivered to the DC, a less potent immune response is induced. However, this hypothesis is unlikely to be true, since the size difference is less than 20 nm. To confirm it, transfersomes of the same size as liposomes (60 nm) have to be evaluated.

Another possible explanation is that their ultradeformability could have negatively influenced their stability, resulting in a decreased depot effect at the administration site. Since one of the most important assets of nanoparticles for their immunogenicity is their potential to exert depot effect, this results in a decreased immunostimulation.

In parallel to the experiments aiming to detect local and systemic immunity induced by the TC delivery of our liposomal constructs, we used a mechanistic approach to investigate the migration of skin DCs subtypes to the lymph nodes. Fluorescent liposomes were obtained by addition of a lipophilic dye, DiI, in the lipid bilayer. DiI is routinely used for long term tracking of cells and liposomes (Litzinger *et al.*, 1994; Shah *et al.*, 2015; Yefimova *et al.*, 2014). When fluorescent liposomes are taken up by DCs, the cells become fluorescent. In addition to mannose which aims at preferentially targeting DCs, fluorescent liposomes incorporated a TLR agonist. MPLA was chosen arbitrarily since the result of the immunogenicity of the constructs were not clear yet.

No fluorescent skin cells could be detected in the lymph nodes. Interestingly, we could detect fluorescent skin DCs in the epidermis as well as in the dermis (data not shown) confirming that our results were not due to a lack of liposome uptake in the skin. Besides, the increased numbers of skin DCs in the lymph nodes 48h after TC application of liposomes, confirmed that the lack of fluorescence was not due to an absence of migration of DC that have internalized the fluorescent liposomes either. Therefore, we speculate that DiI dye could be unstable at the endosomal pH for long periods of time.

Skin DC subsets that were found to preferentially migrate to the lymph nodes are LCs and Lang^{-} dDCs. Both of these populations are capable of antigen cross-presentation to CD8^{+} T cells, and were reported to induce CTL differentiation. Stoitzner *et al.* showed that LCs can induce OVA-specific CTLs (Stoitzner *et al.*, 2006) . Lang^{-} as well as lang^{+} dDCs were found to be involved in T cell cross-priming (Nizza and Campbell, 2014). In humans, LCs and *in vitro*-generated dDCs were found to capture and cross-present melanoma antigens to CD8^{+} T cells(Cao *et al.*, 2007). Naturally occurring human LCs and Lang^{-} dDCs were also found to induce CD8^{+} T cell priming against two different melanoma antigen peptides (the melanoma differentiation antigen MART-1 peptide, and the glycoprotein gp100 peptide). However, LC were found to be more efficient since the generated CTL had an increased cytotoxic activity (Klechevsky *et al.*, 2008).

Surprisingly, both plain liposomes and those incorporating MPLA and mannose induced the migration of skin DCs to the lymph nodes in similar numbers. Two interpretations are possible. The first hypothesis involves a technical problem that may have occurred during the formulation step, and that may have resulted in liposome contamination with molecules capable of inducing DC activation, such as bacterial products. This hypothesis will be verified by repeating the experiment under more rigorous conditions to ensure the liposomes are devoid of any DC activating molecule. Another hypothesis is that liposome constituents may have the capacity to activate DCs. However, in contrast to cationic liposomes capable of inducing DC maturation and secretion of pro-inflammatory cytokines (Lonez *et al.*, 2012; Soema *et al.*, 2015), it is improbable that our anionic constructs are capable of exerting such effects. Previous studies in our laboratory have shown that they do not induce the maturation of human DCs *in vitro* (Espuelas *et al.*, 2005b, 2008). This hypothesis may be verified by investigating the maturation state of the migrating skin DCs, by assessing the expression of maturation markers, such as CD80 and CD86. LCs and dDCs were described to have the capacity to migrate to draining lymph nodes after antigen uptake, without acquiring a mature state. (Sparber *et al.*, 2010). Therefore, we speculate that even if plain liposomes induce the migration of skin DCs to the lymph nodes, for an unknown reason yet, they do not induce their maturation, in contrast to TLR agonist-containing liposomes.

In conclusion, we report herein the development of a TC vaccination strategy involving the use of liposome-based constructs and resulting in the induction of local and systemic immune

responses. Altogether, our results provide a rationale for the use of the liposome technology to develop and improve cancer vaccines for TC delivery.

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The work reported in this first chapter allowed us to ascertain some keypoints usefull for future investigation:

First, the formulation process is well controlled across newly introduced variations. All our liposome construct variants were prepared using the lipid film hydration technique. They differed by the TLR agonist (TLR2/6 agonist, Pam_2CAG, or TLR 4 agonist, MPLA), the presence of a DC-targeting molecule (di-mannose) and the physicochemical properties of the lipid vesicle, resulting in either conventional liposomes or more flexible ones called transfersomes. All the formulations were monodispersed with diameter < 100 nm, and the peptide coupling rates were high, indicating that our formulation technique is robust and reproducible.

Second, TC administration of our constructs proved to induce similar immune responses to those induced by the SC route. Our attempts to further optimize the liposome formulation showed the TLR2/6 agonist to be superior to the TLR4 agonist, since it induced a local and a systemic immune response, whereas the latter induced only a systemic one. Both di-mannose addition to the constructs, and the replacement of the conventional liposome vesicle with a transfersome, did not improve the immune response.

Interestingly, we showed through these findings that our liposomal constructs are promising tumor-specific vaccines for the TC route and defined the composition of the most optimal formulation in our model, thereby, reaching the first objective of this work.

To pursue the development of our liposome-based vaccines for the TC tumor-specific vaccination, their tumor-specific efficacy by the TC route has to be evaluated in mice bearing ErbB2-expressing tumors. On another level, it would be interesting to optimize the vaccine formulation by varying further one or more of its elements. The danger molecule can be optimized by combining multiple agonists which may have a synergistic effect. The $CD4^+$ T cell epitope can be varied by choosing peptides derived from the target TAA, and, finally, the carrier liposomal vesicle can be replaced with a lipid nanocapsule which currently seems promising for TC immunization.

To further develop the liposome constructs in the view of an ultimate human use, we first need further insights into the different immune activation potentials of the various formulations. Therefore, it appears necessary for us to run comparative studies of the

migration of skin DC subpopulations induced by liposomes and transfersomes. They are expected to inform us about the identity, the number and the maturation state of migrating skin DC subpopulation in addition to the kinetics of this migration. Correlation of this information with the induced immune response may contribute to further understanding of the differences observed in the detected immune responses. Responses induced in animal models may deviate partially or totally from those observed later in clinical trials. Therefore, always in the view of a human use, evaluating the liposome constructs in a context that is more predictive of the human immune response is crucial.

Chapter 2: NOD-SCID-IL2r gamma null mice engrafted with human splenocytes show promise for the evaluation of liposome-based candidate vaccines

My host laboratory in the Lebanese University has significantly contributed to the development of a humanized mouse model in which immunodeficient NSG mice are engrafted with normal human splenocytes (Hu-SPL-NSG). These splenocytes derive from a bank generated from spleens of organ donors or of individuals who have undergone clinically indicated splenectomy. In previous works, the Hu-SPL-NSG mouse model had proven its capability to mount primary human immune responses. It was found to respond to immunization with the Merozoite Surface Protein (MSP)-3 of *Plasmodium falciparum* and to the F protein of the Respiratory Syncytial Virus (RSV) by secreting specific human antibodies (Bouharoun-Tayoun et al., 2004; Chamat et al., 1999). Therefore we chose the Hu-SPL-NSG mouse model as a surrogate for the evaluation of the human immune response and tested the immunogenicity of our liposome-based constructs in it.

The ultimate goal of this part of my thesis was to verify whether our liposomal constructs can induce a $CD8^+$ T cell response in the Hu-SPL-NSG mouse. However, the work in this model faces several challenges and implies various difficulties and complications. From choosing precious human spleens that have a suitable MHC class II genotype capable of presenting the $CD4^+$ T cell peptide epitope to euthanizing the Hu-SPL-NSG mice and verifying their reconstitution and the induction of a human immune response, more than two months may run out. In addition, the ability of the Hu-SPL-NSG mouse model to mount human immune responses is established mostly for the humoral responses. Finally, humoral responses can be assessed regularly in mice sera in the ongoing experiment whereas cellular immune responses can be evaluated in secondary lymphoid organs only after animal euthanasia. For all these reasons, in a first proof of concept, we replaced the $CD8^+$ T cell epitope with a B cell epitope in our liposomal platform incorporating a $CD4^+$ T cell epitope and a TLR agonist and verified its ability to induce human humoral and $CD4^+$ T cell immune responses in the Hu-SPL-NSG mouse after administration *via* a conventional vaccination route. The chosen B cell epitope was derived from the pilin of *Pseudomonas aeruginosa* strain K (PAK), and the B cell epitope-bearing-constructs were

previously evaluated in a conventional mouse model (Heurtault *et al.*, 2009). The results of this section are presented in the following **scientific article#2** (in preparation).

Scientific article #2:

NOD-SCID-IL2r gamma null mice engrafted with human splenocytes show promise for the evaluation of liposome-based candidate vaccines

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In preparation

Introduction

In the preclinical development stage of biomedical products, including vaccines, the rodent models are the most widely used ones because they are convenient, easy to manipulate and they largely mirror the human biology at different levels, including the immune system. However, divergence of clinical results from the ones expected based on preclinical trials frequently interrupts the vaccine development pipeline, at a high logistic and financial loss (Mestas and Hughes, 2004). Such occurrences underline the fact that murine immune responses do not always reflect the human ones. Therefore, even though mice have always been and will remain an attractive *in vivo* model for preliminary studies of human immunology, the development of complementary models for further investigation of promising candidates is of a primordial importance. Mice with severe combined immunodeficiency (SCID) reconstituted with human cells have been used to study the human immune response to a wide variety of antigens. Our team has contributed to the development of a humanized mouse model, in which SCID mice are engrafted with human splenocytes (Hu-SPL-SCID) (Brams *et al.*, 1998, 1998; Chamat *et al.*, 1999). We have shown that in this model it is possible to induce primary human responses against several antigens, including the F protein of the Respiratory Syncitial Virus (Chamat *et al.*, 1999),the horse ferritin (Brams *et al.*, 1998) and the Merozoite-surface-protein-3 of *Plasmodium falciparum* (Bouharoun-Tayoun *et al.*, 2004). Nonetheless, the immunization protocol relied on the use of whole proteins or large peptides mixed with potent adjuvants such as complete Freund's adjuvant, which cannot be used in clinic.

The current trend in modern vaccination is to avoid the use of large antigens and replace them with minimal pathogen-derived antigenic sequences that would reduce the risk of vaccine toxicity, reactogenicity and off-target effects. These peptide-based vaccines require the co-administration of adjuvants that provide danger signals needed for dendritic cell (DC) maturation, the latter being central for the induction of adaptive immune responses. Modern adjuvants are pathogen-derived components, chosen to be Microbe-Associated Molecular Patterns (MAMPs), such as bacterial lipopeptides (Tang *et al.*, 2012). To deliver the vaccine peptides and the adjuvant simultaneously to the same DCs, a new strategy using lipid nanoparticles such as liposomes is rapidly developing (Schwendener, 2014).

Liposomes have been used as viral vaccine delivery vectors for more than 15 years in clinic and are, in this case, termed virosomes. Epaxal, a hepatitis A vaccine consisting of virosomes displaying inactivated viral particles on their lipid bilayers, was shown to induce rapid and long lasting immunity (Ambrosch *et al.*, 1997; Bovier, 2008). Another virosome-based vaccine, Inflexal, directed against influenza, was recently approved for clinical use. It proved to be more immunogenic than the conventional influenza vaccine (Fan and Zhang, 2013).

Our team has developed liposome-based vaccine candidates that co-deliver a target peptide containing a B cell or a $CD8^{+}$T cell epitope, as well as a universal $CD4^{+}$ T cell epitope derived from the hemagglutinin of the influenza virus (HA), and a TLR agonist as an adjuvant (Heurtault *et al.*, 2009; Thomann *et al.*, 2011). One of these vaccines targets *Pseudomonas aeruginosa*, an opportunistic human pathogen that causes respiratory and urinary tract infections in patients with impaired immunity. The adherence of *P. aeruginosa* to the host cells is mediated by polar pili on the bacterial surface (Campbell *et al.*, 2003; Salter, 2015). Therefore, antibodies directed against certain regions of these pili are capable of blocking bacterial infections.

The liposome vaccine contains a 17 aminoacid B cell epitope peptide derived from the C-terminal receptor-binding region (residues 128–144) of *P. aeruginosa* pilin protein strain K (PAK) (Campbell *et al.*, 2003; Lee *et al.*, 1989). The native peptide PAKcys (KCTSDWDEQFIPKGCSK) contains two cysteine residues and therefore, is cyclic and yields low coupling rates on functionalized liposomes (Heurtault *et al.*, 2009). In a previous work, our team has found that replacing one of the two cysteine residues with a serine, resulting in a linear PAKser peptide (KCTSDWDEQFIPKGSSK) yields improved coupling rates. In BALB/c mice, we have shown that intraperitoneal administration of PAK peptide-containing constructs resulted in the induction of high anti-PAK IgG titers with both PAKcys and PAKser peptides, while intranasal administration induced IgA antibodies. Additionally, PAKser induced higher specific antibody titers as compared to PAKcys, and those antibodies were found to recognize to native peptide Therefore, we chose PAKser to incorporate in our construct during this work (Heurtault *et al.*, 2009).

In the present work, we aimed to evaluate the suitability of a humanized mouse model for preclinical testing of liposome-based vaccines, using the liposomal PAK vaccine candidate. We used NOD-SCID-γnull (NSG) mice, which are more severely imunodeficient that SCID mice and

therefore more receptive for human xenografts (Ito *et al.*, 2012; Lepus *et al.*, 2009). We first assessed the safety of different liposome formulations incorporating diverse TLR agonists towards human splenocytes *in vitro.* The most suitable TLR agonist was then incorporated into liposomes bearing HA and PAK peptides, and used to immunize Hu-SPL-NSG mice. We evaluated the effect of concomitant liposomes injection on the capacity of human splenocytes to survive in the mice, home to the spleen and remain functional. Finally, the liposomes potential to induce humoral and cellular responses was determined.

Material and methods

1. Immunodeficient animals

NOD.Cg-Prkdc^{scid}-IL2rg^{tm1Wjl} /SzJ (NSG) mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed and bred in sterile microisolator cages in our facilities at the Lebanese University according to the US animal care and experimentation guidelines. All food, water, caging and bedding was autoclaved before use. Six- to 8-week old NSG mice were included in the experiments.

2. Proteins and peptides

The following peptides and conjugates were obtained from Genosphere (Paris, France): *P. aeruginosa* strain K (PAK) pilin-derived PAKSer (KCTSDWDEQFIPKGSSK) peptide (Campbell *et al.*, 2003); influenza virus haemagglutinin-derived HA 307–319 (PKYVKQNTLKLAT-C) (O'Sullivan *et al.*, 1991); Keyhole limpet hemocyanin-conjugated PAK (PAK-KLH); Bovine serum albumin-conjugated PAK (PAK-BSA). All products purity, as assessed by HPLC, was > 80%.

3. Formulation and characterization of liposomal constructs

3.1. Lipids and adjuvants

Egg yolk L-α-phosphatidylcholine (PC) and cholesterol (Chol, recrystallized in methanol) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France); their purity exceeded 99%. L-α-phosphatidyl-DL-glycerol transesterified from egg yolk PC (PG) was purchased from Avanti Polar lipids (Alabaster, AL). The lipopeptides S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteinyl-alanyl-glycine (Pam_2CAG) and S-[2,3-bis (palmitoyloxy)-(2R)-propyl]-N-palmitoyl-(R)-cysteinyl-alanyl-glycine (Pam_3CAG) and the thiol-functionalized lipid anchor dipalmitoylglycerol maleimide (DPGMal) were synthesized in our laboratory as previously described (Espuelas *et al.*, 2003, 2008; Heurtault *et al.*, 2009; Roth *et al.*, 2004). The lipopolysaccharide derivative Monophosphoryl Lipid A (MPLA) was purchased from Invivogen (San Diego, CA). All reagents were conserved under argon at -20°C.

3.2. Formulation of liposomal SUVs (liposomes)

Multilamellar vesicles (MLVs) were prepared by lipid film hydration technique. Briefly, chloroform/methanol (9/1 v/v) solutions containing PC, PG, Chol, DPG-mal and adjuvant (table 1) were mixed in a round-bottom Pyrex tube, and slowly evaporated under a continuous flow

of argon. The molar proportions of each of the constituents is shown in table 1. The resulting lipid film was completely dried under high vacuum for 1 h. It was then hydrated in 10 mM Hepes buffer (pH 7.4) containing 5% w/v sorbitol by rigorous vortex mixing, to yield a phospholipid concentration of 15 mM. The resulting MLV suspension was sonicated (1 sec cycle every 3 sec) for 1 hour at room temperature under a continuous flow of argon, using a Vibra Cell 75041 ultrasonicator (750 W, 20 kHz, Fisher Bioblock Scientific, Illkirch, France) equipped with a 3 mm-diameter tip probe (40% amplitude). The resulting SUV preparations were centrifuged twice at 10,000 g to remove the titanium dust originating from the probes. Formulations were snap frozen in liquid nitrogen after addition of 5% glucose as cryoprotectant and stored at -80°C until use.

3.3. Peptide conjugation to SUV

Potential disulfide bonds of cysteine residues between peptides that may result in peptide dimerisation, were reduced with 0.7 M eq. tris(2-carboxyethyl) phosphine (Interchim, Montluçon, France), for 15 minutes under argon. Equimolar quantities of $CD4^+$ and $CD8^+$ T cell epitope peptides were then coupled to freshly prepared SUVs by Michael addition in 10 mM Hepes buffer (pH 6.5) containing 5% (w/v) sorbitol (0.5 molar eq of each peptide *vs* surface accessible thiol-reactive maleimide functions) according to a two-step procedure. In a first step, 0.5 molar eq. of B epitope peptide (PAK) *vs* surface accessible thiol-reactive maleimide function of DPGMal (final molar ratio of 2.5%) were added and incubated for 2 h. In a second step, the PAK-coupled formulations were incubated with the $CD4^+$ T cell epitope (HA) for 2 h. Incubations were performed under argon at room temperature. A 10-fold excess of β-mercaptoethanol was then added for 30 minutes to inactivate all unreacted maleimide groups on internal and external surfaces of SUVs. Then, the formulation was extensively dialyzed (Spectra/Por, exclusion limit of 12–14 kDa, Spectrum laboratories, DG Breda, Netherlands) against a 10 mM Hepes buffer (pH 7.4) containing 5% (w/v) sorbitol to eliminate unreacted reagents and peptides.

Table 1: Composition of formulated liposomes.

	Formulations	Composition	Molar Proportion
Non-peptide incorporating constructs	Lp MPLA 2%	PC/PG/Chol/MPLA	78/20/50/2
	Lp Pam_3CAG 2%	PC/PG/Chol/ Pam_3CAG	78/20/50/2
	Lp Pam_2CAG 2%	PC/PG/Chol/ Pam_2CAG	78/20/50/2
	Lp Pam_2CAG 0.2%	PC/PG/Chol/ Pam_2CAG	78/20/50/0.2
Peptide incorporating constructs	Lp Pam_2CAG 2%/HA/PAK	PC/PG/Chol/DPG-Mal/Pam_2CAG/ HA/PAK	73/20/50/5/2/1.25/1.25
	Lp Pam_2CAG 0.2%/HA/PAK	PC/PG/Chol/DPG-Mal/Pam_2CAG/ HA/PAK	75/20/50/5/0.2/1.25/1.25

PC: phosphatidylcholine, PG: phosphatidylglycerol, Chol: cholesterol, DPG-Mal: dipalmitoyl glycerol-maleimide, MPLA: monophosphoryl lipid A, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, Pam_3CAG: tripalmitoyl-cysteine-alanyl-glycine.

3.4. Physicochemical characterization of liposome constructs

3.4.1. Nanoparticle size measurement by dynamic light scattering

The average size of formulated SUVs was measured by dynamic light scattering using a Zetasizer Nano-ZS (Malvern instruments, Orsay, France) with the following specifications: sampling time: 30 sec; viscosity: 1.014 cP; refractive index: 1.34; scattering angle: 90°; temperature: 25°C. SUVs were diluted at 1/100 in 10 mM Hepes buffer (pH 7.4) containing 5% (w/v) sorbitol, and the results were the average of three consecutive measurements. Data were analyzed using the multimodal number distribution software included with the instrument. Particle size is expressed in intensity. Samples are considered monodispersed when the polydispersity index (PDI) is <0.3.

3.4.2. Phosphatidylcholine content

The PC content of formulated SUVs was determined using an enzymatic assay with the LabAssay™ Phospholipid kit (Wako Pure Chemical Industries Ltd, Richmond, VA). Briefly, 1-2 µL of SUV preparation were incubated in triplicates in a 96-well plate with 200 µL of the enzymatic reagent. The reagent contains a phospholipase C (0.47 U/mL) that releases the choline, which, in turn, is oxidized by the choline oxidase. The reaction produces hydrogen peroxide needed by the peroxidase (2.16 U/mL) to convert a chromogen into a blue product. After 10 min at 37 C, absorbance was measured at 590 nm using a microplate reader (Safas SP2000, Xenius 5801, Monaco). A standard curve of choline chloride served to establish a calibration curve.

3.4.3. Quantification of conjugated peptides

The quantification of conjugated peptides in each formulation was performed after acid hydrolysis using a fluorometric assay with fluorescamine (4-phenyl-spiro [furan-2(3H), 1'-phthalan] -3,3' –dione (Sigma-Aldrich) (Boeckler *et al.*, 1999; Böhlen *et al.*, 1973). Briefly, amino acids were generated after formulation hydrolysis at 110 C for 12 h in a heating module (Pierce Reacti Therm III™, Pierce, Breviere, France). After neutralization by the addition of sodium hydroxide, 40 µL of the hydrolysis solution was added to 1.5 mL of 50 mM sodium borate buffer (pH 9), followed by the addition of 500 µL of fluorescamine solution in dioxane (300 mg/mL. Fluorescence was measured immediately at $\lambda_{excitation}$ = 400 nm and $\lambda_{emission}$ = 480 nm. A physical mixture of plain liposomes and peptides served to establish a calibration curve. Coupling yields were calculated relative to the quantity of surface-exposed maleimide functions.

4. Human spleen cells

4.1. Human spleen cell sources

Anonymized human spleen fragments from deceased organ transplant donors were provided, following an ethical agreement by the National Organization for Organ and Tissues Donation and Transplantation (NOOTDT), a governmental organization affiliated to the Lebanese Ministry of Health. The informed consent to donate organs for transplantation or scientific research was signed by the donors themselves during their lifetime or by their parents following their death.

4.2. Preparation and cryopreservation of human spleen cell suspensions

Donors were screened for HIV, hepatitis B and C and syphilis. The spleen fragments were processed within 24 hours after surgical excision as previously described (Bouharoun-Tayoun *et al.*, 2004). Briefly, splenic tissue was dissected and forced through a stainless steel mesh. Red blood cells were lyzed in Gey's solution for 5-10 min at 25^{o}C. The leukocyte-enriched cell suspension was washed and suspended in ice-cold medium consisting of 37% fetal calf serum (FCS), 10% culture-grade dimethyl sulfoxide (DMSO) and 53% RPMI 1640 (all purchased from Sigma Aldrich, Saint Louis, MA), and stored in liquid nitrogen until use.

5. Cell proliferation assay

After thawing, spleen cells were washed twice with RPMI-10%FCS, distributed at 5.10^5 cells/well in a 96-well plate in complete culture medium consisting of RPMI1640 supplemented with 2 mM glutamine, 2 mM sodium pyruvate, non-essential amino acids, 50 µg/mL gentamicin and 10% Fetal Calf Serum (Sigma). Different liposomal constructs incorporating only Pam_2CAG (TLR2/6 agonist), Pam_3CAG (TLR2/1 agonist) or MPLA (TLR4 agonist) were added to a final concentration of 0.1, 1 and 10 µM of each TLR agonist. Cells cultured with medium served as negative control, and cells cultured with 10 µg/mL of concanavalin-A (Con A, Sigma Aldrich) or lipopolysaccharide (LPS, Invivogen) served as positive controls for T cell proliferation and B cell proliferation, respectively. Cultures were made in triplicates in a final volume of 200 µL. After incubation for 72 hours at 37°C, 5% CO_2, an MTS assay was performed according to the manufacturer protocol (Promega, Madison, WI). Briefly, 150 µL of the supernatant were discarded, and 20 µL/well MTS were added for 4 h at 37°C, 5% CO_2. The optical density was assessed at 490 nm using a microplate spectrophotometer (Multiskan FC, Thermo Scientific, Ratastie, Finland).

6. In vitro priming of human splenocytes with liposome-displayed peptides

Antigen priming was done essentially as described previously, with minor modifications (Bouharoun-Tayoun *et al.*, 2004; Brams *et al.*, 1998; Chamat *et al.*, 1999). Briefly, splenocytes were cultured at 4×10^6 cells/mL (day 0) for three days in complete culture medium either alone or in the presence of 1 µg/mL PAK-KLH or with liposomes. Liposomes incorporating only Pam_2CAG (Lp Pam_2CAG), serving as negative control, or liposomes incorporating Pam_2CAG and peptides, (complete peptide constructs, Lp Pam_2CAG/HA/PAK) serving as vaccine constructs were added at a dilution of 1/150. This results in a final concentration or 0.1 µM of Pam_2CAG with control or peptide constructs, and of 1 µg/mL PAK and 0.8 µg/mL of HA with peptide constructs. On day 1, recombinant human IL-2 was added at 25 IU/mL (Gibco Invitrogen, Carlsbad, CA).

7. NSG mice reconstitution with primed human splenocytes

After 3 days, antigen-primed splenocytes were washed and resuspended in Hank's balanced salt solution (HBSS, Sigma-Aldrich). NSG mice were reconstituted by an intraperitoneal injection (ip) of 30×10^6 primed splenocytes. Four days later (day 7), the reconstituted Hu-SPL-

NSG mice received a booster ip injection of the same type of antigen that was used in the priming step. They received either 10 µg of PAK-KLH antigen in 200 µL of HBSS- Montanide ISA 720 adjuvant (v/v), or of 100 µL of liposomes. Lp Pam_2CAG 2% and 0.2% liposome doses contain 25 µg and 2.5 µg of Pam_2CAG respectively, whereas Lp-Pam_2CAG-HA-PAK contain, in addition to, 12 µg of HA and 15 µg of PAK. A second booster was performed 2 weeks later (day 21). Mice were tail-bled one week after each booster and the serum collected from clotted blood was tested for total human IgG concentration and anti-PAK IgG antibody titer.

8. Assessment of the engraftment of human leukocytes in the spleen of Hu-SPL-NSG mice

Hu-SPL-NSG mice were euthanized at day 28. Spleens were harvested in ice-cold complete culture medium and spleens of the same mouse group were pooled. Single cell suspensions were obtained after organs were crushed and dissociated onto a 70 µm nylon mesh cell strainer. After washing, the spleen cell pellets were resuspended in HBSS containing 1% bovine serum albumin (Sigma-Aldrich), in addition to mouse antibodies specific for human cell surface antigens CD45 (leukocyte marker) (Abcam, Cambridge, UK) or CD3 (T cell marker) (Abcam). After 1h on ice, cells were washed and the secondary antibody, fluorescein isothiocyanate (FITC)-coupled goat anti-mouse Ig (Invitrogen) was added. The percentage of fluorescent human leukocytes was evaluated by fluorescence microscopy.

9. Evaluation of the cellular immune response by ELISPOT

Nitrocellulose-bottomed ELISPOT 96-well plates were coated overnight at 4° C with 5 µg/mL monoclonal antibodies specific for human cytokine IFN-γ, IL-4 or IL-10. The spleen cells were added in triplicates at 3 x 10^5 cells/well and incubated in presence of HA peptide (5 µg/mL), concanavalin A (Con A, 5 µg/mL) (Sigma-Aldrich) or complete culture medium alone. After 36h at 37° C, 5% CO_2, plates were washed twice with deionized H_2O and 3 times with PBS containing 0.05% Tween® 20, and the corresponding biotinylated anti-human cytokine antibody was added for 2h at room temperature. After washing, horseradish peroxidase-conjugated streptavidin was added at 1/100. After 1 h incubation, the spots were revealed by the addition of the horseradish peroxidase substrate 3-amino-9-ethylcarbazole. The colometric reaction was allowed to develop for 30-60 minutes. To stop the reaction, plates were extensively washed with water and dried overnight before analysis. The reaction volume was 100 µL/well. All reagents were purchased from BD Biosciences (San Jose, CA).

10. Measurement of total human immunoglobulin concentration and specific anti-PAK IgG titer in hu-SPL-NSG serum by ELISA

For the detection of total human Ig concentration or specific anti-PAK IgG titer in mouse sera, flat-bottomed microassay plates (Nunc-Thermo Scientific) were coated overnight at 4°C with 2 µg/mL of purified goat anti-human IgG (H + L) (Invitrogen) or PAK-BSA antigen at 2.5 µg/mL respectively, in 0.1 M sodium carbonate buffer, pH 9.5. After washing (PBS, pH 7.2), the plates were saturated with PBS containing 3% skimmed milk (Regilait) for the determination of total human Ig concentration, or containing 3% human serum albumin (Sigma) for the detection of anti-PAK antibodies (dilution buffer). Test sera were then added, two-fold serially diluted in PBS containing 0.05% Tween 20 and either 3% skimmed milk for the determination of total human Ig concentration, or 3% human serum albumin for the detection of anti-PAK antibodies (dilution buffer). Negative controls consisted of preimmune mouse serum of the same animals, whereas positive controls consisted of a standard human IgG solution (Zymed, Invitrogen) for the total human Ig detection, or of mouse anti-PAK serum revealed with appropriate secondary antibody for antibody titers. After an incubation of 1 h, bound human Igs were revealed by subsequent addition of horseradish peroxidase-conjugated goat anti-human IgG (H + L) (Invitrogen) for 1 h, followed by the peroxidase substrate Tetramethylbenzidin (Amresco, Solon, OH). The colorimetric reaction was stopped with 3 M HCL. All incubation steps were performed at room temperature, and the reaction volume was 50 µL/well. The plates were read at 450 nm with substraction of readings at 492 nm (Multiskan FC, Thermo Scientific).

11. Measurement of total human immunoglobulin concentration and specific anti-PAK IgG titer in hu-SPL-NSG serum by dot blot

For the detection of anti-PAK and anti-KLH IgG antibodies, two microliters of a 0.125 µg/µL solution of PAK-BSA or KLH-HA respectively were spotted onto nitrocellulose bands and allowed to dry. The bands were saturated with TBS containing 5% skimmed milk (Regilait). Test sera were then diluted in TBS (Tris 100 mM, NaCl 1.5 M) containing 5% skimmed milk and 0.05% Tween 20 (dilution buffer) to a concentration of 0.025 mg/mL of human IgG for anti-KLH detection, and 1mg/mL of human IgG for anti-PAK detection. Negative controls consisted of preimmune mouse serum of non-reconstituted, and of mouse serum from animal reconstituted with cells from the same spleen donor. After an incubation of 1 h, bound human

Igs were revealed by subsequent addition of alkaline phosphatase-conjugated goat anti-human IgG (H+L) diluted at 1/7500 (Promega) for 1 hr, followed by the addition of NBT/BCIP (Nitro-Blue Tetrazolium / 5-Bromo-4-Chloro-3'-Indolyphosphate) (Promega) diluted in revelation buffer (Tris 100mM, NaCl 100 mM, MgCl2 50mM, pH 9.5). The colorimetric reaction was stopped by washing the strips in distilled water. All incubation steps were performed at room temperature. The color intensity was then evaluated using the GelAnalyzer 2010a.

Results

Our team has previously designed a liposome-based *Pseudomonas aeruginosa* construct that co-delivers 2 peptides, namely PAK (a B cell epitope) and HA (a $CD4^+$ T cell epitope), and incorporates a TLR2/6 agonist (Pam_2CAG) as a danger signal. In BALB/c mice, these constructs were found to induce the production of anti-PAK antibodies when administered by the intranasal or by the intraperitoneal route (Heurtault *et al.*, 2009). Our objective in this part of the work was to evaluate whether a liposome-based vaccine expressing PAK and HA epitopes is able to induce a human cellular and/or humoral immune response in the Hu-SPL-NSG model.

In our experimental approach, we first prepared various liposomes which incorporated only a TLR agonist; they differed by the nature and the concentration of the TLR agonist on their surface. We made a primary assessment of the safety profile of these preparations by testing their toxicity towards human splenocytes *in vitro*. Selected preparations were completed by incorporating the PAK and HA epitopes and were investigated in Hu-SPL-NSG mice for cellular and humoral immune response.

1. Formulation and physicochemical characterization of different liposomal constructs

To identify the most suitable TLR agonist for the analysis of human immune response in Hu-SPL-NSG-mice, we first formulated liposomes incorporating either the original TLR2/6 agonist (Pam_2CAG), previously tested in the Balb/c mouse, a TLR4 agonist (MPLA), or a TLR2/1 agonist (Pam_3CAG), all at 2% mol/mol (2 mol of TLR agonist to 100 mol of phospholipids). The results of the *in vitro* toxicity test (figure 1) suggested Pam_2CAG to be the most appropriate immunostimulatory molecule for incorporation into the liposomal constructs for *in vivo* evaluation. Therefore, we subsequently prepared formulations incorporating Pam_2CAG at two different molar ratios, either 2% or 0.2 %, and expressing or not PAK and HA peptides. The physico-chemical characteristics of all these constructs are summarized in table 2.

Liposome-based SUVs, resulting from the sonication of MLVs, had a mean diameter of 59-85 nm, with a polydispersity index always lower than 0.3, reflecting the monodispersity of the liposome diameter distribution. Their size does not seem to be affected neither by the TLR agonist nor by the peptide coupling step. Liposomes incorporating Pam_2CAG at a molar ratio of 2% exhibited a mean diameter of 82-85 nm, and those incorporating Pam_2CAG at a molar ratio of 0.2% had a mean diameter of 59-64 nm. Peptide coupling yield to the surface-exposed

maleimide function was 100% Physico-chemical characteristics of the liposome-based constructs (n=3)

Table 2: Physicochemical characteristics of the liposome-based constructs (n=3)

Composition	Average diameter ± width (nm)	PDI	Peptide coupling rate (%)
PC/PG/Chol/MPLA 2%	74 ± 12	0.210	-
PC/PG/Chol/Pam$_3$CAG 2%	68 ± 9	0.230	-
PC/PG/Chol/Pam$_2$CAG 2%	85 ± 13	0.187	-
PC/PG/Chol/Pam$_2$CAG 2%/ DPG-Mal/HA/PAK	82 ± 17	0.179	100
PC/PG/Chol/Pam$_2$CAG 0.2%	64 ± 13	0.200	-
PC/PG/Chol/Pam$_2$CAG 0.2%/ DPG-Mal/HA/PAK	59 ± 9	0.238	100

Average size was measured by the dynamic light scattering method, and the peptide coupling rate was determined after acid hydrolysis using a fluorometric assay with fluorescamine. (n=3 preparations, with 3 measurements on each preparation). PDI: polydispersity index, PC: phosphatidylcholine, PG: phosphatidylglycerol, Chol: cholesterol, DPG-Mal: dipalmitoyl glycerol-maleimide, MPLA: monophosphoryl lipid A, Pam$_2$CAG: dipalmitoyl-cysteine-alanyl-glycine, Pam$_3$CAG: tripalmitoyl-cysteine-alanyl-glycine.

Our results indicate that the formulation technique is reproducible and robust. Such a characteristic is of a primordial importance in our work because it allows us to obtain comparable constructs, thus, ensuring that a constant amount of peptides is delivered per vaccine dose. These amounts are equivalent to 15 µg of PAK and 12 µg of HA/100 µL of liposome suspension.

2. Assessment of the safety profile of liposome-bound TLR agonists in vitro: Pam$_2$CAG is the molecule of choice

To identify the most suitable TLR agonist for the analysis of human immune response in Hu-SPL-NSG mice, we first assessed the *in vitro* safety profile of liposomes incorporating the different TLR agonists. For this, splenocytes from different donors were cultured for 72 h either alone or in the presence of liposomes incorporating 2% Pam$_2$CAG, Pam$_3$CAG, or MPLA at a TLR agonist at final concentration of 0.1, 1 or 10 µM. At a concentration of 10 µM, the three molecules were found to be toxic, inducing a high mortality rate (data not shown). At 0.1 µM and 1 µM, all liposomes addition resulted in an increase in the number of viable cells, suggesting a polyclonal proliferation of the splenocytes. The proliferation index (OD with liposomes/OD without liposomes) was the highest for the TLR2/6 agonist (Pam$_2$CAG). Indeed,

addition of these liposomes at 0.1 and and 1 µM final resulted in an index equal to 1.5 (figure 1).

These results suggested Pam_2CAG to be the most adapted TLR agonist for evaluation in the Hu-SPL-NSG mouse model. Concentrations of 0.1 and 1 µM are both suitable for the *in vitro* priming of human splenocytes.

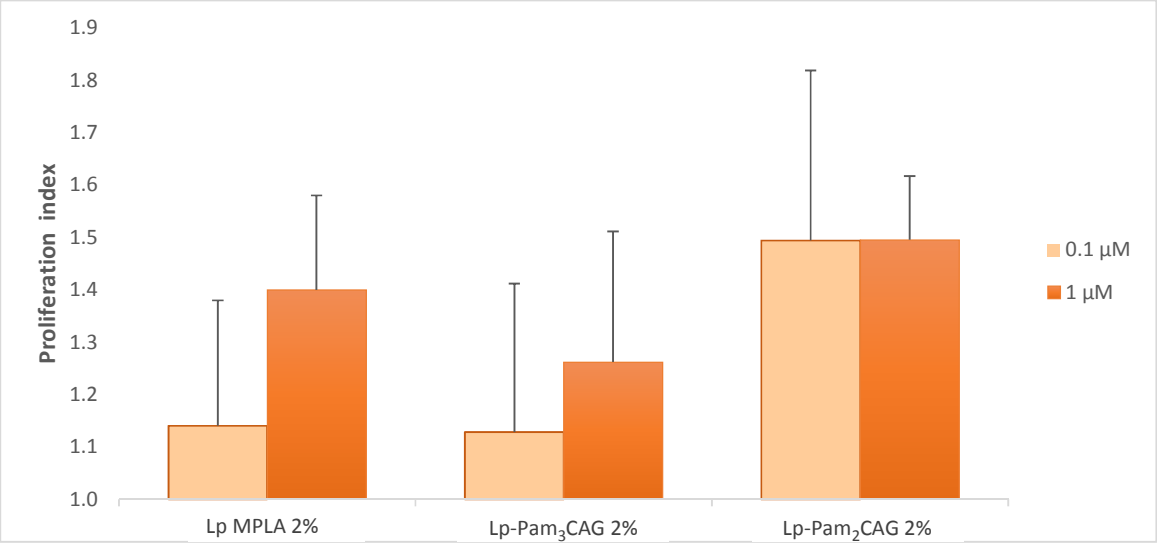

Figure 1: Proliferation of human spleen cells in the presence of liposomes incorporating different TLR agonists. *Spleen cells of different donors were incubated for 72 hours at 37° C with liposomes (Lp) incorporating MPLA (2 donors), Pam_3CAG or Pam_2CAG (5 donors), at a final concentration of 0.1 µM or 1 µM. The number of viable cells in each culture was indirectly estimated using the MTS assay, which was revealed by measuring the optic density at 490 nm. The proliferation index was obtained by dividing the optic density values of each culture condition over that of cells cultured in the absence of liposomes. Results are expressed as mean+/- SD. MPLA: monophosphoryl lipid A, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine, Pam_3CAG: tripalmitoyl-cysteine-alanyl-glycine.*

3. Liposomes incorporating Pam_2CAG at a molar ratio of 2% are toxic for Hu-SPL-NSG mice

To evaluate the specific human immune response induced by the liposomal constructs containing Pam_2CAG as a danger signal, NSG mice were reconstituted with splenocytes from one donor (donor #1) and immunized with liposomal constructs incorporating PAK and HA peptides, in addition to the TLR 2/6 agonist (Lp Pam_2CAG 2%/HA/PAK) at a molar ratio of 2%. Negative controls received liposomes that incorporated only 2% Pam_2CAG (Lp Pam_2CAG2%).

To this end, human splenocytes from donor #1 were primed *in vitro* with Lp Pam_2CAG 2%/HA/PAK or Lp Pam_2CAG 2% at a final concentration of 1 µM of Pam_2CAG in spleen cell cultures. After reconstitution with primed splenocytes, NSG mice received intraperitoneal booster injections of the same constructs that were used in the *in vitro* priming at days 7 and 21 (figure2).

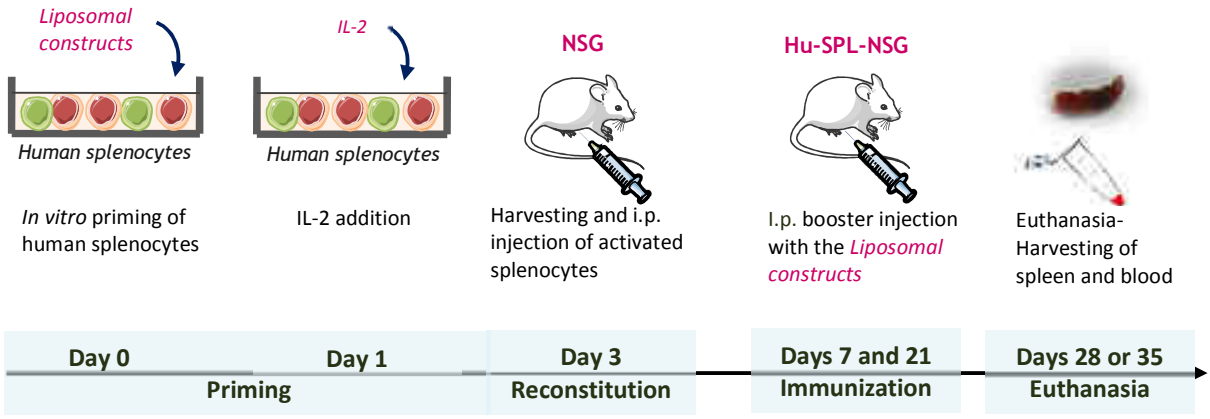

Figure 2: Reconstitution and immunization of Hu-SPL-NSG mice. *Human splenocytes are primed* in vitro *with the liposomal constructs for 3 days. After human IL-2 addition at day one, activated splenocytes are harvested and injected into the NSG mice at day 3. The resulting humanized mice are named Hu-SPL-NSG, and receive booster injections of the same construct that was used in the priming step at days 7 and 21. Hu-SPL-NSG mice are killed at day 28 or 35 and their spleens and blood are harvested to evaluate their reconstitution and the human immune response.*

Unexpectedly, these constructs induced significant morbidity and a high mortality rate in the Hu-SPL-NSG mice, regardless of the presence of peptides in the construct (table 3). Clinical signs appeared after the 2nd boost injection, and included weight loss, anemia, hunched posture, fur loss and reduced mobility. Only 53% of the animals remained alive at day 35. Human IgG concentrations in their serum were low (0.1-0.5 mg/mL) suggesting mortality of the injected human cells. Therefore, the evaluation of Lp Pam_2CAG 2%/HA-/PAK formulations in Hu-SPL-NSG mice was discontinued and the amount of TLR agonist was reduced ten-fold in the following experiments.

Table 3: Mortality of Hu-SPL-NSG mice immunized with liposomal constructs incorporating Pam_2CAG 2%.

Liposomal construct	Engrafted mice (n)	Mortality (%)
Lp Pam_2CAG 2%	7	4 (57%)
Lp Pam_2CAG 2%/HA/PAK	8	3 (37.5%)
TOTAL	**15**	**7 (47%)**

Mortality rates refer to mice that either died spontaneously, or were morbid and euthanized for humane reasons before the experiment end point. Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine,

4. Evaluation of liposomal constructs incorporating Pam_2CAG 0.2% in Hu-SPL-NSG mice

4.1. Liposomal constructs incorporating Pam_2CAG 0.2% are not toxic

Subsequent immunization experiments were performed with liposomal constructs incorporating PAK and HA peptides in addition to the TLR 2/6 agonist (Pam_2CAG), at a molar ratio of 0.2%. The negative control consisted of liposomes that incorporated only 0.2% Pam_2CAG. The positive control consisted of the PAK peptide covalently coupled to the carrier protein KLH (PAK-KLH) and adjuvanted with montanide.

The HA peptide contains 12 amino acids and consists in a single $CD4^+$ T cell epitope. This epitope can bind to the DR1, DR2, DR5 and DR7 alleles of the MHC DRB1 gene (O'Sullivan *et al.*, 1991). Therefore, human spleens were first genotyped and those with one or two alleles recognizing the HA peptide were included in the study.

NSG mice received an intraperitoneal injection of primed human splenocytes as described in the "Material and Methods" section, followed by 2 booster injections of liposomes or PAK-KLH at days 7 and 21 (figure 2). No signs of morbidity or mortality were recorded in 25 mice reconstituted with splenocytes of 4 donors (donors #2 to #5) and immunized with liposomes incorporating 0.2% Pam_2CAG, nor in 9 mice reconstituted with splenocytes of 2 donors (donors #4 and #5) and immunized with PAK-KLH.

4.2. Human cells remain functional and secrete IgG in the Hu-SPL-SCID mice

The Hu-SPL-SCID mice were bled at days 14, 28 and 35. Human IgG were detectable in the serum of all the mice, and their concentration increased with time. Furthermore, variations were observed. For instance, at day 28, in mice reconstituted with cells of donor # 1, the serum concentration was 8.2 mg/mL, whereas in those reconstituted with cells of donor # 4, it was 0.76 mg/mL. Results for day 28 are shown in figure 3.

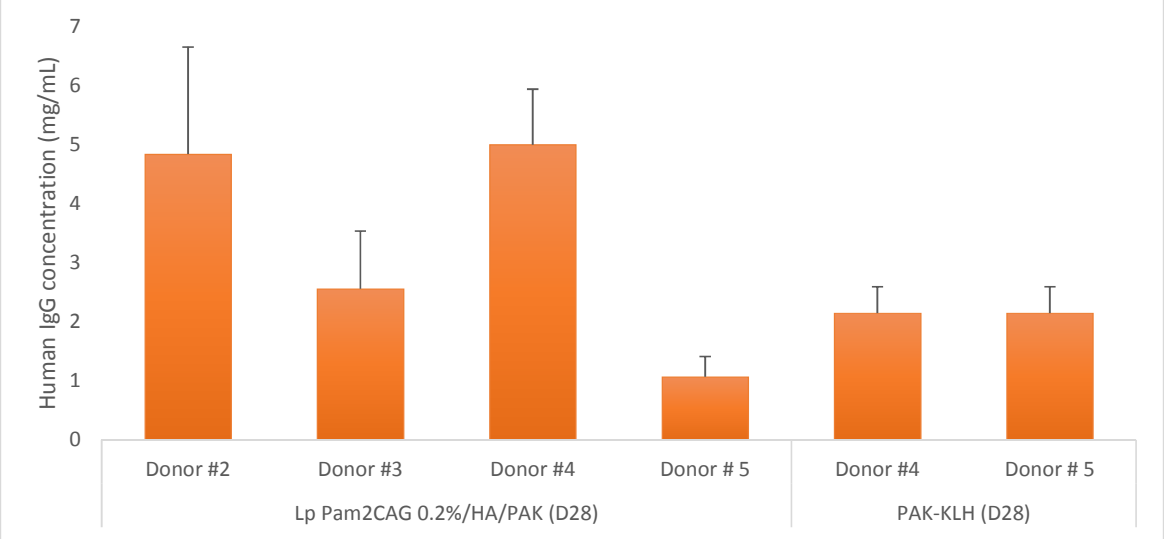

Figure 3: Concentration of human IgG in Hu-SPL-NSG mice sera at day 28 (D28). *NSG mice engrafted with* in vitro*-primed splenocytes of 4 different donors received booster injections of Lp Pam_2CAG 0.2%/HA/PAK or PAK-KLH at days 7 and 21. Spleen cells of donor #2 were used to engraft 6 mice. Those of donor# 3 were used to reconstitute 7 mice. Spleen cells of donor #3 were engrafted in 10 mice. Finally, cells of donor #4 were used to engraft 11 mice. The concentration of human IgG was evaluated by ELISA using a standard of human IgG. Results are expressed as mean +/- SD of values obtained in sera of mice engrafted with the same human spleen cells. Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine.*

All these results indicate that the human cells remained alive and functional in the reconstituted animals. Additionally, we noted that the human IgG concentration varied among different spleen donors.

4.3. Human cells home to the spleens of NSG mice

Previous results reported by our team indicate that successful reconstitution of immunodeficient mice with human splenocytes is dependent on efficient homing of the

human cells to the animal's spleen following their intraperitoneal injection. Indeed, the spleen architecture provides the optimal environment for the cooperation between different cell populations needed for mounting an efficient immune response (Ghosn, 2015). Therefore, we investigated the presence of viable human splenocytes in the spleens of reconstituted mice.

NSG mice engrafted with spleen cells of the 4 different donors (donor #2: 13 mice; donor # 3: 12 mice; donor #4: 6 mice; donor #5: 6 mice) were euthanized at day 28 or 35 and their spleens were harvested for analysis.

Macroscopic observation revealed significant increase in the spleen size: before engraftment of human cells, spleens of NSG mice are very thin and less than 1 cm long, while almost one month after reconstitution, we noticed that the spleens of the Hu-SPL-NSG mice became noticeably thicker and reached 1.5 to 2.5 cm long (figure 4).

(A) **(B)**

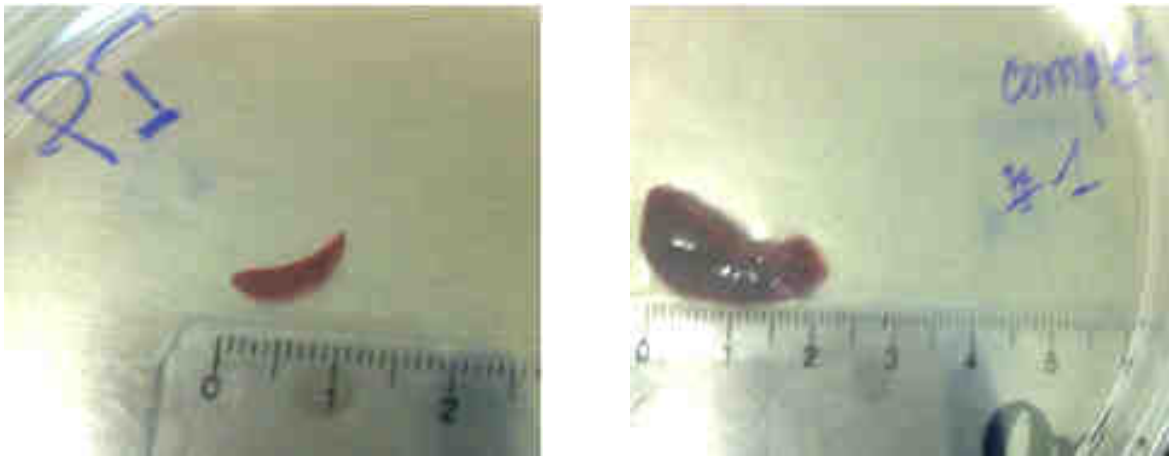

Figure 4: Spleens of (A) a non-reconstituted NSG mouse and (B) a Hu-SPL-NSG mouse. *One month after engraftment, the spleens of Hu-SPL-NSG mice increased in size, suggesting a homing of human splenocytes.*

Spleens of animals of the same group were then pooled. Cell suspensions were tested in indirect immunofluorescence to evaluate the percentage of human leucocytes and T lymphocytes. Cells were incubated with FITC-labeled anti-human CD45 and anti-human CD3 antibodies and observed under a fluorescent microscope. In Figure 5, panels A and B show the fluorescent human cells in a representative experiment. Figure 5C represents the percentage of human leucocytes and human T lymphocytes in the spleens of Hu-SPL-NSG mice

reconstituted with splenocytes of the 4 different donors. These spleens were found to harbor 35-55% of human leucocytes ($CD45^+$), among which the majority are T cells ($CD3^+$, 26-35% of total cells).

These results show that human splenocytes remain viable one month after engraftment and home to the spleens of Hu-SPL-NSG mice. The homing of leucocytes, and especially of T lymphocytes seems independent of the human donor.

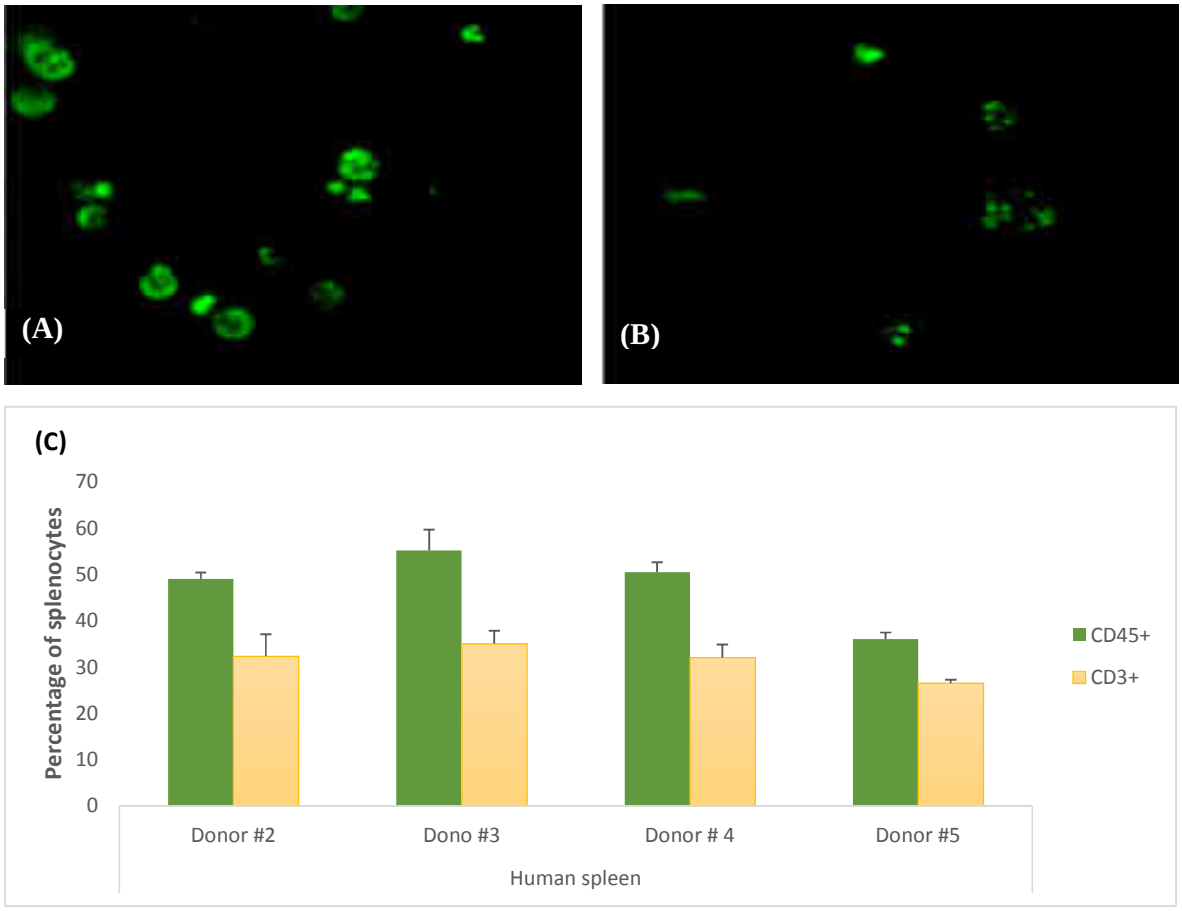

Figure 5: Human leucocytes and T lymphocytes in the spleens of Hu-SPL-NSG mice. *Spleens of Hu-SPL-NSG mice reconstituted with human splenocytes were harvested at day 28 or 35. Pooled splenocytes were then labeled with FITC-conjugated anti-human CD45 and anti-human CD3 antibodies. (A) and (B) corresponds to a representative experiment showing fluorescence of a spleen cell suspension of Hu-SPL-NSG for CD45 and CD3, respectively. (C) Percentage of human leucocytes and T lymphocytes in the spleens Hu-SPL-NSG mice. Results are expressed as mean +/- SD of experiments performed with 4 different human spleen cell donors.*

4.4. Immune response to liposomes incorporating PAK, HA and 0.2% Pam_2CAG

4.4.1. Liposomes HA-PAK-Pam_2CAG 0.2% induce a CD4+ T cell immune response in Hu-SPL-NSG mice

To assess the capacity of our liposomal constructs to induce a human cellular immune response in the Hu-SPL-NSG mouse model, pooled spleen cells of animals immunized either with the Lp Pam_2CAG 0.2%/HA/PAK or with the negative control Lp Pam_2CAG 0.2% were challenged *in vitro* with the HA peptide. ELISPOT assays were performed to evaluate the number of human T cells producing IFN-γ, IL-4 or IL-10. Mice reconstituted with the cells of donor # 4, Lp Pam_2CAG 0.2% /HA/PAK elicited a high number of HA-specific IFN-γ producing cells, as compared to Pam_2CAG-bearing liposomes (figure 6). These results suggest that Lp Pam_2CAG 0.2%/HA/PAK formulations are able to induce a cellular immune response in the Hu-SPL-NSG mouse model with a Th1 profile. No specific secretion of IL-4 (Th2 profile) or IL-10 (Treg profile) was detectable.

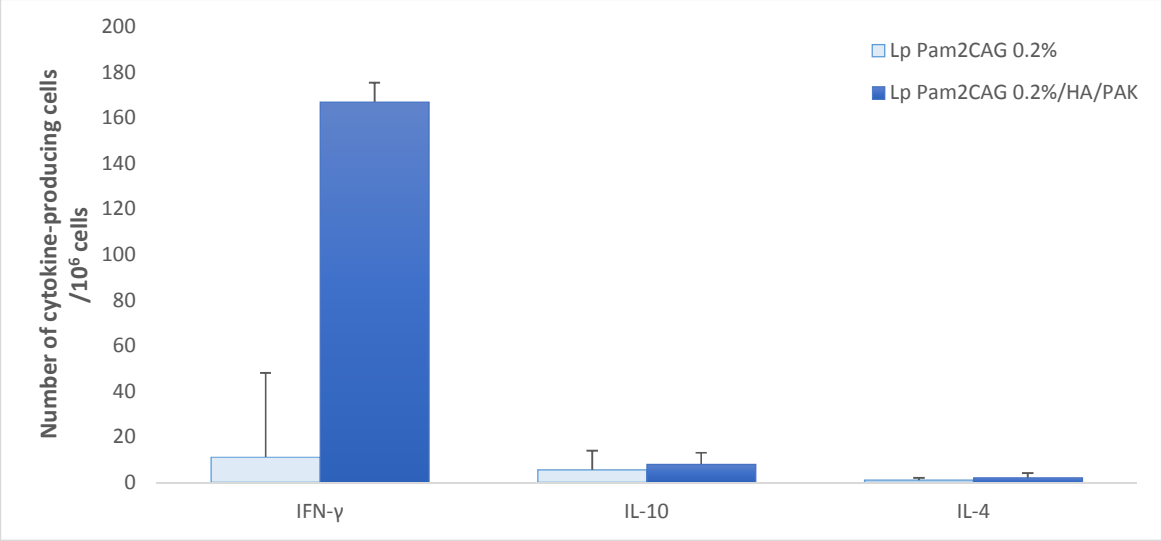

Figure 6. HA-specific IFN-γ, IL-10 and IL-4-production induced by the liposomal constructs in the Hu-SPL-NSG mouse model. *NSG mice were reconstituted with spleen cells of donor #4 and immunized at days 7 and 21 with Lp Pam_2CAG 0.2% or Lp Pam_2CAG/HA/PAK formulations. Spleens were collected on day 28 and cells were cultured either alone or in the presence of HA (5 µg/mL). The number of IFN-γ-secreting cells/10^6 cells was measured by direct ELISpot assay. Results are expressed as mean +/- SEM of ELISpot triplicates. IFN: interferon, IL: interleukin, Lp: liposome, Pam_2CAG: dipalmitoyl-cysteine-alanyl-glycine.*

4.4.2. Liposomes Pam_2CAG 0.2%/HA/PAK do not induce detectable human anti-PAK antibodies in Hu-SPL-NSG mice

To evaluate the capacity of our liposomal constructs to induce a specific humoral immune response in the Hu-SPL-NSG mouse model, we assessed by ELISA the presence of anti-PAK antibodies in the sera of mice immunized with either Lp Pam_2CAG 0.2%/HA/PAK or PAK-KLH/montanide.

Unexpectedly, and despite a successful engraftment of functional human splenocytes in the NSG mice, no humoral response against PAK peptide was detected, neither in mice immunized with the liposomal constructs, nor in those receiving PAK-KLH. Even more surprisingly, the search for antibodies directed against the carrier protein KLH in mice immunized with PAK-KLH was also negative by ELISA (table 4). These results prompted further investigations of the validity of the antibody detection technique. Therefore, we assessed the presence of these antibodies using a different method, the dot blot. This method revealed the presence of anti-KLH and anti-PAK antibodies in sera of mice immunized with PAK-KLH. However, sera of mice immunized with Lp-Pam_2CAG 0.2%/HA/PAK did not show any signal (figure 7).

Table 4: Reconstitution and immune response of mice immunized with Lp Pam_2CAG 0.2%/HA/PAK or PAK-KLH.

Immunizing preparation	Spleen cell donor	Number of immunized Hu-SPL-NSG mice	HA-specific IFN-γ - production	[Human IgG] at D28 (mg/mL)	ELISA		Dot-blot	
					Anti-PAK	Anti-KLH	Anti-PAK	Anti-KLH
Lp Pam_2CAG 0.2%/HA/PAK	#2	3	-	3.4 - 6.3	-	/	-	/
	#3	4	-	1.6 – 4.2	-	/	-	/
	#4	3	+	4.2-4.6	-	/	-	/
	#5	3	-	1.2-1.5	-	/	-	/
PAK-KLH/ Montanide	#4	4	/	2.4-3	-	-	+	+
	#5	5	/	0.7-0.9	-	-	+	+

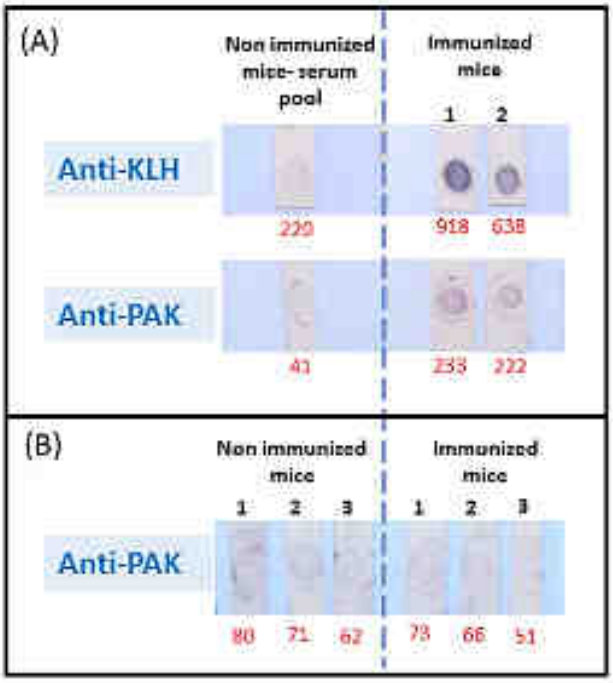

Figure 7. Human anti-KLH and anti-PAK antibodies in Hu-SPL-NSG mice immunized with (A) PAK-KLH, or with (B) Lp Pam$_2$CAG 0.2%/HA/PAK. *NSG mice engrafted wi- primed splenocytes of donor #4 received booster injections of Lp PAK-KLH or Pam$_2$CAG 0.2%/HA/PAK at days 7 and 21. Sera were collected at day 28 and the presence of anti-KLH and anti-PAK antibodies was assessed by dot blot. Results are representative of two different experiments.* Red numbers under the dots represent the coloration intensity recorded *by the GelAnalyzer 2010 a.*

The dot blot results show that the ELISA technique used for the detection of anti-KLH and anti-PAK antibodies is not reliable. They further confirm that the Hu-SPL-NSG can mount a primary humoral response against PAK peptide when it is coupled to a carrier protein and administered with a strong adjuvant, however, our liposomal constructs do not seem to be able to induce a humoral immune response against this small peptide in the Hu-SPL-NSG mouse model**.**

Discussion:

Despite its extensive use and utility as a first-line model for the evaluation of candidate vaccines, the classical rodent model presents a major pitfall in that immune responses elicited in mice frequently diverge from those observed in humans (Mestas and Hughes, 2004). The humanized mouse model appears as an attractive alternative for testing vaccine candidates in development. The NSG mouse reconstituted with human splenocytes has proven its worth and ability to mount human responses to highly immunogenic antigen preparations (Bouharoun-Tayoun *et al.*, 2004; Brams *et al.*, 1998; Chamat *et al.*, 1999) and to be more predictive of human responses to vaccine candidates than the classical murine model (Ghosn *et al.,* in preparation). However, there are very few reports in the literature about its potential in evaluating modern vaccine candidates containing minimal antigen structures and mild adjuvants.

Our team has developed a liposome-based construct which seems promising as a vaccine candidate against *P. aeruginosa*. This construct contains a B cell epitope of *P. aeruginosa* (PAK), a universal $CD4^+$ T cell epitope (HA) and a TLR2/6 agonist (Pam_2CAG) at a proportion of 0.2% mol/mol. It induces a specific anti-PAK humoral immune response after intranasal and intraperitoneal administration to BALB/c mice (Heurtault *et al.*, 2009). Our objective was to determine whether the Hu-SPL-NSG model is suitable for preclinical testing of this construct and applicable for evaluation of liposome-based vaccines in general.

Our first aim was to select the TLR agonist with the most optimal potential as a danger signal to be used as an adjuvant of liposome constructs in human vaccines. Variants of the original liposome were designed, carrying three different immunostimulatory molecules, namely a TLR 4 agonist (MPLA), a TLR2/1 agonist (Pam_3CAG) or the original TLR 2/6 agonist (Pam_2CAG). MPLA is the nontoxic derivative of bacterial lipopolysaccharides (LPS) and is currently incorporated into adjuvant systems developed by GlaxoSmithKline. It has been incorporated into liposomes (Boks *et al.*, 2015; Cluff, 2010) and evaluated in several vaccine clinical trials, where it proved to efficiently induce both humoral and cellular immune responses by human cells (Alving and Rao, 2008; Alving *et al.*, 2012). Pam_2CAG and Pam_3CAG are di- and tri-acylated derivatives of the N-terminal moiety of *E. coli* lipoprotein that interact with TLR2/6 and TLR2/1 heterodimers, respectively (Espuelas *et al.*, 2005; Oliveira-Nascimento *et al.*, 2012; Omueti *et al.*, 2005; Roth *et al.*, 2004; Takeda *et al.*, 2002). When incorporated into liposomal constructs,

they were both found to induce human monocyte-derived DCs maturation *in vitro*, as revealed by the expression of CD80, CD83, CD86 and HLA-DR molecules (Espuelas *et al.*, 2005). Pam_2CAG and Pam_3CAG were both found to promote protective tumor-specific responses *in vivo* in conventional preclinical mouse models (Heurtault *et al.*, 2009; Thomann *et al.*, 2011).

In our constructs, peptides were anchored to the liposome surface using an amphiphilic thiol reactive anchor inserted in the lipid bilayer. Cysteine-containing peptides were subsequently covalently linked to the maleïmide group of the anchor by the Michael addition (Schelté *et al.*, 2000). This soft coupling step in aqueous medium preserves the narrow size distribution of the preformed liposomes resulting in reproducible liposome-based constructs.

The physicochemical characteristics of our formulations were highly controlled and reproducible. This is advantageous for their *in vivo* evaluation, especially in Hu-SPL-NSG mice. Since the animals are reconstituted with human spleen cells of donors having different genetic backgrounds, the homogeneity of the formulations is essential to minimize the variations inherent to the experimental conditions.

We first assessed the safety profile of the 3 different TLR agonists toward human splenocytes by adding different concentrations of liposomes incorporating these TLR agonists to spleen cell cultures. At the high concentration of 10 µM, all three TLR agonists were found to be toxic, inducing high mortality rates in human splenocyte cultures. In a previous report, human monocyte-derived DCs were cultured in presence of liposomal Pam_3CAG or a functionalized Pam_2CAG derivative at a lipopeptide concentration of up to 50 µM and no toxic effect was reported (Espuelas,2005). Similarly, free water-soluble analogs of Pam_2CAG and Pam_3CAG could be added at concentrations of 5 µM to a murine DC cell line without inducing a toxic effect (Spanneda 2010). These results suggest that the toxic effect we noted is exerted on a different cell population than DCs in the spleen cell suspension, probably the lymphocytes.

At lower concentrations, we observed that MPLA, Pam_3CAG and Pam_2CAG exhibit a TLR agonist activity by inducing stimulation indexes >1. Pam_2CAG seemed be the most appropriate TLR agonist because it elicited the highest antigen independent proliferation rate. It is to be noted that TLRs, including TLR2 and TLR4 are also expressed on T (Kabelitz, 2007) and B lymphocytes, even if TLR4 expression is low on B lymphocytes (Buchta and Bishop, 2014). It is

well documented that signaling through these TLRs induces B cell antigen-independent proliferation (Buchta and Bishop, 2014). Concerning T cells, it is widely accepted that the role of TLR signaling is restricted to amplifying responses induced by TCR engagement (Rahman *et al.*, 2009). Our results agree with previous observations in mice in which Pam_2 C-type lipopeptides appeared to be more efficient than the Pam_3-C type ones in inducing the proliferation of murine splenocytes (Metzger *et al.*, 1995). Conversely, Spanneda *et al.* and Boeglin *et al.* found no differences on the proliferation of murine B cells cultured with either Pam_2C-type or Pam_3C-type lipopeptides (Boeglin *et al.*, 2011; Spanedda *et al.*, 2010). Boeglin *et al* additionally reported that signaling through TLR4 results in the same proliferation index (Boeglin *et al.*, 2011).

Based on these results we selected Pam_2CAG-incorporating complete liposomes for *in vivo* evaluation in Hu-SPL-NSG mice. According to our immunization protocol, human splenocytes were primed with complete liposomes *in vitro* before being engrafted in the NSG mice and the animals received 2 boosters of those same constructs. Liposomal formulations incorporating Pam_2CAG at a 2% molar ratio (equivalent to the *in vitro* experimental condition of 1 μM) were first used in the hope to elicit the most potent response. However, these constructs were found to be toxic, as they resulted in the death of approximately 50% of the immunized mice.

Hu-SPL-NSG mice immunized with complete liposomes incorporating 0.2% Pam_2CAG had a very high viability, indicating the lack of toxicity of this adjuvant at the chosen concentration. Moreover, the engraftment rate of human leucocytes in their spleens was reproducibly high, with the majority of these cells being $CD3^+$ T cells. In parallel, a spontaneous production of high amounts of human IgG, with a serum concentration always exceeding 1 mg/mL, was noted in all animals. Altogether, these results indicate a successful engraftment and functionality of human splenocytes in the Hu-SPL-NSG mice. They are in accordance with results usually obtained with classical vaccine preparations in our team (Ghosn, 2015). We can therefore conclude that the concomitant intraperitoneal administration of liposomal constructs incorporating Pam_2CAG 0.2% in addition to a $CD4^+$ and a B cell epitope together with the human cells to the NSG mice does not alter the injected human cell viability or behavior and, specifically, does not impair their homing to the spleen. A recently published paper by Majji *et al.* shows that NOD RAG-deficient γ-null (NRG) mice humanized with hematopoietic stem cells and vaccinated with liposomes that express a peptide antigen and

incorporate MPLA, undergo a successful engraftment of injected cells and generate human $CD4^+$, $CD8^+$ and B cells (Majji *et al.*, 2016). However, to our knowledge, there are no reports on engraftment of differentiated human lymphocytes in immunodeficient mice in the presence of liposomes. This information is therefore important for further consideration of the Hu-SPL-NSG model for evaluation of liposome based vaccines.

In our experiments, we immunized mice reconstituted with the spleen cells of 4 different donors**. We assessed the capacity of our liposomal constructs to induce a human cellular immune response** by evaluating the number of human lymphocytes specific for the $CD4^+$ T cell peptide (HA) in pooled spleens of mice of each experimental group. Our results show a potent cellular immune response in the spleen pool of mice reconstituted with splenocytes of one donor. These results seem promising because the detected immune response is characterized by the production of IFN-γ, which corresponds to a Th1 profile. This response profile is particularly desirable in the case of vaccines against cancer and intracellular pathogens.

Retrospectively, we suspect that our experimental conditions may have led to an underestimation of the response rate (1/4 donors). Indeed, recent results obtained in our laboratory indicate that a higher sensitivity may be obtained by testing each mouse spleen cell suspension separately with the $CD4^+$ peptide in the ELISpot assay. Since we tested the responses of pooled spleens of mouse groups, we speculate that low individual responses might have been diluted and became undetectable when the spleens wee pooled. Moreover, we can improve the sensitivity of $CD4^+$ T cell response detection by performing an indirect or cultured ELISpot. In this assay, the cells are cultured for up to 12 days in the presence of the peptide and human IL-2 to induce the proliferation of antigen-specific lymphocytes before performing the ELISpot assay.

In accordance with our results, the study reported by Majji *et al.* shows that immunization of humanized mice with liposomes encapsulating an influenza A matrix protein-derived peptide and incorporating MPLA elicits vaccine specific $CD8^+$ T cells. It is to be noted that in our liposome constructs, each vaccine dose delivers 15 µg of the B peptide, 12 µg of the HA peptide and 2.5 µg of Pam_2CAG, while their vaccine dose comprises more than 7-fold the amount of the immunizing peptide (200 µg) (Majji *et al.*, 2016). In another recent study

published in June 2017, immunodeficient mice reconstituted with human hematopoietic stem cells received an intranasal administration of cationic liposomes encapsulating a complete *Mycobacterium tuberculosis* protein and CpG oligodeoxynucleotide (ODN), a nucleotide-based adjuvant that signals through TLR9. These mice responded by producing $CD4^+$ and $CD8^+$ T cells secreting cytokines known to be required for protective immunity. In addition, the elicited immune response limited infection in these mice upon bacterial challenge (Grover *et al.*, 2017). Interestingly, the administered vaccine dose comprised only 2 µg of *Mycobacterium tuberculosis* protein, as compared to 15 and 12 µg of our single-epitope B and $CD4^+$ T cell peptides, respectively.

The ability of liposomal constructs to induce a humoral immune response in the humanized mouse model has been described in a few reports. An early study conducted in 1995 described the immunization of SCID mice reconstituted with human peripheral blood lymphocytes with a liposome encapsulating a ganglioside and the tetanus toxoid (TT) protein. The authors reported production of high titers of ganglioside-specific IgG and IgM antibodies but indicated that a pre-existing immune memory against TT was required for the induction of this response (Ifversen *et al.*, 1995). Another study in which Hu-PBL-SCID mice were immunized with multilamellar liposomes entrapping the model protein ovalbumin also showed their capability to produce high IgG and IgM titers (Walker and Gallagher, 1994).

Surprisingly, we failed to detect anti-PAK antibodies in the sera of immunized mice, in both ELISA and dot blot techniques. We extensively attempted to optimize the ELISA assay, for instance by testing different coating conditions in order to increase the sensitivity of the test, and several blocking reagents in order to minimize background signal. However, no specific signal could be detected for anti-PAK antibodies.

The ability of human splenocytes to respond against the PAK peptide was confirmed by immunizing the mice with the PAK peptide coupled to the carrier protein keyhole limpet hemocyanin (PAK-KLH). Mice were found to produce human anti-PAK and anti-KLH antibodies, that were detectable by dot blot but not by ELISA.

These results suggest that the liposomal constructs are unable to induce PAK-specific humoral responses in the Hu-SPL-NSG mouse model, however this conclusion should be considered

with caution due to the confirmed invalidity of the ELISA assay for the detection of anti-PAK antibodies, in addition to the low sensitivity of the dot blot technique.

In conclusion, we demonstrated through this work that minimalist liposome-based constructs incorporating a B-cell and a $CD4^+$ T cell peptides and a TLR2/6 agonist are safe in the Hu-SPL-NSG mouse model up to a ratio of 0.2% Pam_2CAG mole /mole of phospholipid. Moreover, the present paper is one of the first reports that provide a proof of concept for the ability of such constructs to induce primary cellular immune responses in the Hu-SPL-NSG model.

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The liposomes used in this part of the work were prepared using the same techniques as in the chapter 1 and had similar physicochemical properties. Our liposomal platform proved to be immunogenic in the Hu-SPL-NSG mouse since it induced a cellular immune response against the $CD4^+$ T cell epitope. However, it was unable to induce a humoral response against the chosen B cell epitope.

Since the humoral response was not the main goal of the current part of this work, the presence of a $CD4^+$ T cell response justifies further evaluation of the capacity of the liposomal formulations to induce a $CD8^+$ T cell response in the Hu-SPL-NSG mouse model. Subsequently, the efficacy of this immune response against tumors has to be evaluated. To this end, human tumor models expressing the TAA from which the $CD8^+$ T cell epitope is derived have to be developed.

GENERAL DISCUSSION
AND
PERPECTIVES

Despite extensive efforts joined worldwide to develop efficient cancer vaccines, there is only one such vaccine available on the market nowadays, which is Sipuleucel-T, an approved DC-based vaccine against prostate cancer. The development of these vaccines is hampered mainly by the lack of a reliable predictive animal model, indeed many candidates have revealed no efficacy in clinical trials, despite promising preclinical data. These facts highlight the need to improve many elements of the preclinical evaluation phase. To address this issue, we aimed in this work to assess three main factors that influence vaccines efficacy, namely the vaccine administration route, the vaccine composition, which is partly dictated by the administration route, and the preclinical model in which these vaccine preparations are evaluated.

To this end, we first chose liposome-based formulations that were previously developed by our team (Thomann *et al.*, 2011). They incorporate a TLR2/6 agonist (Pam_2CAG), a universal $CD4^+$ T cell epitope peptide derived from the hemagglutinin of the influenza virus (HA 307–319, PKYVKQNTLKLAT-C) (O'Sullivan *et al.*, 1991), in addition to a target $CD8^+$ T cell peptide derived from the human TAA ErbB2 (p63–71, CG-TYLPTNASL) (Nagata *et al.*, 1997), expressed on a number of cancers of epithelial origin (Penault-Llorca, 2003). Since these formulations have proved their efficacy by the SC and the intranasal routes in the BALB/c mouse, we aimed to evaluate their immune potential by TC administration. To assess whether it is possible to use a more predictive preclinical model of the immune response to evaluate liposome-based constructs, we administered a model liposomal formulation to Hu-SPL-NSG mice (Bouharoun-Tayoun *et al.*, 2004). In this formulation, the $CD8^+$ T cell epitope peptide was replaced with a B cell peptide derived from the pilin of *P. aeruginosa* (PAK, 128–144, KCTSDWDEQFIPKGSSK) (Campbell *et al.*, 2003; Lee *et al.*, 1989), in order to allow the evaluation of both cellular and humoral immunity. We then evaluated the liposomes for their effect on mice reconstitution on one hand, and for their immune potential on the other hand.

1. A robust formulation technique that yields homogeneous liposome-based constructs

In order to adapt the original formulation for the TC route, we first optimized the immunostimulatory molecule. Therefore, we replaced the previously evaluated TLR2/6 agonist, Pam_2CAG, with a TLR4 agonist, MPLA. We also investigated the effect of the addition of a DC targeting molecule, di-mannose, and that of the lipid vesicle fluidity by replacing the

conventional liposomal vesicle with a transfersome. Additionally, to evaluate the model construct in the Hu-SPL-NSG mouse, we also optimized the immunostimulatory molecule. Therefore, we incorporated in the liposomes either MPLA, or Pam_2CAG, or Pam_3CAG, a TLR2/1 agonist

To formulate the liposomal constructs, we adopted the lipid hydration technique that results in MLVs, followed by sonication and peptide conjugation of the SUV surface. In the case of transfersomes, it has been demonstrated by our team that Tf-MLV sonication results in polydisperse formulations (Kakhi, 2015). Therefore, sonication was replaced with manual extrusion. Extrusion is currently one of the most common methods that produce controlled monodisperse SUVs, and allow a strict control of the resulting SUV size through the choice of the pore size of the filter across which the vesicles are forced, and of the number of passages through this filter (Lapinski *et al.*, 2007). Our formulation technique allowed us to obtain homogenous populations (<100nm) that were also monodisperse and showed a narrow size distribution (PDI<0.3 and low CV). Thus, we succeeded at maintaining virtually constant physicochemical properties all along our work, independently of the lipid vesicle composition, of the incorporation of several types of immunostimulatory molecules and of peptide addition to the formulation. Concerning the peptide anchoring strategy, it was found to be more efficient than the conventional encapsulation method (non-published observations) and has previously proved its efficacy in several works (Heurtault *et al.*, 2009; Roth *et al.*, 2005; Thomann *et al.*, 2011)

In conclusion, the preparation of various liposomal constructs, with homogeneous physico-chemical properties all along this work highlighted the robustness of the employed strategy, comprising the formulation technique and the peptide conjugation. This is a particularly interesting asset for their *in vivo* evaluation that minimizes the variations inherent to the experimental conditions.

2. Feasibility and immunogenicity of TC vaccination with liposome-based constructs

2.1. The TC route is as potent as the SC route in inducing tumor-specific immune responses: a proof of concept

First, we compared the immune responses induced by the TC and SC administration of the original liposomal formulation. Our results showed similar amplitudes of the local and systemic immune responses against both vaccine peptides, meaning that our liposomal constructs are able to induce similar responses whether by the subcutaneous or TC routes. This indicates that applying conventional liposomes by massage on intact skin is followed by the passage of these liposomes through the skin barrier towards internal skin layers rich in DCs. These results provide one more undeniable evidence that the TC route is capable, under the right conditions, to drive the cutaneous immune system to mount a potent cancer-specific immune response.

Previous reports have proven the ability of the TC route to induce tumor-specific responses. Using a model CTL epitope derived from ovalbumin, Stein *et al.* have shown that TCI induces a potent response which is, at least, as potent as that induced by the subcutaneous and the oral routes (Stein *et al.*, 2014), that resulted in the rejection of the antigen-expressing tumor cells. Similarly, Rechtsteiner *et al* found that TC immunization can induce potent immune responses characterized by a cytotoxic activity against cancer cells (Rechtsteiner *et al.*, 2005).

However, this work is among the first reports where liposomes where used a vaccine delivery vectors for the TC route. This proof of concept substantiates further optimization of the formulations.

2.2. Vaccine composition modulates the induced immune response

2.2.1. The TLR2/6 agonist Pam_2CAG is superior to the TLR4 agonist MPLA for the TC route

Comparing the response induced by liposomes expressing TLR2/6 and TL4 agonists by the TC route, we proved the efficacy of both liposomal TLR2/6 agonist (Pam_2CAG) and TLR4 (MPLA) agonist to induce cellular responses of the Th1 type, which is known to be protective against

cancer. As for the choice of the TLR agonist, we showed that the TLR2/6 agonist is potent in eliciting both local and systemic responses, whereas the TLR4 agonist induced only systemic responses. These results are of a great interest since they can be applied in different ways in cancer vaccination. Induction of a systemic response means that liposomes bearing both TLR agonist are theoretically worth investigating for the treatment of internal tumors. Various cancers of epithelial origin expressing ErbB2, such as breast cancer and ovarian cancer, can be targeted by TC application of our liposome-based formulations. Moreover, ErbB2 is chosen herein as a model TAA and, therefore, can be replaced with any other target TAA.

However, local immune responses are particularly needed in the case of melanoma, where the lymphocytes need to be sufficiently recruited to the vaccination site. Several attempts of TC vaccination against melanoma have been conducted in preclinical models and melanoma TC vaccination was recently translated into clinical testing (Ott *et al.*, 2014; Ozao-Choy *et al.*, 2014). For instance, a TC vaccine consisting of a mixture of melanoma-derived peptides dissolved in DMSO administered to melanoma patients after barrier disruption by tape stripping was found to increase overall survival (55.8 months for patients who responded to all vaccine peptides *vs* 20.3 months for partial responders) (Fujiyama *et al.*, 2014). Another attempt was conducted against the NY-ESO-1 melanoma TAA, where combination of TC administered immunostimulatory molecules, such as resiquimod, a potent TLR7/8 agonist, with intradermal administration of NY-ESO-1 protein emulsified in montanide resulted in $CD8^+$ T cell responses in 3/12 patients (Sabado *et al.*, 2015). Therefore, it seems that the TLR2/6 agonist is superior to the TLR4 agonist.

The different efficacy profiles of these 2 TLR agonists also suggest that they target different DC populations. It is admitted that TLR2/6 is expressed on both LCs in the epidermis and dDCs. However, it has been frequently reported that TLR4 functionality is impaired on LCs, but not on dDCs (Flacher *et al.*, 2006; Oosterhoff *et al.*, 2013). Our results suggest therefore that the liposomal constructs have reached the dermis to activate dDCs through TLR signaling. Alternatively, TLR4 functionality on LCs may not be impaired as it is thought to be.

Interestingly, using flow cytometric analysis of the DC sub-populations after TCI with liposomes incorporating theTLR4 agonist in the lipid bilayer, we showed that they induce the migration of skin DCs, preferentially LCs and $Lang^-$ dDCs. However there was no difference

between their effect and that of the plain formulations. This means that MPLA might not be capable of inducing efficient skin DC migration and is in line with our previous result.

- **What other TLR agonist are strong candidates for TC vaccination?** After providing the proof of concept about the feasibility and the immune potential of TC vaccination using TLR2/6 and TLR4 agonists, it is interesting to expand our investigation to include other immunostimulatory molecules in the liposomal constructs. Therefore, it appears necessary for us to incorporate in their composition a TLR agonist of the imidazoquinoline family. Imidazoquinolines are potent TLR agonists that seem promising for application in cancer adjuvant immunotherapy (Shukla *et al.*, 2012, 2012; Vasilakos and Tomai, 2013). Imiquimod, an imidazoquinoline that signals through the TLR7, has been approved by the FDA for the therapy of basal cell carcinoma and genital warts (Vacchelli *et al.*, 2012), and is currently investigated in several studies in preclinical and clinical development (Chi *et al.*, 2017). Imiquimod is used as a topical cream which is applied alone on the cancerous lesion. It induces a non-specific immune activation that promotes tumor-specific responses thus, resulting in tumor regression. Indeed, it was shown to enhance tumor-specific $CD4^+$ and $CD8^+$ immune responses and to induce objective responses in melanoma, in various skin-involving metastatic cancers such as breast cancer and in intravesical therapy of bladder cancer (Adams *et al.*, 2012; Dewan *et al.*, 2012; Hayashi *et al.*, 2010; Narayan *et al.*, 2012). Due to his potential, imiquimod is being tested as a cancer vaccine adjuvant. For instance, a bivalent therapeutic vaccine for Human Papillomavirus (HPV) delivered intradermally was adjuvanted with transcutaneously applied imiquimod. This vaccine was found to induce HPV-specific $CD8^+$ T cell responses and tumor eradication in C57BL/6 mice (Esquerré *et al.*, 2017). Therefore, we expect imiquimod incorporation into our liposomal constructs to amplify the specific immune responses against vaccine-peptide, especially that it is adapted for the TC route.

2.2.2. Skin DC targeting by mannose addition to liposomal constructs does not significantly improve immunogenicity

Next we evaluated whether mannose addition to the liposomal constructs delivered through the TC route would increase their immunogenicity by improving their uptake. Previous results of SC immunizations had shown that mannose addition increases vaccine immunogenicity when the amount of TLR agonist is very low (Thomann *et al.*, 2011). In TC immunization, a favorable effect of mannose was far from being as evident. Indeed, mannose addition was

found to exert variable and slightly significant effect on the local and systemic immune response. While mannose receptor expression is demonstrated on dDCs, it is still debatable on LCs (Condaminet *et al.*, 1998; de Koning *et al.*, 2010; Plzáková *et al.*, 2004; Polak *et al.*, 2014; Wollenberg *et al.*, 2002). According to our results, we may speculate that the mannose receptor is indeed absent on LCs, or that it is not suitable for skin DC targeting. Additionally, its expression on keratinocytes (Szolnoky *et al.*, 2001) may result in a preferential uptake of mannose-expressing liposomes by the numerous keratinocytes instead of the rare DCs. Therefore, we conclude that adding a mannose residue to our liposomal construct does not provide a beneficial effect in targeting skin DCs.

- **How to improve skin DC targeting?** Several authors have addressed the utility of targeting vaccines or proteins to skin DCs through endocytic receptors, such as langerin, DEC-205/CD205 and Dendritic Cell-Specific ICAM-3 Grabbing non-Integrin (DC-SIGN). These C-type lectin receptors are not expressed equally on all skin DCs. For instance, langerin expression is restricted to LCs (Valladeau *et al.*, 2000) and a small subset of dDCs (Henri *et al.*, 2010), while DC-SIGN expression is mostly on dDCs (Fehres *et al.*, 2015b). DEC-205/CD205 expression is high on DCs and low on LCs and its targeting through monoclonal antibodies injected in the dermis was found to induce preferential uptake by dDCs (Fehres *et al.*, 2015c; Stoitzner *et al.*, 2014), but also by LCs (Flacher *et al.*, 2006, 2009, 2010). Interestingly, targeting of LCs through the langerin receptor, but not through the DEC-205/CD205 receptor was suggested to induce tolerance (Flacher *et al.*, 2010; Idoyaga *et al.*, 2008). Altogether, these findings can be of interest for future improvements of the formulations, using 2 different strategies. For analytical purposes, adding only one of these molecules to the liposome constructs would target one or few subsets of skin DCs and would reveal their differential contribution to the induced immune response. Alternatively, several molecules may be combined in one vaccine formulation to simultaneously target a broader selection of skin DC subpopulations.

2.2.3. Transfersomes are not superior to conventional liposomes in TC vaccination

We next assessed the immune potential of transfersome-based vaccines. We speculated that, being ultradeformable, transfersomes would be better able to cross the skin barrier, thus reaching skin DCs in increased amounts and inducing a more potent immune response. Indeed, transfersomes are characterized by their capacity to increase skin penetration and are

drawing a growing attention to be used for TC delivery of drugs and vaccines (Benson, 2006, 2009). Our results show however that transfersome-based constructs do not exert an improved immunostimulatory potential as compared to their liposomal counterparts. On the contrary, they seem to have a negative influence by impairing the $CD4^+$ T cell responses. It may possible that the conditions in which we performed the TCI with the nanoparticles, i.e ethanol application on the mouse skin and massage of the formulation, are not favorable for transfersomes to fully exert their skin crossing activity. Alternatively, another reason why transfersomes were inferior to liposomes may reside elsewhere than in their skin barrier crossing potential. Due to their ultradeformability, transfersomes are less stable than liposomes (Kakhi, 2015), which results in a shorter residence time inside the skin layers (depot effect) leading to decreased interactions with the DCs. Therefore, we conclude that transfersomes are not superior to liposomes in our vaccination conditions.

- **Can other lipid vesicles than transfersomes improve the constructs immunogenicity?** In the future experiments it would be interesting to test another type of ultrafluid variants of liposomes, called ethosomes. They contain an high amount of ethanol in their composition, up to 50 % v/v, which increases the fluidity of their lipid bi-layers (Touitou *et al.*, 2000). Their efficiency in skin barrier crossing is being progressively established over the last few years. Ethosomes were proved to have a high capacity for drug delivery through the skin barrier (Bragagni *et al.*, 2012; Ghanbarzadeh and Arami, 2013; Zhang *et al.*, 2012), and are currently being investigated in TC vaccine delivery. For instnace, Zhang *et al.* reported that ovalbumin encapsulation inside ethosomes induces more potent humoral responses than in other lipid vesicles (Zhang *et al.*, 2017). Therefore, incorporating the three elements of our liposomal constructs into ethosomes may also constitute a promising approach to optimize them for TCI purposes.

2.3. Beyond this project: what other factors may influence the immune potential of liposome-mediated TC vaccination?

Besides vaccine composition, several factors may influence the immune potential of liposome-based vaccines delivered by the TC route and need to be addressed in the future.

As a first-line TC application technique, we have chosen the massage technique for its convenience and simplicity. Now that we have evaluated the effect of TLR agonists, mannose

targeting molecules, and the lipid vesicles on the immune response, it is worth considering another application technique than massage, such as the use of occlusive patches or microneedles. It has been reported that TC vaccination using occlusive patches, increases vaccine penetration by creating a hydration gradient through the skin that is associated with an improved penetration of hydrophilic molecules and liposomes (Trauer *et al.*, 2014). The efficacy of this approach has been proved in preclinical as well in clinical experiments of TC delivery. For instance, a Respiratory Syncytial Virus vaccine was administered using a patch to mice and conferred them protection against viral challenge (Hervé *et al.*, 2016). In a clinical trial of TC vaccination against tetanus toxoid and diphtheria toxoid, a patch was used to deliver the two vaccine components and was shown to induce high titer antibodies (Hirobe *et al.*, 2012). For all these reasons, it appears necessary for us to investigate the immune potential of our transfersome-based constructs under occlusive conditions or using patches, in an attempt to harness their full potential. The administration of the conventional liposomes using these same strategies seems also interesting to perform.

The efficacy of TC vaccination can further improved by the use of microneedles that would better deliver the vaccine into the internal skin layers. Since a few years, various types of microneedle are being developed for TC vaccine delivery (DeMuth *et al.*, 2013; Esser *et al.*, 2016; Kim and Prausnitz, 2011; van der Maaden *et al.*, 2014), among which self-resorbing biodegradable microneedles are the most adapted for clinical use. Interestingly, the efficacy of microneedle-mediated TC vaccination has recently proved its worth, especially with the recent success of a phase I clinical trial of an influenza vaccine which is delivered through the TC route using a microneedle patch (Rouphael *et al.*, 2017).

In the first steps of TC immunization testing, we chose to apply the formulations in their original fluid state. Now that we have demonstrated the proof of concept, it appears necessary for us to optimize their physical form. Semi solid formulations, or gels, are more convenient and practical for TC application and increase the residence time of the formulations on the skin, thus improving TC penetration (Boyapalle *et al.*, 2012; Priprem *et al.*, 2016; Sardana *et al.*, 2017). Our liposome-based constructs were previously found to be suitable for incorporation into a hydroxypropyl methylcellulose (HPMC) gel, without alteration of their physicochemical properties (Kakhi, 2015). Therefore, incorporating the currently optimized

liposomes into semisolid, HPMC or other gel formulations, is expected to enhance their TC passage.

In conclusion, by combining different strategies that have each proven to be efficient in partially enhancing skin penetration, it would probably be possible to obtain an additive or synergistic effect that greatly enhances vaccine delivery to skin DCs, resulting in high efficacy of liposome vaccination by the TC route

2.4. Beyond this project: after the proof of immunogenicity of the liposomal constructs by the TC route, it is time to assess their efficacy?

The present work is one of the first reports that demonstrate the ability of liposomal constructs incorporating two single-epitope peptides and a TLR agonist delivered by the TC route to induce local and systemic immune responses. Because the ultimate goal is to develop a cancer vaccine with a therapeutic potential, our results justify the evaluation of our liposomal constructs capacity to provide protection against cancer progression in tumor-bearing mice following TC immunization. We have already initiated this part of the project.

My host team at the University of Strasbourg has a mouse renal carcinoma cell line transfected to express the human ErbB2 protein (RenCa-ErbB2), the target TAA of our liposomal preparations. It has been demonstrated that following intravenous injection, RenCa-ErbB2 cells migrate to the mouse lungs where they form pulmonary tumors. This cell line has been used to evaluate the protective effect of ErbB2-bearing constructs administered by the subcutaneous and the intranasal routes. In the current project, we evaluated the potential of liposomal constructs administered (D 2, 6 and 10) therapeutically via the TC route to inhibit tumor growth in BALB/s mice previously injected with RenCa-ErbB2 cells (D0). Unfortunately, we noticed that a behavioral divergence had occurred in the tumor cell line leading to variable aggressivity. With the same number of injected cells, the number of pulmonary nodules varied extensively from a very low count, to an overwhelming number of adjacent nodules that led to rapid death of the mice before the experiment end point. Nonetheless, in one experiment where the number of lung tumors was within the desired range, liposomal constructs incorporating the TLR4 agonist MPLA showed a partial efficacy. Interestingly, the size of the

pulmonary nodules was also reduced (data not shown). These results correlate with MPLA capacity to promote a systemic immune response following TCI. It is to be noted however, that production of IFN-γ in the spleen was more important in mice which received the TLR2/6 agonist, without a noticeable effect on tumor development. At this stage, these results have not yet been reproduced and we are still unable to confirm their significance.

We are currently addressing this difficulty by subcloning the RenCa-ErbB2 cells to obtain a stable reliable cell line before repeating the tests.

2.5. Beyond this project: application of the TC vaccination with liposomal constructs to melanoma

The first cancer type that may be targeted with TC vaccination is, obviously, melanoma. In this case, TC vaccination is expected to induce a protective local immunity in which the tumor-specific lymphocytes home to the skin. Indeed, following their priming in the secondary lymphoid organs by DCs, lymphocytes express homing molecules to the sites where these DC encountered the antigen for the first time This has been described with DCs of several origins, including cutaneous, intestinal and pulmonary DCs (Mikhak *et al.*, 2013; Mora *et al.*, 2005). Additionally, melanoma cells express a set of TAAs which are melanoma differentiated antigens. Because of their high specificity, these TAA constitute attractive targets for cancer vaccination that have been already investigated in a number of clinical trials using different strategies (Adams *et al.*, 2008; Blanchard *et al.*, 2013; Chen *et al.*, 2015; Ott *et al.*, 2014; Sabado *et al.*, 2015).

For all these reasons, it appears interesting to use the liposome-based vaccine platform that we have adapted for the TC route in order to develop a melanoma vaccine. To this end, the first step will be the choice of a CD8+ target peptide, derived from a melanoma differentiation antigen, to be incorporated into our liposomal formulations. An attractive mouse model to be used is the B16 melanoma model, which has been adapted for the evaluation of other melanoma vaccines. It consists of C57BL/6 mice injected with B16 melanoma cells, either subcutaneously to form local tumors, or intravenously to form pulmonary tumors (Damsky and Bosenberg, 2010; Fedosova *et al.*, 2015; Li *et al.*, 2007; Overwijk and Restifo, 2001).

Evaluating the efficacy of our liposome constructs following TC immunization towards local and pulmonary tumors will not only be interesting for its therapeutic potential but will also provide important information the immune mechanisms underlying the protective effect.

3. Liposomal constructs are immunogenic in the Hu-SPL-NSG mouse model

After evaluating several liposome-based constructs in the conventional murine model, the promising results prompted us to further investigate their potential for human vaccination. The discrepancy between mouse and human immune responses to antigens can be a major limiting factor in vaccine development. This is particularly true for peptide vaccines that rely on recognition of single epitopes by lymphocyte antigen receptors, given that these receptors are species specific.

For this evaluation, we selected the Hu-NSG-SPL model, in which NSG mice are reconstituted with human splenocytes. As discussed in the chapter 4 of the introduction, this model presents several advantages over more common humanized models for the evaluation of vaccine candidates. Indeed, in mice reconstituted with peripheral blood lymphocytes (Hu-PBL-SCID), a strong GVHD occurs at high rates leading to premature death and decreasing the experiment time window (Ito *et al.*, 2009; King *et al.*, 2009; Rijn *et al.*, 2003). Moreover, a massive activation of human PBL against mouse xenoantigens (King *et al.*, 2009; Tary-Lehmann *et al.*, 1994)masks specific responses induced by weak immunizing peptides. In the humanized mouse model where mice are reconstituted with hematopoietic stem cells (HSC), ethical and practical considerations limit the access to these cells. Additionally, this model presents T cells that are restricted to murine MHC, resulting in the induction of weak responses (Ishikawa *et al.*, 2005; Shultz *et al.*, 2007; Traggiai *et al.*, 2004). Moreover, the Hu-SPL-NSG model that we used represents several advantages: a large amount of cells is obtained from one human spleen, thus allowing to repeat the experiment several times with cells of the same donor. Additionally, engrafted mice do not develop signs of strong graft *versus* host disease (GVHD) (Ghosn, 2015) and the human T cells are restricted to the human MHC.

Since the potential of TC immunization with liposomes has not yet been significantly investigated in humanized immunodeficient mice, we selected to use, prior to the cancer

specific liposome constructs another model liposome in which a B epitope is inserted instead of the $CD8^+$ epitope ErbB2. Our rationale for this choice was that an induced humoral immune response would be easier to follow up than the cellular immune response. We selected for liposomes expressing, besides the $CD4^+$ peptide (HA), the PAK peptide specific for *P. aeruginosa*.

3.1. The TLR2/6 agonist Pam_2CAG is suitable to be used with human splenocytes

We carried our experiments on 3 different agonists, namely a TLR4 agonist (MPLA), a TLR 2/1 agonist (Pam_3CAG) and a TLR2/6 agonsit (Pam_2CAG). In vitro comparison of their potential to induce splenocyte proliferation allowed us to select Pam_2CAG for further investigation in vivo. Intraperitoneal injection of the liposomes incorporating Pam_2CAG to the humanized mice induced HA-specific $CD4^+$ IFN-γ secreting cells indicating a Th1 profile immune response. This is one of the few reports on the capability of liposomes incorporating single-epitope peptide to induce detectable responses in the humanize moue model.

Regarding the comparison of the three TLR agonists, the selection of the Pam_2CAG was made based upon the in vitro preliminary assays. Indeed, when splenocytes of different donors were cultured in presence of liposomes incorporating the three immunostimulatory molecules, Pam_2CAG resulted in the highest proliferation rate. These results are somehow in agreement with our findings for the TC immunization of BALB/c mice, where Pam_2CAG was superior to MPLA.

It has been shown that TLR agonists induce antigen-independent B cell, but not T cell, proliferation. Since MPLA is a TLR4 agonist, and this receptor is weakly expressed on B cells (Buchta and Bishop, 2014), this explains the low proliferation index induced by MPLA. It is to be noted that MPLA is currently used in a number of vaccines such as the viral vaccines Fendrix and Cervarix, respectively against Hepatitis B and Human Papillomavirus, in addition to a number of cancer vaccine candidates in clinical trial development pipeline (Didierlaurent *et al.*, 2009). MPLA efficacy in viral and cancer vaccines stems from its capacity to induce Th1 responses. Therefore, for the purpose of evaluating a cancer-specific liposomes in the humanized mice, MPLA remains a good immunostimulatory candidate.

3.2. A model liposome-based construct induces a cellular but not a humoral immune response in the Hu-SPL-NSG mouse

Concerning the specific immune response of the Hu-APL-NSG mice to the liposomes incorporating PAK, HA and Pam_2CAG, we detected a high-amplitude response of the Th1 profile against the $CD4^+$ T cell peptide. To our knowledge, we are the first to demonstrate that liposomal constructs delivering epitope peptides and adjuvanted with a TLR agonist can elicit a potent cellular immune response in the Hu-SPL-NSG mouse model. Concerning other humanized models, very few reports have addressed this issue (Majji *et al.*, 2016).

No humoral response to the PAK peptide was, however, detected. Two explanations are possible for this finding:

a) PAK peptide contains a single B-cell epitope, meaning that we cannot expect high specific antibody titers in the Hu-SPL-NSG mouse model.
b) The induced immune response is of the Th1 profile, which is less efficient in inducing antibodies than the Th2 profile. For potential application of this liposomal model in vaccine trials in which a humoral immune response in desired, it is worth investigating other TLR agonists more appropriate for the desired profile.

In previous works that demonstrated the ability of the humanized mice to respond to immunizations with liposomes, whole proteins were usually used (Ifversen *et al.*, 1995; Walker and Gallagher, 1994), or small peptides in excessively high amounts (Majji *et al.*, 2016). Therefore, the ability of the Hu-SPL-NSG mouse model to response to single-epitope peptides incorporated in liposomal constructs makes it a strong candidate prototype for liposomal cancer vaccine evaluation. For this purpose, it needs to be further investigated for its capability to induce $CD8^+$ T cell responses following liposome administration.

3.3. Beyond this project: evaluation of the efficacy of liposomal constructs against cancer in the Hu-SPL-NSG mouse

To evaluate whether it is possible to elicit cancer specific CTL responses in this model, mice will be immunized with liposomes bearing, in addition to a $CD4^+$ T cell epitope peptide and a TLR agonist, a $CD8^+$ T cell peptide (ErbB2) instead of the B cell epitope peptide.

As for the choice of $CD4^+$ T cell epitope to be included in the liposomes, new alternatives should be explored. Throughout this thesis work, all tested liposomal vaccines HA peptide derived from the hemagglutinin protein of the influenza virus. This peptide was selected because virtually all humans are expected to have memory $CD4^+$ T cells to the hemagglutinin protein. , since influenza virus infection is ubiquitous. Additionally, the $CD4^+$ T cell epitope (307–319, PKYVKQNTLKLAT-C) is promiscuous and can bind to MHC DRB1 molecules of several haplotypes (O'Sullivan *et al.*, 1991). However, we were only able to detect a cellular response in mice reconstituted with cells of one spleen donor out of four. Therefore, it might be beneficial to replace HA with another universal $CD4^+$ peptide, such as a tetanus toxoid-derived peptide. According to the literature, peptides derived from the tetanus toxoid are frequently used to provide help for vaccine formulations lacking $CD4^+$ epitope sequences (Cruz *et al.*, 2014; Rueda *et al.*)

To subsequently evaluate the protective effect of the liposomal constructs, a Hu-SPL-NSG-tumor model is needed. We are currently preliminary experiments in order to select a suitable human tumor cell line that highly expresses ErbB2 and can induce solid tumors. Two cell lines candidates are being considered, namely MCF-7 and SKBR-3, both derived from breast cancer adenocarcinoma (Comşa *et al.*, 2015; Holliday and Speirs, 2011).

4. A humanized mouse model for TC cancer vaccination: time to think of the next generation model

The ultimate goal of the current project is to develop a humanized Hu-SPL-NSG model for TC immunization, in which animals are engrafted both with human skin explants and human immune cells, and in which immune responses following vaccine application on the human skin graft can be evaluated. The two major requirements for the success of this model are first to conserve the human skin architecture and physiological conditions in the host and, second, to develop an immune cell engraftment protocol that allows lymphocyte priming by the skin dendritic cells.

Models of human skin transplantation into humanized mice are currently being developed in the aim of studying skin-related conditions, such as psoriasis, or organ graft rejection. Soria *et*

al. transplanted NSG mice with human skin and assessed the architectural, immune, and functional integrity of the transplanted grafts. They demonstrated that the skin retains its integrity and its revascularization for several weeks after transplantation. Additionally, they showed that the skin preserves its immune architecture, with a persistence of LCS, dDCs, and dermal macrophages. Using intradermally injected fluorescent nanoparticles, they found that various skin DCs are capable of nanoparticle uptake. Moreover, both LCs and dDCs were found to conserve their capacity of antigen presentation. Indeed, after an intradermal injection of an attenuated vaccinia virus coding for the gag protein of human immunodeficiency virus, the human specimen was excised and it was demonstrated that activated LCs and dDCs were both capable of priming gag-specific lymphocytes *in vitro* (Soria *et al.*, 2014).

Our goal is to further develop this model to assess the immune response to the liposomal vaccine candidates in vivo. Therefore, the two human tissues, respectively the spleen and the skin, will need to derive from the same donor. The skin explants will need to be obtained from deceased spleen donor. Alternatively, skin explants may be obtained from surgical waste tissue following plastic surgeries. In the latter case, to prevent skin allograft rejection, MHC-matching would be needed.

Another challenge would be to avoid interference of the mouse innate immune system. Indeed, upon skin transplantation, NSG mice show high levels of mouse cellular infiltrates in the graft, mostly comprised of neutrophils (Kirkiles-Smith *et al.*, 2009). Racki *et al* have efficiently reduced this infiltration, which is detrimental for the graft survival, by administrating to the Hu-PBL-NSG mice antibodies targeting, GR1, a neutrophil marker. This strategy dramatically decreased skin graft infiltration with murine granulocytes, without affecting the subsequent engraftment of allogeneic human PBL (Racki *et al.*, 2010).

In conclusion, we proved in this work the feasibility and the efficacy of TC vaccination using versatile liposome-based constructs that serve as platforms for designing innovative vaccines, thus providing a further rationale for the development of cancer vaccines for TC delivery. We also found that the Hu-SPL-NSG mouse model can indeed be suitable for the evaluation of liposome-based vaccines. Therefore, the ultimate perspective of this work

resides in developing a humanized mouse model for TC vaccination that complements the conventional mouse models, thus facilitating the selection of the best candidate cancer vaccines for human use.

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Hanadi SALIBA

Development of innovative liposome-based constructs for non-invasive cancer immunotherapy in humans

Résumé

La voie d'administration d'un vaccin et le modèle préclinique dans le lequel il est évalué sont des facteurs majeurs qui contribuent à son succès chez l'homme. Dans ce contexte, la découverte que la voie transcutanée (TC) induit une réponse immunitaire puissante a fait de la vaccination antitumorale TC une stratégie prometteuse. Une évaluation complémentaire du candidat vaccin dans un modèle de souris humanisée (Hu-SPL-NSG), plus prédictif de la réponse humaine, est aussi nécessaire.

L'objectif de cette thèse est i) d'optimiser des constructions liposomiques peptidiques incorporant un agoniste de TLR pour la voie TC et ii) d'évaluer leur immunogénicité dans le modèle Hu-SPL-NSG.

Ainsi, nous avons fait varier la nature de l'agoniste de TLR et la déformabilité de la vésicule liposomique, et avons rajouté une molécule de ciblage des cellules dendritiques. L'immunogenicité de ces formulations par voie TC a ensuite été évaluée chez la souris. Enfin, nous avons testé la capacité d'une construction liposomique modèle à induire une réponse cellulaire et humorale dans le modèle Hu-SPL-NSG.

L'ensemble de ces travaux a fourni une première preuve de concept sur la faisabilité de la vaccination antitumorale TC par des liposomes et de son applicabilité chez l'homme.

Mots-clés : vaccin antitumoral, liposome, voie transcutanée, souris humanisée

Résumé en anglais

A vaccine administration route and the preclinical model in which it is evaluated are major factors that contribute to its success in humans. In this context, the discovery that the transcutaneous (TC) route induces a powerful immune response has made theTC tumor-specific vaccination a promising strategy. Further evaluation of candidate vaccines in a humanized mouse model (Hu-SPL-NSG), more predictive of the human response, is also needed.

The objective of this thesis is to (i) optimize liposomal constructs incorporating peptides and a TLR agonist for the TC pathway and (ii) evaluate their immunogenicity in the Hu-SPL-NSG model.

Thus, we have varied the nature of the TLR agonist and the deformability of the liposomal vesicle, and have added a dendritic cell targeting molecule. Immunogenicity of these formulations by the TC route was then evaluated in mice. Finally, we tested the ability of a model liposomal construct to induce a cellular and humoral response in the Hu-SPL-NSG model.

All of this work provided a first proof of concept on the feasibility of TC tumor-specific vaccination by liposomes and its applicability in humans.

Keywords: tumor-specific vaccine, liposome, transcutaneous route, humanized mouse