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Étude des basophiles dans l'inflammation allergique de la peau

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À mes parents,
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RESUMÉ

L'allergie est un état dans lequel l'hôte réagit de manière excessive à des antigènes autrement inoffensifs (allergènes) en induisant une inflammation de type Th2. Les maladies allergiques, y compris la dermatite atopique, l'asthme, la rhinite allergique et les allergies alimentaires, sont des maladies inflammatoires étroitement liées impliquant différents sites corporels. La prévalence des maladies allergiques a considérablement augmenté, avec près de 30% de la population mondiale touchée par une ou plusieurs allergies, représentant ainsi un problème émergent de santé publique. Un certain nombre d'acteurs cellulaires et moléculaires ont été impliqués dans l'inflammation allergique de la peau, cependant, leurs interactions au sein du réseau inflammatoire restent mal définies, ce qui empêche considérablement la compréhension de la pathogenèse allergique et le développement d'approches thérapeutiques et de préventions efficaces.

L'un des objectifs de mon laboratoire est de mieux comprendre les réseaux inflammatoires dans la peau allergique. Les basophiles, un type de granulocytes représentant 1% des leucocytes sanguins, sont un composant cellulaire caractéristique dans l'inflammation allergique de la peau. Les basophiles complètent leur maturation dans la moelle osseuse, circulent dans le sang puis migrent vers les tissus sous des conditions inflammatoires. Ils ont été reconnus pour leurs fonctions effectrices dans d'allergie, comme la libération d'histamine et de leucotriènes. Récemment, de nouveaux anticorps détectant spécifiquement les basophiles et des réactifs / outils permettant leur déplétion ont été développés chez la souris. Ceci a permis de mettre en évidence ce type cellulaire et de révéler des rôles importants impliquant les basophiles dans l'immunité contre certains pathogènes et troubles immunologiques, y compris les infections parasitaires et les allergies, bien que certains résultats demeurent contradictoires. Malgré ces progrès, la façon dont les basophiles sont recrutés dans le tissu inflammé, comment ils sont activés et la manière dont ils interagissent avec d'autres cellules dans le micro-environnement inflammatoire local restent encore largement méconnus.

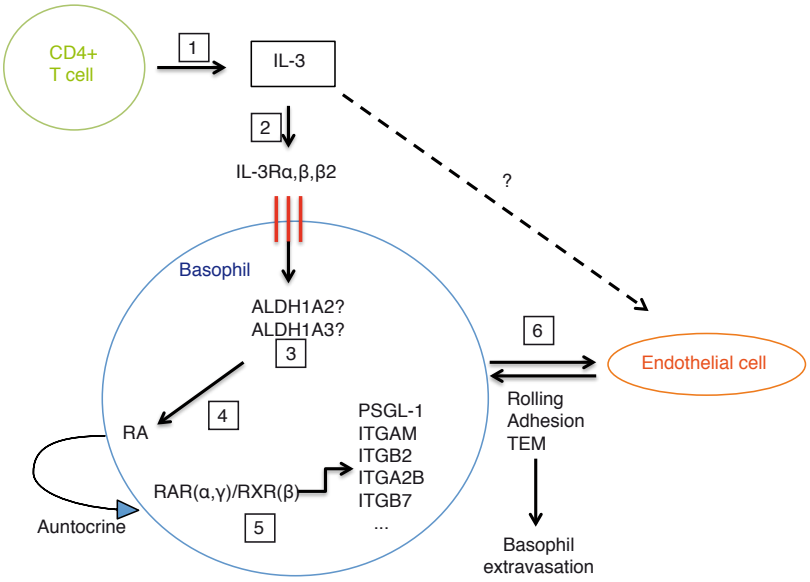
L'objectif de ma thèse de doctorat est d'étudier le recrutement, l'activation et la fonction des basophiles dans le réseau inflammatoire de la peau allergique. Pour aborder ce sujet complexe, j'ai tout d'abord caractérisé et optimisé un modèle murin physiopathologique robuste de dermatite atopique allergique (DAA) induite par un haptène, dans lequel les souris de type Balb/c ont été sensibilisées dans un premier temps avec de l'isothiocyanate de fluorescéine (FITC) (un haptène dissous dans du phtalate de dibutyle-DBP et de l'acétone) sur leurs oreilles gauches, suivi d'un « challenge » avec le FITC sur l'oreille droite. Les cellules immunitaires dans la peau de l'oreille droite ont été caractérisées et analysées à l'aide d'une immunohistochimie (IHC) et de cytométrie en flux, montrant un infiltrat marqué des basophiles, accompagné de cellules T, d'éosinophiles, de neutrophiles et de mastocytes. Les analyses de RT-qPCR ont montré une augmentation des cytokines typiques de type Th2 : IL-4, IL13 et IL-3. De plus, en utilisant une souris double reportrice *Il4/Il13*, j'ai constaté que les basophiles, en plus des cellules T de type Th2, produisaient de grandes quantités d'IL-4 et d'IL-13, tandis que le marquage intracellulaire a montré que l'IL-3 était principalement produite par les cellules T.

1) L'IL-3 joue un rôle clé dans l'extravasation des basophiles vers la peau allergique

Des études de mon laboratoire ont récemment démontré que, dans un modèle murin de dermatite atopique (DA) induite par la surexpression de la « lymphopoiétine thymique stromale» (TSLP), une cytokine clé favorisant les réponses immunitaires de type Th2, le recrutement des basophiles vers la peau souffrant d'une DA implique des mécanismes d'immunité innée et adaptative. Cependant, j'ai constaté que le recrutement des basophiles dans la DAA induite par le FITC était totalement dépendant de l'immunité adaptative, car le recrutement des basophiles a été supprimé chez les souris *Rag1*$^{-/-}$ (ne contenant pas de cellules T et B). Puisqu'il a récemment été montré que l'IL-3 est importante pour le recrutement des basophiles dans un modèle d'infection parasitaire, j'ai donc ensuite examiné l'inflammation allergique induite par le FITC chez des souris *Il3*$^{-/-}$. Les analyses de la peau par cytométrie en flux ont montré que le nombre de basophiles dans le derme était largement diminué chez les souris *Il3*$^{-/-}$ traitées par le

FITC, alors que les autres types cellulaires (cellules T, éosinophiles, neutrophiles et mastocytes) n'étaient pas affectés, ce qui suggère que l'IL-3 joue un rôle clé et spécifique dans le recrutement des basophiles. De manière inattendue, lorsque j'ai effectué une coloration IHC pour détecter les basophiles, j'ai observé un phénomène intéressant montrant que les basophiles étaient bloqués dans les vaisseaux sanguins de la peau des souris *Il3*$^{-/-}$ traitées avec le FITC. Une analyse cinétique à différents laps de temps suivant le challenge avec le FITC a montré un phénotype similaire, ce qui suggère que cela n'est pas dû à un retard dans le recrutement des basophiles. D'autre part, l'analyse du sang par cytométrie en flux a montré un nombre plus élevé de basophiles chez les souris *Il3*$^{-/-}$ traitées par le FITC, indiquant que les basophiles circulant n'ont pas diminué en l'absence d'IL-3. Collectivement, mes résultats suggèrent que l'extravasation des basophiles vers la peau souffrant d'une DAA induite par le FITC est défectueuse chez les souris *Il3*$^{-/-}$. De plus, en générant des souris CD4-Cre$^{Tg/0}$/Il3$^{L2/L2}$ dans lesquelles l'IL-3 est sélectivement invalidé dans les lymphocytes T, j'ai montré que l'IL-3 produite précisément par les lymphocytes T était responsable de l'extravasation des basophiles dans la peau allergique. Pour étudier plus précisément les mécanismes régulant ce phénomène, j'ai trié les basophiles et les cellules endothéliales par cytométrie en flux à partir de la peau de souris CT et *Il3*$^{-/-}$ traitées par le FITC (sachant que les basophiles dans la peau des souris *Il3*$^{-/-}$ sont ceux bloqués dans les vaisseaux sanguins) et j'ai analysé l'expression de différentes molécules connues pour être impliquées dans l'extravasation des leucocytes. D'une manière intéressante, j'ai constaté que PSGL-1 ainsi que ITGAM, ITGB2, ITGA2B et ITGB7, connues pour être impliquées dans le processus de roulement et l'adhésion des leucocytes aux cellules endothéliales précédant leur extravasation vers les tissus, ont été considérablement diminuées dans les basophiles provenant des souris *Il3*$^{-/-}$ traitées par le FITC, ce qui suggère que ces molécules pourraient être responsables de l'extravasation des basophiles. En outre, des études ont montré que l'acide rétinoïque (AR) peut agir sur l'extravasation des leucocytes, en induisant l'expression d'intégrines à leur surface. J'ai donc testé cette hypothèse en appliquant sur la peau des souris *Il3*$^{-/-}$ traitées par le FITC de l'AR (all-trans RA), et les résultats préliminaires ont montré que l'AR restaure l'extravasation des basophiles qui était défectueuse dans la peau de ces souris. De

plus, en analysant l 'expression des enzymes impliquées dans le mécanisme de synthèse de l'AR, j'ai constaté que l'enzyme RALDH3 (rétinaldehyde dehydrogenase) était diminuée dans les basophiles triés de la peau des souris *Il3*$^{-/-}$. Ainsi ces résultats proposent un mécanisme par lequel, l'IL-3 produite par les lymphocytes T agit directement sur les basophiles, qui expriment le récepteur à l'IL-3 (IL-3R) en induisant l'expression de RALDH3, qui va ensuite permettre la synthèse de l'acide rétinoïque par les basophiles. Cet acide rétinoïque va, de manière autocrine, sur l'hétérodimer RAR/RXR, ce qui va entrainer l'expression des gènes PSGL-1, ITGB2, ITGAM, ITGA2B et ITGB7, qui vont permettre ainsi l'interaction des basophiles avec les cellules endothéliale. Cependant, les basophiles pourront passer la barrière des vaisseaux sanguins pour arriver dans la peau et être activés (voir schéma ci-dessous).



2) L'épuisement des basophiles dans le modèle de souris *MCPT8*^{DTR} conduit à une réduction systémique des éosinophiles et des neutrophiles

Récemment, des souris *Mcpt8*^{DTR} ont été développées comme outil puissant pour l'étude de la fonction des basophiles *in vivo*. Dans ce modèle, le récepteur de la toxine diphtérique (DTR) est exprimé sous le contrôle du gène MCPT8 ; par conséquent, l'administration de la toxine diphtérique (TD) conduit à épuiser spécifiquement les

basophiles. À l'aide des souris *Mcpt8*^{DTR}, j'ai cherché à étudier le rôle des basophiles dans l'inflammation allergique de la peau. Dans un premier temps, j'ai constaté que l'appauvrissement des basophiles a entraîné une diminution des éosinophiles et des neutrophiles dans la peau enflammée. Bien que cette découverte est concomitante avec des concepts récents que les basophiles jouent un rôle essentiel dans le recrutement des éosinophiles dans la peau, mes recherches supplémentaires ont révélé de manière surprenante que l'injection de la TD provoquait une diminution systémique des éosinophiles et des neutrophiles dans le sang, la rate et la moelle osseuse. Une caractérisation cinétique des souris *Mcpt8*^{DTR} injectées avec la TD a montré une réduction des éosinophiles et des neutrophiles en fonction du temps dans ces organes. De plus, j'ai découvert que les progéniteurs des granulocytes et des macrophages (GMPs), dérivés des cellules souches hématopoïétiques, et donnant naissance aux neutrophiles et aux éosinophiles, ont été épuisés lors de l'injection de la TD. En outre, j'ai constaté que les GMPs expriment le gène MCPT8, ce qui n'a jamais été signalé auparavant, fournissant une explication plausible de la réduction systémique des neutrophiles et des éosinophiles. Mes résultats suggèrent que l'interprétation des études basées sur des souris utilisant le promoteur du gène MCPT8 pour supprimer les basophiles, devrait être soigneusement ré-évaluée, et avertissent que le temps de l'injection et la dose de TD doivent être considérés dans la caractérisation des cellules à base du système DTR. Mes résultats suggèrent également que, contrairement aux récentes publications, les basophiles ne jouent pas un rôle essentiel dans le recrutement d'éosinophiles ou de neutrophiles dans la peau allergique.

En conclusion, les études de ma thèse de doctorat fournissent de nouvelles notions et ajoutent des informations importantes sur le recrutement des basophiles, leur activation et leur fonction dans l'inflammation allergique de la peau, ce qui aide à une meilleure compréhension de cette pathogenèse inflammatoire le développement de stratégies plus efficaces pour traiter les troubles inflammatoires associés.

Introduction sur les Basophiles

Les basophiles ont été découverts par Paul Ehlrich en 1879. Ils représentent les granulocytes les moins abondants (moins de 1% des leucocytes du sang périphérique) chez les mammifères. Ils ont longtemps été négligés en raison de leur rareté, de leur courte durée de vie, de leurs similitudes avec les mastocytes et aussi pour le manque d'outils pour leur détection et leur analyse fonctionnelle. Récemment, des anticorps détectant spécifiquement les basophiles, et des réactifs / outils pour invalider les basophiles ont été développés, permettant ainsi leur caractérisation fonctionnelle *in vivo,* ce qui a révélé des rôles non redondants et importants des basophiles dans l'immunité.

Développement

Les basophiles sont un type de granulocytes caractérisés par leurs granules basophiles dans le cytoplasme. Chez les souris, les basophiles représentent une étroite relation lignagère avec les mastocytes (Metcalf, Ng, Baldwin, Di Rago et Mifsud, 2013), tandis que les basophiles humains semblent être plus proches des éosinophiles (Grundstrom et al., 2012). Cependant, il est important de souligner que la plupart des études sur le développement des basophiles proviennent de modèles murins. Les basophiles proviennent de la moelle osseuse du granulocyte et monocyte progénitor (GMP) et leur spécification de lignée dépend de l'expression du facteur de transcription GATA-binding protein 2 (GATA2) et de CCAAT / enhancer-binding protein-α (C / EBPα), puis ils entrent dans la circulation sanguine sous un phénotype mature (H. Iwasaki & Akashi, 2007). Il a été démontré que la protéine IKZF1 de la famille Ikaros régulait négativement le développement des basophiles en inhibant C / EBPα (Rao, Smuda, Gregory, Min et Brown, 2013), tandis que le transducteur de signal et l'activateur de la transcription (STAT 5) s'est révélé être crucial pour le développement des basophiles en augmentant l'expression de GATA2 et C / EBPα (X. Qi et al., 2013). Dans des conditions stables, les basophiles terminent leur maturation dans la moelle osseuse, puis circulent dans le sang périphérique oubien vont dans la rate, mais ne prolifèrent pas davantage. Les basophiles ont une durée de vie très courte de 60 heures chez la souris (Ohnmacht & Voehringer, 2009).

Il a été démontré que d'autres facteurs agissent sur le développement et la différenciation des basophiles. La lymphopoïétine stromale thymique (TSLP) et l'IL-3 sont deux cytokines qui peuvent favoriser l'expansion des basophiles dans le corps en activant la voie STAT5 et en réduisant l'apoptose (Didichenko, Spiegl, Brunner et Dahinden, 2008). La TSLP peut favoriser le recrutement des basophiles dans la peau dans un modèle de dermatite atopique et peut induire une basophilie indépendamment de l'IL-3 (Siracusa et al., 2011). Il a été montré que l'IL-3 induisait la basophilie et l'accumulation de basophiles dans le ganglion lymphatique dans un modèle d'infection avec des helminthes (S. Kim et al., 2010). Les progéniteurs GMPs traités à l'IL-3 ont montré une différenciation en basophiles fortement impliqués dans l'immunité IgE (Ohmori et al., 2009), tandis que TSLP a amélioré la survie des basophiles (Siracusa et al., 2011). De plus, le facteur stimulant les colonies de granulocytes-macrophages (GM-CSF) et l'IL-5 qui partagent la même chaîne βc avec l'IL-3, induisent également une expansion des basophiles chez la souris, lorsque l'IL-3 n'est pas bien exprimée (Schneider et al., 2009). Globalement, IL-3 et TSLP semblent être les principales cytokines contribuant au développement des basophiles.

Activation

Les basophiles sont bien connus pour leur fonction effectrice marquée par la libération de médiateurs lors de leur activation. Des signaux différents peuvent activer les basophiles. Parmi ces signaux, des anticorps, des cytokines mais aussi des protéases et des allergènes peuvent médier l'activation des basophiles. En conséquence de leur activation, les basophiles libéreront des molécules préformées telles que l'histamine et le leucotriène C4, le facteur d'activation plaquettaire (PAF), l'acide rétinoïque, des cytokines incluant IL-4, IL-13, IL-6, TSLP, mais aussi des peptides antimicrobiens. Toutes ces molécules seront impliquées dans des réactions immédiates ou tardives du système immunitaire (Steiner, Huber, Harrer, & Himly, 2016).

L’activation par les anticorps

IgE. Les basophiles expriment à leur surface le récepteur Fc présentant une haute affinité pour les IgE, FcεRI. Les anticorps IgE spécifiques de l'antigène générés par les réponses humorales sont capturés par les basophiles circulants à travers le FceRI (Siracusa, Kim, Spergel, & Artis, 2013). Ainsi, lors de la ré-exposition à l'antigène, en réponse à la liaison des IgE à leur récepteur, les basophiles dégranulent et sécrètent un certain nombre de médiateurs, comme l'histamine, les leucotriènes (Schroeder, 2011), les médiateurs lipidiques, les prostaglandines et diverses cytokines. , IL-3, IL-4, IL-13, IL-9, IL-25 et des chimiokines comme RANTES (Chirumbolo, 2012). L'IL-4 joue un rôle clé dans l'induction de la différenciation des cellules Th2 à partir de cellules T CD4 + naïves (Gomez et al., 2014). En outre, l'IL-4, sécrétée conjointement avec l'IL-6 des basophiles, a montré qu'elle activait les lymphocytes B dans les réponses humorales en améliorant leur prolifération et la sécrétion d'immunoglobulines (Denzel et al., 2008). L'IL-4 sécrétée par les basophiles aide également à la migration des éosinophiles en régulant positivement la chimiokine CCL11 dans les fibroblastes (Nakashima et al., 2014) et conduit la différenciation des monocytes inflammatoires en macrophages M2, dans l'inflammation cutanée allergique (Egawa et al., 2013).

IgG. Il a été démontré que les basophiles peuvent favoriser l'anaphylaxie en réponse à des complexes IgG-antigène qui se lient à FcγRIIIA chez la souris et FcγRIIA chez l'humain (Tsujimura et al., 2008) et produisent ainsi un facteur d'activation plaquettaire (PAF).

IgD. IgD est une classe d'anticorps qui est produite dans la phase précoce du développement des cellules B et qui est connu pour activer les basophiles, ce qui entraînera la sécrétion des peptides antimicrobiens par les basophiles et inhibera la croissance des bactéries. Cependant, l'activation médiée par les IgD n'a pas d'impact sur la libération d'histamine par les basophiles, mais augmente leur production d'IL-4 (K. Chen et al., 2009).

L’activation par les cytokines

Récemment, des études ont montré que les basophiles peuvent être très hétérogènes, car leur développement peut être influencé par différentes cytokines, principalement l'IL-3 et la TSLP, bien qu'elles puissent se développer à l'état physiologique en l'absence des deux cytokines.

IL-3. Les basophiles activés par l’IL-3 contribuent à la réponse immunitaire (acquise) dépendante des IgE (Karasuyama et Yamanishi, 2014). IL-3 favorise le développement des basophiles à la fois *in vivo* et *in vitro*, en outre, il favorise également l'activation des basophiles (Siracusa et al., 2013), et a été montré pour améliorer leur production d'IL-4 et IL-13 chez l’humain (Schroeder, Chichester , & Bieneman, 2009; Siracusa et al., 2011). En réponse à l'infection par les helminthes, l'IL-3 a été jugée cruciale pour l'expansion des basophiles périphériques (Lantz et al., 2008) et pour améliorer la production d'IL-4 et d'IL-13 par les basophiles activés par l’IgE (Gibbs et al. ., 1996). Les basophiles eux-mêmes peuvent produire de l'IL-3 et créer ainsi une boucle de rétroaction positive pour leur activation (Marone, Borriello, Varricchi, Genovese et Granata, 2014). Il a également été montré que l'IL-3 était importante pour le recrutement des basophiles dans le ganglion lymphatique mésentérique en lors d’une infection par des helminthes (S. Kim et al., 2010) et des ganglions lymphatiques drainant la peau dans un modèle de souris de dermatite atopique induit par une surexpression de TSLP (Leyva-Castillo et al., 2013). Cependant, le fait que les basophiles puissent se développer en l'absence de signalisation IL-3-IL3R montre que d'autres médiateurs sont capables de réguler leur développement et leur fonction.

TSLP. La TSLP (lymphopoïétine stromale thymique) est une cytokine clé dans l'inflammation allergique, elle est dérivée de l'épithélium et favorise les réponses immunitaires de type Th2 (S. Kim et al., 2010, Y. J. Liu, 2006, Ziegler, 2010). Les basophiles activés par TSLP sont différents des basophiles induits par IL-3. Les basophiles induits par TSLP contribuent à des réponses immunitaires (innées) indépendantes des IgE (Karasuyama et Yamanishi, 2014). Il a été démontré que les basophiles induits par TSLP jouent un rôle dans la pathogenèse de l'oesophagite éosinophilique indépendamment des IgE (Noti et al., 2013). Par conséquent, TSLP s'est

également révélée être élevée dans l'œsophage des patients atteints d'œsophagite à éosinophiles. Les basophiles induits par TSLP ont contribué à la pathogenèse de la dermatite atopique (Leyva-Castillo et al., 2013, Siracusa et al., 2013) et à l'allergie alimentaire (Muto et al., 2014, Noti et al., 2014). TSLP a été signalé pour promouvoir l'hématopoïèse des basophiles indépendamment de l'IL-3 (Siracusa et al., 2011). Suite à l'infection des souris par *Trichinella spiralis*, les réponses des basophiles dans le ganglion lymphatique drainant l'intestin étaient fortement dépendantes des interactions TSLP-TSLPR et non de celles de l'IL3-IL3R (Giacomin et al., 2012).

Des études non publiées de mon laboratoire ont récemment démontré que dans un modèle de dermatite atopique murin, induit par la surexpression de TSLP, le recrutement de basophiles à la peau souffrant de dermatite atopique implique à la fois des voies IL-3-dépendante et indépendante. Comment les basophiles sont recrutés sur le site de l'inflammation et comment ils sont activés reste controversé; de plus, la manière dont les basophiles sont recrutés lors de l'inflammation cutanée allergique est loin d'être claire.

IL25 et IL33. Ces cytokines sont des initiateurs importants des réponses Th2. Les basophiles humains expriment IL17RB et ST2, qui sont respectivement des récepteurs pour IL-25 et IL-33. Lorsqu'ils sont amorcés avec IL-33, les basophiles activent la voie NFkB et sécrètent IL-4 et IL-13. De plus, il a été montré que l'IL-25 régulait positivement l'IL-3Ra et CD203c à la surface des basophiles. (Smithgall et al., 2008, H. Wang et al., 2010).

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MAIN ABBREVIATIONS

A
A2Ar: adenosine receptor 2A
Ab: antibody
ACD: allergic contact dermatitis
ACK: ammonium-chloride-potassium
AD: atopic dermatitis
AIM: apoptosis inhibitor of macrophage
ALDH: aldehyde dehydrogenase
ALR: AIM-2 receptor
AMP: adenosine monophosphate
APC: antigen presenting cell
At-RA: all-trans retinoic acid
B
BAL: bronchoalveolar lavage
BB1: basogranulin
BIR: baculovirus inhibitor repeat domain
BM: bone marrow
C
CAI: chronic-allergic inflammation
CARD: caspase activation and recruitment domains
CC: β chemokine
CD: cluster of differentiation
CDSN: corneodesmosin
CLP: common lymphoid progenitor
CLR: c-type lectin receptor
CMP: common myeloid progenitor
CT: control
CTD: c-terminal domain
CTLA4: choline transporter-like protein 4
CTLD: c-type lectin-like domain
CXC: α chemokine
D
DBP: dibutyl phthalate
DC: dendritic cell
dDC: dermal dendritic cell
DETC: dendritic epidermal T cell
DT: diphtheria toxin
DTR: diphtheria toxin receptor
E
EC: endothelial cell
ESAM-1: endothelial cell-specific adhesion molecule
F
FCεRI: high affinity IgE receptor
FCS: foetal calf serum

FITC: fluorescein isothiocyanate
FLG: filaggrin
FLT3/FLT3L: fms-like tyrosine kinase 3 (L for ligand)
FMLF: formyl-methionyl-leucyl phenylalanine

G

GATA: Trans-acting T-cell-specific transcription factor
GM-CSF: granulocyte-macrophage – colony stimulating factor
GMP: granulocyte –macrophage progenitor

H

H&E: haematoxylin & eosin
HDM: house dust mite
HLA: human leucocyte antigen
HSC: hematopoietic stem cell
HSV: herpes simplex virus

I

ICAM-1: Intercellular adhesion molecule 1
IFN: Interferon
Ig: Immunoglobulin
IHC: Immunohistochemistry
IKZF: Ikaros family zinc finger protein
IL: Interleukin
ITG: Integrin

K

KC: keratinocyte

L

LacZ: beta-D-galactosidase
LC: Langerhans cell
LFA-1: lymphocyte function associated antigen 1
LRR: Leucine-rich repeat receptor
LTB4: Leukotriene B4

M

MAC-1: macrophage-1 antigen
MBP: major basic protein
MC: mast cell
MCPT8: mast cell protease 8
MDP: macrophage and dendritic cell progenitor
MEP: megakaryocyte-erythroid progenitor
MHC: major histocompatibility complex

N

N.b.: *Nippostrongylus brasiliensis*
NADP: Nicotinamide adenine dinucleotide phosphate
NET: neutrophil extracellular trap
NK cell: natural killer
NKG2D: natural killer group 2, member D
NKT cell: natural killer T cell
NLR: nucleotide-binding oligomerization domain-like receptor
NOD: nucleotide-binding oligomerization domain

O
OVA: ovalbumin
P
PAMP: pathogen-associated molecular pattern
pDC: plasmacytoid dendritic cell
PRR: pattern-recognition receptor
PSGL-1: P-selectin glycoprotein ligand 1
PYD: pyrin domain
R
RA: retinoic acid
RANTES: regulated upon activation, normal T cell expressed and secreted
RAR: retinoic acid receptor
RARE: retinoic acid receptor response elements
RD: repressor domain
RIG: retinoic acid-inducible gene
RLR: RIG-like receptor
ROR: RAR-related orphan receptor
RXR: retinoic X receptor
S
SLE: systemic lupus erythematosus

STAT: Signal transducer and activator of transcription
T
TCR: T cell receptor
TEM: trans-endothelial migration
TFH: T follicular helper
TGF: transforming growth factor
TH: T helper
TLR: tool-like receptor
TNF: tumour necrosis factor
Treg: regulator T cell
TSLP: thymic stromal lymphopoietin
V
VCAM-1: vascular cell adhesion molecule 1
VLA-4: very late antigen 4
W
WT: willdtype
Y
YFP: yellow fluorescent protein

INTRODUCTION

INTRODUCTION

The immune system is a complex network of organs, tissues and cells that interact with each other to defend the body against attacks by pathogenic microorganisms, thus creating a state of protection from infectious diseases. It is recognized for its diversity and specificity to respond against a wide range of pathogens. A deficiency in the immune response, or an overreaction to pathogens can cause harmful damages. Thus, the key challenge of the immune system is to keep a balance between inoffensive and damaging responses.

1. The Immune system

The immune system in mammals has been divided into innate and adaptive immunity, each with a different function and role. All immune cells are derived from precursors in the bone marrow, through a process called haematopoiesis. During evolution, the innate immune system appeared before the adaptive immune system, and represents the first line of defence against pathogens. On the contrary, the adaptive immune system is characterized by a slower and highly specific immune response.

1.1 Haematopoiesis

The cells of the immune system derive from precursors in the bone marrow, where they develop and for some of them, get mature. Self-renewal and multipotent hematopoietic stem cells (HSCs), the origin of all hematopoietic cells in the body, are able to differentiate and give rise to two distinct lineages:

1) The myeloid lineage, through the common myeloid progenitors (CMPs), which will either differentiate into granulocyte-macrophage progenitors (GMPs) and be the origin of granulocytes, such as neutrophils, eosinophils, basophils and mast cells, or differentiate into monocyte and dendritic cell progenitors (MDPs) to give the monocytes and macrophages, then common dendritic cell progenitors (CDPs) which will provide the

dendritic cells; CMP also differentiate into megakaryocyte-erythroid progenitors (MEPs) that is the origin of megakaryocytes and platelets.

2) The lymphoid lineage, through the common lymphoid progenitors (CLPs), who will be the origin of T lymphocytes, B lymphocytes and natural killer (NK) cells (figure 1).

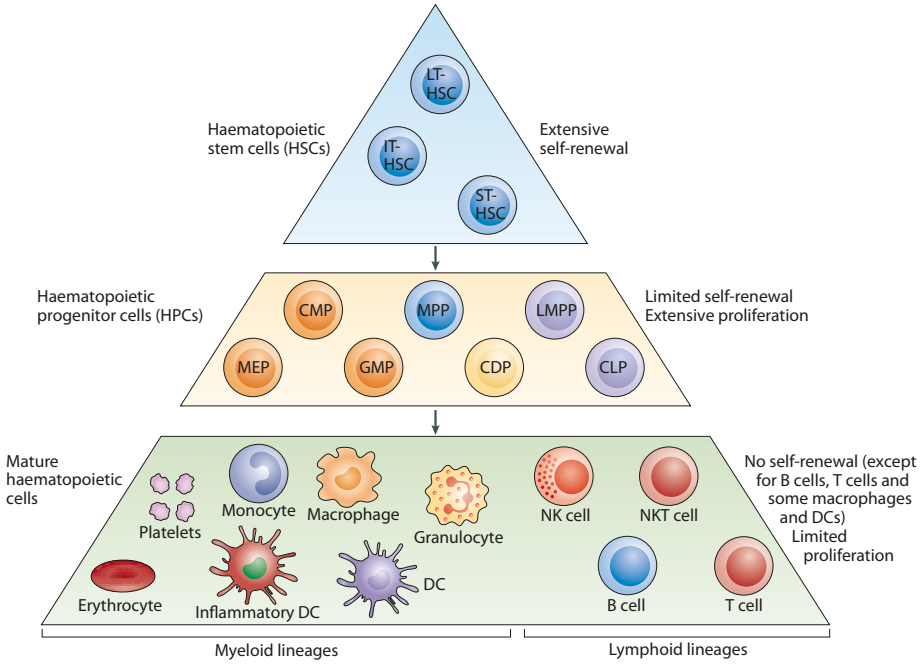

Figure 1: Hierarchy of the haematopoietic system.
The haematopoietic system is characterised by self-renewal of Long-term(LT), short-term(ST) and intermediate-term (IT) hematopoietic stem cells (HSCs). HSCs will generate several types of haematopoietic progenitor cells, capable of proliferation, self-renewal, and differentiation into multiple types of mature blood cells. From Manz and Boettcher (2014)

1.2 Principles of innate and adaptive immunity

1.2.1 Innate immunity

For an organism to survive, it depends on its ability to recognize infectious agents and induce suitable defence responses. These protective mechanisms that a host undergoes form the innate immune system. This includes constitutive mechanisms that comprise physical barrier (body surface and cavities) as well as biochemical barriers (mucosal epithelia, tears) that are all present at the sites of interaction with microbes and have capability to destruct pathogens without affecting host cells and tissues. However, the important and most reliable mechanisms are inducible and comprise cytokines,

chemokines, lipid and soluble mediators, released by innate immune cells. For activating these mechanisms, a pathogen must be specifically recognized by its host. The strategy of the innate immune response resides in recognizing pathogen-associated molecular patterns (PAMPs) expressed on the surface of pathogens when they bind to pattern-recognition receptors (PRRs) on innate immune cells.

1.2.1.1 Pattern-Recognition Receptors (PRRs)

Detection of pathogens is accomplished through germline-encoded pattern-recognition receptors (PRRs). It is the ability for the innate immune cells to bind PAMPS on the surface of microbes (nonself agents) and distinguish them from self-molecules, to activate the corresponding immune responses. Five families of PRRs have been identified, based on protein domain homology: the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-like receptors (RLRs), and the AIM2-like receptors (ALRs) (Brubaker, Bonham, Zanoni, & Kagan, 2015) (table 1). The innate immune response induced by PRRs leads to the production of proinflammatory cytokines and interferons (IFN), important for initiating innate and adaptive immune responses. PRR activation also leads to phagocytosis, autophagy and cell death (Deretic, Saitoh, & Akira, 2013; Lamkanfi & Dixit, 2014).

Family	Members	Shared domains	Receptor locations
TLR	1–10 in humans, 1–9 and 11–13 mice	LRR, TIR	Cell surface, endosomal compartments
CLR	Dectin-1, Dectin-2, . . . etc. (reviewed in Reference 4)	C-type lectin	Cell surface
NLR	NOD1 (NLRC1), NOD2 (NLRC2), NLRC3–5, NLRP1–9 and 11–14, NAIP1, -2, -5, -6	Nucleotide binding, LRR	Cytoplasm, plasma, and endosomal membrane associated
RLR	RIG-I, MDA5, LGP2	DExD/H helicase	Cytoplasm
ALR	AIM2, IFI16	PYRIN, HIN-200	Cytoplasm, nucleus (IFI16)

Table 1: Pattern-recognition receptor families (Brubaker et al., 2015)

Toll-like receptors (TLRs)

The first receptor of the Toll family was described in drosophila. Members of this family have a key role in induction of immune and inflammatory responses in mammal, mainly through the NF-κb signalling pathway. TLRs are expressed in innate immune cells such

as dendritic cells (DCs) and macrophages but also non-immune cells such as fibroblasts and epithelial cells. They are divided into two subfamilies based on their localization: cell surface TLRs and intracellular TLRs. Cell surface TLRs comprise TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, which can recognize microbial components such as lipids, lipoproteins, and proteins, while intracellular TLRs are localized in the endosome, they include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 and can recognize bacterial and virus nucleic, and also self-nucleic acids in case of autoimmune diseases (Celhar & Fairhurst, 2014; Kawai & Akira, 2010).

C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) are associated with carbohydrate binding through conserved motifs present in the C-type lectin-like domain (CTLD), important for binding mannose, glucose or galactose. They also recognize lipids and proteins. They are expressed mainly on myeloid cells and were conventionally associated with anti-fungal immunity, but recently new roles were identified in homeostasis, autoimmunity, allergy or host defence against microbes (Dambuza & Brown, 2015).

Nucleotide oligomerization domain (NOD)-like receptors (NLRs)

NLRs are intracellular, cytoplasmic sensors that are principally expressed by lymphocytes and antigen-presenting cells (APCs), like macrophages and dendritic cells, but also on epithelial and mesothelial cells. They are composed of a variable N-terminal protein-protein interaction domain, defined by the caspase recruitment domain (CARD), which mediates caspase-independent interactions, a pyrin domain (PYD), CARD and PYD are both involved in apoptosis, a baculovirus inhibitor repeat domain (BIR), a central nucleotide-binding oligomerization (NOD) domain, which mediates self-oligomerization that occurs during activation, and C-terminal leucine-rich repeat (LRR) that recognizes PAMPs. NOD1 and NOD2, the two well-characterized NLRs induce activation of NF-κB and MAPK pathways, which result in upregulation of transcription and production of pro-inflammatory molecules (Chen, Shaw, Kim, & Nunez, 2009).

Retinoic acid-inducible gene (RIG)-like receptors (RLR)

The RLR family contains three members: the founding member and the best

characterized RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5) and LGP2 (laboratory of genetics and physiology 2). They are cytoplasmic and composed of three distinct domains, an N-terminal region comprising caspase activation and recruitment domains (CARD), a central DExD/H box RNA helicase domain which hydrolyzes ATP and a C-terminal repressor domain (RD) embedded within the C-terminal domain (CTD). They can detect a variety of viruses and signal the production of IFN and interferon stimulated genes (ISG) product (Loo & Gale, 2011).

Apoptosis inhibitor of macrophage 2 (AIM2)-like receptors (ALRs)

ALRs family plays an important role in activating the inflammasome and the STING-dependent IFN response. They were originally identified as a family of IFN-inducible genes in mice. They contain a Pyrin domain for protein-protein interactions and a hematopoietic interferon-inducible nuclear (HIN) domain for DNA binding. Eights ALRs are described in mice and five in humans, however, most of their functions are still unknown (Brunette et al., 2012)

1.2.1.2 Innate immune cells

The innate immune system is the first line of defence against invading pathogens. Innate immune cells are of from both haematopoietic and non-haematopoietic origins. Hematopoietic cells include natural killer (NK) cells, mast cells, basophils, neutrophils, eosinophils, macrophages and dendritic cells, while non haematopoietic cells include epithelial cells from skin, respiratory, gastrointestinal, and genitourinary tracts.

Epithelial cells

Originally, the mucosal epithelia from the skin, airways, reproductive and gastrointestinal tracts were known to serve only as a passive barrier against invading pathogens. However, adding to the fact that it's the first surveillance system encountered by pathogens entering the host, the epithelial cells are often the target of replication by a variety of pathogens and recent studies showed that epithelial cells are capable of playing crucial roles in recognition of microbes and orchestrating an inflammatory

response to activate myeloid cells in the submucosal layers (Cutler & Jotwani, 2006; Weindl, Wagener, & Schaller, 2010). Epithelial cells present PRRs on their surface and can produce cytokines, growth factors and chemokines able of recruiting leucocytes to the inflammatory site (A. Iwasaki & Medzhitov, 2015).

Natural killer (NK) cells

NK cells generally develop in the bone marrow, but can also derive from lymph nodes and liver. They represent 10% of peripheral blood mononuclear cells of circulating human lymphocytes. Even though morphologically, they are characterized as large, granular, bone marrow-derived lymphocytes, they are still considered to be a part of the innate immune system. Their main function is immuno-surveillance of our body. NK cells have been implicated in various disease conditions, in particular in cancer and viral infection. They are known to produce cytokines such as interferon gamma (IFN-γ), and to possess a high cytotoxic activity (Mandal & Viswanathan, 2015).

Mast cells

Mast cells originate from the bone marrow, circulate in the blood then enter different tissues, where they reside and develop into mature cells under the influence of certain local growth factors and cytokines. Mature mast cells contain secretory lysosomes that occupy most of their cytoplasm. Once activated, mast cells release their granule constituents such as histamin, heparin, proteases (chymase, tryptase), lipid mediators (prostaglandins and leukotrienes) and cytokines (TNFα, IL-4, etc). Mast cells play protective roles against bacterial infection and detrimental roles in allergy such as asthma (Wernersson & Pejler, 2014).

Basophils

Unlike mast cells, basophils complete their maturation in the bone marrow then circulate in the blood under physiological conditions. They are granulocytes that account for less than 1% of peripheral blood leukocytes. They have long been neglected due to their short life span, their rarity and their similar phenotype with mast cells, including surface expression of the high affinity IgE receptor (FcεRI) and the presence of basophilic granules in their cytoplasm. However, newly developed tools allowed the functional

analysis of basophils and revealed important and non-redundant roles in immunity. Basophils are known to secrete large quantities of histamines, leukotriene, IL-4 and IL-13 in response to various stimuli. They have been shown to play key roles in allergy, parasite infection and autoimmune diseases (Karasuyama & Yamanishi, 2014).

Eosinophils

Eosinophils are released into the peripheral blood in a phenotypically mature state, they are capable of being activated and recruited into tissues in response to stimuli, in particular to the cytokine interleukin-5 (IL-5) and the eotaxin (CCL11) and RANTES (CCL5) chemokines. They are implicated in various inflammatory diseases including helminth infections, hypereosinophilic syndromes and allergy. In response to external stimuli, eosinophils will release the contents of their specific granules into the extracellular space, including pro-inflammatory cytokines and chemokines, granule proteins (Major Basic Protein, MBP) and lipid mediators (prostaglandins, leukotrienes). These pro-inflammatory molecules will induce mucus secretion, regulation of vascular permeability and activation of other immune cells (Rosenberg & Foster, 2013).

Neutrophils

Polymorphonuclear neutrophils are the most abundant type of granulocytes and play their major role during acute inflammation. Neutrophils are short-lived cells with a half-life of approximately 8 hours in mice and human. They are the first type of leukocytes to be recruited to inflammatory sites where they use different mechanisms to eradicate pathogens. Neutrophils can eliminate pathogens by three different mechanisms: phagocytosis, by killing the pathogens using NADPH oxygenase-dependent mechanisms (reactive oxygen species) or antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme) released from the neutrophil granules, when highly activated, neutrophils can eliminate extracellular microorganisms by releasing neutrophil extracellular traps (NETs), which will immobilize pathogens and prevent them from spreading (Kolaczkowska & Kubes, 2013).

Macrophages

Macrophages derive from differentiation of monocytes within the tissue. Tissue-resident macrophages are mainly known for their fundamental role as immune sentinels in the first line of tissue defence against pathogens or environmental challenges, initiating inflammatory responses, clearing inflammatory debris and restoring homeostatic tissue environments. They have wide roles in clearance (degradation of erythroid nuclei, senescent erythrocytes, apoptotic cells and pulmonary surfactant), development (bone degradation and angiogenesis) and the regulation of metabolism (regulation of insulin sensitivity and adaptive thermogenesis in adipose tissue) (Davies, Jenkins, Allen, & Taylor, 2013).

Dendritic cells

Dendritic cells (DCs) are a large group of related innate immune cell type that are antigen-presenting cells (APCs). DCs express various PRRs, and thus play a key role between innate and adaptive immunity, since they recognize pathogens, capture them and present them on the surface of naive T cells, which will be activated.

DCs express CD11c and MHC class II, however, when they are activated MHC–peptide complexes and co-stimulatory molecules will be much expressed on their surface, allowing them to effectively activate T cells. There are four major populations of DCs: conventional DCs (cDCs), Langerhans cells, monocyte-derived DCs and plasmacytoid DCs (pDCs). These subsets are associated to each other as they have a common myeloid progenitor. cDCs in lymphoid organs contain two major subpopulations, that can be distinguished by the expression of CD8α or CD4. There are tissue-resident cells that are equivalent to, and related by lineage to these populations and marked by the expression of CD103 and CD11b, respectively. In the steady state, these cells will migrate to lymph nodes, but following peripheral infection or immunization which will activate DC, they will migrate to a notably greater extent. CD8α+ DCs, CD4+ DCs and pDCs can be differentiated from bone marrow *in vitro* by stimulation with FMS-like tyrosine kinase 3 ligand (FLT3L). Langerhans cells are skin-resident cells that are similar to macrophages, but possess cDC-like properties when they migrate to lymphoid organs. Monocytes can develop into TNF and iNOS-producing (TIP)-DCs at

inflammatory sites, but to what extent these cells symbolize true DCs remains a question. CD11c+MHC class II^{hi} DCs can be grown *in vitro* from bone marrow cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF); these DCs have been reported to be comparable to *in vivo* monocyte-derived DCs, but it is controversial how close they are related to any *in vivo* population. pDCs are a distinct lineage of DCs with more specificity for cytokine production, particularly type I interferon, rather than antigen presentation. However, it is notable that upon activation, all DCs begin to secrete a range of cytokines that will influence the cells that they are interacting with. Moreover, it is probable that additional subsets of DCs with specialized functions are still under discovery (Pearce & Everts, 2015).

1.2.2 Adaptive immunity

The adaptive immune system is characterized by a high-specific response against pathogens. It's the interplay between APCs and T and B lymphocytes, which are the major players of the adaptive immunity.

Two distinct immune responses comprise the adaptive immune system, depending on the kind of the antigen: the humoral response also called antibody-mediated response and the cell-mediated immunity. Humoral immunity involves B cells that will produce antibodies against extracellular microbes and toxins, and cell-mediated immunity involves T cells that act against intracellular microbes and virally infected agents (Bonilla & Oettgen, 2010).

1.2.2.1 Cell-mediated immunity (T cells)

T cells develop from a common lymphoid progenitor in the bone marrow. They complete their maturation in the thymus to become mature naive T cells. There are several known types of T cells such as T helper cells, cytotoxic T cells, regulatory T cells (Treg) and follicular helper T cells (TfH). For a T cell to recognize an antigen, T cell receptor (TCR) has to bind to appropriate molecules of the major histocompatibility complex (MHC), also called human leucocyte antigen (HLA) in human, presented on the surface of APCs. Two classed of MHC molecules exist: MHC class I and MHC class II.

MHC class I molecules are expressed ubiquitously on the surface of all nucleated cells and interact with CD8+ cytotoxic T lymphocytes. They are bound to peptides which are generated from proteasome degradation, during viral infection or cancer transformation. MHC class II molecules are expressed mainly by professional APCs, such as DCs, macrophages and B cells. These molecules bind to peptides that are derived from proteins degraded in the endocytic pathway, thus, they present exogenous antigens and interact with CD4+ helper T cells (Neefjes, Jongsma, Paul, & Bakke, 2011).

T helper (Th) cells

Mature Th cells express the CD4 protein on their surface and are activated by interaction with MHC class II molecules then differentiate into T effector and memory T cells. They are key cells of the adaptive immune system, since they regulate cytotoxic cellular immune response and B-cell antibody production. They also interact with components of the innate immune system. Naive Th cells can differentiate into different subsets, depending on the microenvironment, such as the presence of cytokines and other factors. The first classification was limited to Th1 and Th2 subsets. Th1 cells are induced by IFN-γ and IL-12, secreted primarily by DCs. Th1 express the transcription factor T-bet and can produce IFN-γ. Th2 cells are induced by IL-4, they also secrete IL-4 and express the transcription factor GATA-3. Currently, classification became extended to include Th17 and less studied Th9, Tfh and Th22. Th17 cells are induced by IL-6 or IL21 and TGF-β, they express both transcription factors T-bet and GATA-3 and can secrete IL-17. Recent studies have shown that Th9 cells are induced by IL-4 and TGF-β, they can produce IL10 and IL-21, but their function remains not well known. Th22 is induced by TNF-α and IL-6 and is further promoted by IL-1β and can secrete IL-22 (Ivanova & Orekhov, 2015). Newly discovered Tfh cells are known to provide help for germinal centre B cells. Tfh cells are primed by IL-6 and IL-21 and express the transcription factor BCL6 (H. Qi, 2016) (figure 2).

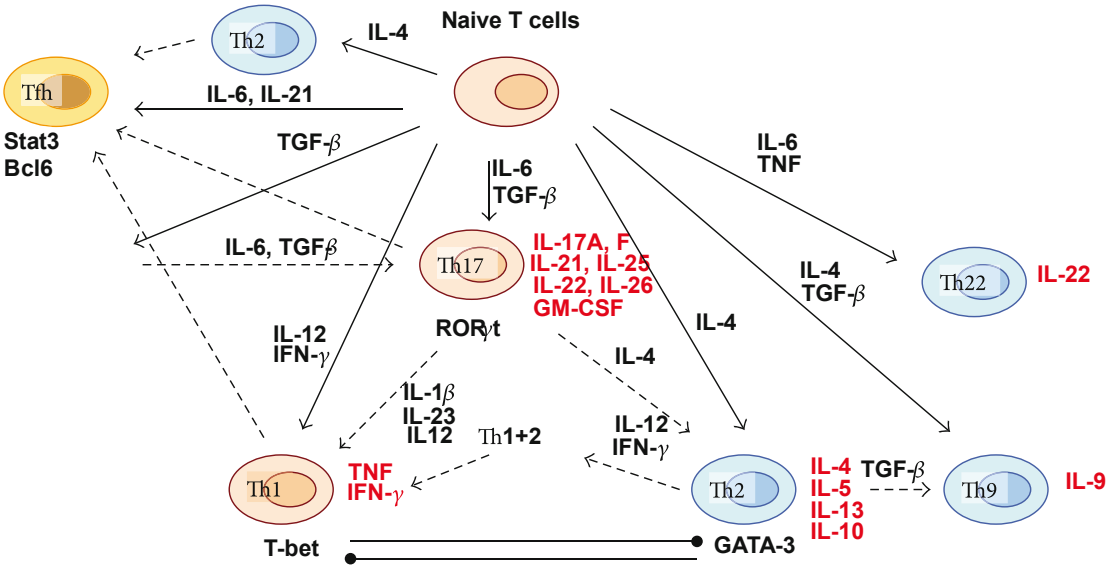

Figure 2: T cell differentiation pathways. Modified from (Ivanova & Orekhov, 2015).

Cytotoxic T cells

Cytotoxic T cells are important for protection against virus infection and intracellular pathogens. Mature cytotoxic T cells express the surface marker CD8 and are activated when recognizing MHC class I molecules. They kill infected cells by promoting their cytolysis. To induce cytolysis, cytotoxic T cells possess two different pathways: the FAS-FAS ligand pathway, which initiate programmed cell death by aggregation of Fas on infected cells, or through the granule-exocytosis pathway, by secretion of pore-forming protein perforin and a family of serine proteinases that are known as granzymes, necessary for the induction of apoptosis.

Moreover, cytotoxic T cells can secrete pro-inflammatory cytokines such as IFN-γ and TNF, but also hold a regulatory role by their secretion of IL-10, which can dampen inflammation and tissue damage (Barry & Bleackley, 2002).

Regulatory T (Treg) cells

CD4+CD25+ Treg cells are known to express the transcription factor FOXP3. Through the influence of local environmental conditions, Treg cells downregulate the immune response and maintain tolerance to self-antigens (Galgani, De Rosa, La Cava, & Matarese, 2016).

Four basic regulation mechanisms of Treg cells exist: secretion of inhibitory cytokines, cytolysis, metabolic disruption and targeting dendritic cells (figure 3).

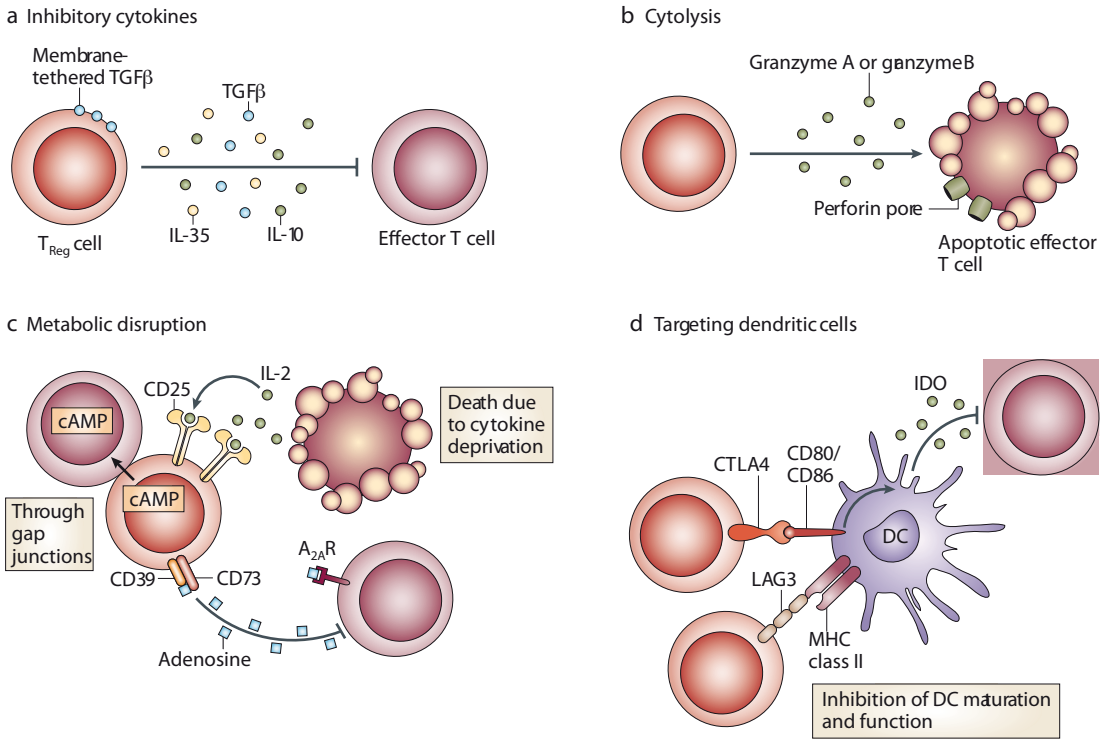

Figure 3: Different mechanisms used by TReg cells.
a) Inhibitory cytokines include interleukin-10 (IL-10), IL-35 and transforming growth factor-β (TGFβ). **b)** Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. **c)** Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor α)-dependent cytokine deprivation- mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A2AR)-mediated immunosuppression. d) Targeting dendritic cells (DCs) includes mechanisms that modulate (DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3; also known as CD223)–MHC-class-II mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DC (Vignali, Collison, & Workman, 2008).

Natural killer T (NKT) cells

NKT cells are activated by endogenous self-lipid antigens and exogenous lipid antigens. These lipids are presented by CD1d molecules on APCs to the T-cell receptor on NKT cells, which distinguish them from NK cells. NKT cells produce cytokines and cytotoxic

proteins and can also be activated in a CD1d-independent manner (Getz & Reardon, 2017).

1.2.2.2 Humoral immunity (B cells)

The humoral immunity is a part of the adaptive immune system. It serves as a protection against an infinite number of pathogens, which are recognized by B cells, due to the B cell receptor (BCR) on their surface. B cells are then activated and will differentiate into antibody-producing plasma cells and memory cells. B cell activation needs the participation of T cells. The main function of B cells is the production of high-affinity, isotype-switched antibodies, necessary for host defence. Different types of antibodies, also called immunoglobulins (Ig) can be generated. Five classes of antibodies (IgM, IgD, IgG, IgA, and IgE) exist, the nature of the response is subjective to the critical helper signals derived from antigen specific CD4 T cells at the site of the B cell encounter. Thus, Th1 cells stimulate the production of immunoglobulin IgG2a, whileTh2 cells induce the production of IgE and IgG1 isotypes.

In mammals, B cells develop from a common lymphoid progenitor in the bone marrow (BM). Immature B cells leave the BM and migrate to secondary lymphoid organs, such as spleen or lymph nodes, where they continue their development and differentiate into naive, follicular or marginal zone B cells (Pieper, Grimbacher, & Eibel, 2013).

When they recognize an antigen, B cells internalize it and deliver it to the endosome so it can be degraded into peptides that are bound to MHC class II molecules, and will be recognized by CD4 T cells to establish the contact with B cells and provide them help in the secondary lymphoid organs. B cells are also known to serve as APCs, they can produce pro-inflammatory cytokines and maintains the structure of secondary lymphoid organs (Hoffman, Lakkis, & Chalasani, 2016).

2. Skin immunity and inflammation

The skin is the largest organ in our body and the first barrier against external pathogens. The skin immune system is composed of both innate and adaptive immune cells. The nature of the responses that are generated in the skin depends on the interaction between the microorganisms colonizing the skin and the immune system. The main role of the skin is to protect the body from external physical and chemical attacks, penetration of pathogens and water loss, by interacting with host epithelial and immune cells. Deregulation of these crosstalks leads to pathogenesis of inflammatory skin diseases.

2.1 Skin components

Two major components compose the skin: the epithelium and the connective tissue. The epidermis is composed of four different layers: The stratum corneum, the stratum granulosum, the spinous cell layer and the basal layer (figure 4) and is mostly composed of keratinocytes (95%). Some differences exist between human and mouse epidermis, while hair follicles are very dense in mouse skin, human skin present a larger areas of interfollicular skin and has a thicker epidermis. Human epidermis is composed of Langehans cells (LC) and CD8+ T cells, while mouse epidermis has dendritic epidermal T cells (DETCs), which don't exist in human skin. The epidermis is avascular and aneural and is nourished by the dermis.

The dermis is tightly connected to the epidermis through a basement membrane and is rich in extracellular matrix and stromal cells, like fibroblasts, blood and lymph vessels. The dermis also contains matrix components such as collagen, elastin and glycosaminoglycans, and is populated by macrophages, mast cells, conventional αβ T cells and innate lymphoid cells (Pasparakis, Haase, & Nestle, 2014).

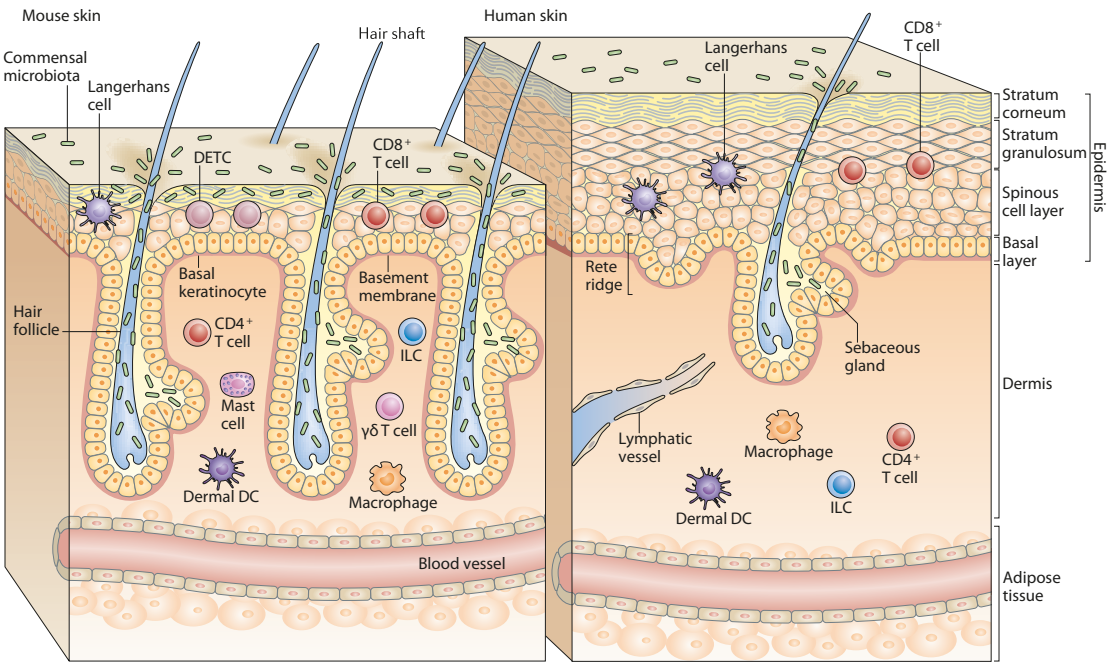

Figure 4: Structure of the skin in mice and human

a) Mouse skin, composed of densely packed hair follicles; the epidermis confine a prominent population of Vγ5+ dendritic epidermal T cells (DETCs), which are absent from human epidermis.
b) Human skin has larger areas of interfollicular skin with sparse hair follicles, a thicker epidermis (with more cell layers) and a thickerdermis than mouse skin. Langerhans cells and CD8+ T cells are prominent in human epidermis.
Human and mouse dermis are populated by macrophages, mast cells, conventional αβ T cells and a small population of innate lymphoid cells (ILCs) (Pasparakis et al., 2014).

2.2 Cell regulation of skin immunity and inflammation

2.2.1 Myeloid cells in skin immunity and inflammation

It is surprising how little is known about the myeloid cell network in skin inflammation. These cells are either generated in the bone marrow then circulate in the blood to enter the skin under inflammatory condition or skin resident myeloid cells.

Dendritic cells. DCs colonising the skin are different in mouse and human. In mouse epidermis, Langerhans cells are well-defined DC population that is maintained by Colony stimulating factor 1 receptor (CSF1R) (Ginhoux et al., 2006). Moreover, hair follicle keratinocytes were shown to produce CCL2 and CCL20 to induce recruitment of Langerhans cells which express the receptors CCR2 and CCR6 respectively during inflammatory conditions (Nagao et al., 2012). In mouse dermis, two populations of DCs

exist: $CD103^+$ DCs, which are the $CD8\alpha^+$ migratory lymph node DCs; and $CD11b^+$ DCs (Tamoutounour et al., 2013). These DCs present in the skin have specialized functions: in mice, Langerhans cells induce Th17 cell response in response to *Candida albicans* stimulus, or cross-presentation of antigens to $CD8^+$ T cells (Igyarto et al., 2011; Stoitzner et al., 2006). $CD103^+$ DCs are important for generation of Th1 cells (Igyarto et al., 2011; Stoitzner et al., 2006). In human , Langerhans cells have been shown to contribute to Treg cell expansion; and the main DC subsets in human dermis are $CD14^+$ but are so week to stimulate T cells (Klechevsky et al., 2008).

Macrophages/monocytes. There are tissue-resident or bone marrow derived monocytes and macrophages. Signalling pathways concerning these cells are poorly understood. However, tissue-resident macrophage are known to be responsible for surveying the tissue and detect early enter of antigens to the body. Circulating monocytes support DCs in the transport of antigens to draining lymph nodes (Jakubzick et al., 2013). M1 macrophages contribute to acute and chronic skin inflammation; in contrast, M2 macrophages secrete IL-10 and TGFβ and contribute to dampening skin inflammation. In a model of IgE-mediated allergic skin inflammation, M2 macrophages were shown to be important for resolution of skin inflammation (Egawa et al., 2013).

Mast cells and basophils. Mast cells (MCs) and basophils possess preformed pro-inflammatory mediators stored in their cytoplasmic granules that will be released upon antigen crosslinking. Basophils have been found in skin lesions of patients with atopic dermatitis, prurigo, bullous pemphigoid, but were absent in psoriasis or systemic lupus erythematosus (SLE) (Ito et al., 2011). In mouse models of atopic dermatitis or helminth infection basophils were recruited to skin and other tissues through a TSLP (thymic stromal lymphopoietin)-dependent mechanism (Giacomin et al., 2012; Siracusa et al., 2011). Moreover a recent study in our lab showed that basophils contribute to Th2 immune inflammation in a mouse model of atopic dermatitis (Leyva-Castillo et al., 2013). In mouse, basophils are also important for IgE-mediated chronic allergic inflammation but mast cell role in skin disorders have been contradictory (Voehringer, 2013).

2.2.2 Lymphoid cells in skin immunity and inflammation

T cells. T cells that can be found inside non-inflamed skin and were shown to be sufficient to induce psoriasis in the absence of circulating lymphocytes (Boyman et al., 2004). The skin comprises two distinct memory T cell subsets: epidermis resident memory $CD8^+$ T cells and recirculating memory $CD4^+$ T cells. Studies of herpes simplex virus (HSV) infection showed that skin-resident $CD4^+$ T cells were highly protective compared to circulating $CD4^+$ T cells (Gebhardt et al., 2009). In addition, another population exist which is tissue-resident T_{reg} cells in the skin. In a model of skin-specific autoimmunity in which ovalbumin is overexpressed by keratinocytes, it was shown that activated Tregs persisted in the tissue to supress the autoimmune responses (Rosenblum et al., 2011).

γδ Tcells. γδ T cells in mice are the first to leave the thymus and localize in the skin. They have important roles in tumour immune surveillance, wound healing and skin inflammation. They are protective against skin tumours through a mechanism involving the NK group 2, member D (NKG2D) receptor and through production of IFNγ and IL-17. In the dermis, a new subset of γδ T cells has been identified which express the IL-7 receptor and the CCR6 and retinoic acid-related orphan receptor- γt (RORγt) that will mostly induce produce IL-17 when challenged with microbes (Gray, Suzuki, & Cyster, 2011). It is well known that IL-17-producing γδ T cells play an important role in the imiquimod-induced model of psoriasis (Flutter & Nestle, 2013). γδ T cells can also produce IL-22 in this model. They depend on the CCR6 expression for homing to the skin and promotion of skin inflammation. IL-36 mediates the interaction between epidermal keratinocytes and DCs, which regulates the recruitment then activation of $CCR6^+$ dermal γδ T cells in psoriasiform dermatitis (Tortola et al., 2012). In psoriatic human skin, a subset of γδ T cells ($Vγ9Vδ2^+$) has been identified and was shown to produce IL-17 as well, and thus mediate pro-inflammatory action, which was correlated with disease severity (Laggner et al., 2011). These findings highlight the importance of these cells in the contribution of inflammation in human and mice. Also, these γδ T cells were shown to be important for epithelial homeostasis through the production of growth factors (FGF7, FGF9), which contribute to keratinocyte survival (Witherden & Havran, 2013).

Innate lymphoid cells. Innate lymphoid cells (ILCs) are present in surface tissues like the intestine, lungs and skin. They are divided in tree subsets: group 1 ILCs (ILC1 and NK cells), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s and lymphoid tissue inducer cells), that is on the basis of cytokine and transcription factor expression (Spits et al., 2013). The three of them are present in human skin, however, ILC2s and ILC3s are more studies in skin homeostasis than ILC1s. ILC2s were shown to be potentially implicated in Th2-type dermatitis, to produce IL-13 that can supress mast cell function *in vitro* (Roediger et al., 2013). A study showed that ILC2s promotes Th2-immune responses in atopic dermatitis mouse model through a mechanism involving TSLP, but not IL-33 or IL-25, which is similar with human atopic dermatitis (B. S. Kim et al., 2013). ILC3s are the major population to be present in human skin. They were shown to be sufficient to induce psoriatic plaque formation in a model of imiquimod-induced psoriasis (Pantelyushin et al., 2012).

2.2.3 Keratinocytes in skin immunity and inflammation

Keratinocytes are strategically present at the interface between the body and the external environment. They receive signals for the environment and transduce them to immune cells in the skin. This connection is achieved through the expression of receptors at the surface of immune cells that will allow them to sense microorganisms or stress factors. Three signalling pathways are known for epithelial contribution to skin inflammation. In human and mice, dysregulation of AP-1 transcription factors (JUN and FOS) was associated with skin inflammation (Briso et al., 2013; Zenz et al., 2008). Another important transcription factor is the signal transducer and activator of transcription 3 (STAT3). Studies in mice specifically expressing STAT3 active form in keratinocytes related its expression with psoriasis (Sano et al., 2005). The third pathway is the TNF pathway, which was confirmed by the efficient treatment of psoriatic skin with TNF-neutralizing antibody even though the correct mechanism remains poorly understood.

2.3 Skin inflammation

2.3.1 Allergic skin inflammation

Allergic pathologies, such as asthma, allergic rhinitis, atopic dermatitis or food allergies are increasingly frequent in the developed countries. Allergy is the susceptibility of certain people to develop reactivity when exposed to some antigens called allergens. One of the common features of allergic disorders is the production of allergen-specific IgE and expansion of allergen-specific T helper 2 (Th2) cells.

Two types of allergens exist and are present the environment, the non-infectious substance that will induce the production of IgE, such as house-dust-mite or certain foods like eggs or peanuts, and the non-infectious substance that will induce an adaptive immune response that is associated with local inflammation and occurs independently of IgE, for example allergic contact dermatitis to nickel. As our lifestyle changes and the standards improve, we have a reduced exposure to pathogenic organisms, which normally promote a proper Th1 immune response instead of Th2, and allow the control of harmful immune response, and a higher exposure to harmless allergens that permits the development of Th2-type response, that is called the hygiene hypothesis.

Many factors can affect the development of skin allergy. Host genetic background, the type and the concentration of the allergen and whether other agents present in the environment enhance the exposure and that are able to promote a Th2 type immune response. Another important factor is the frequency of the exposure to that same allergen. A defective barrier function has been known to be a critical factor in the initiation of atopic dermatitis, but also for the progression from atopic dermatitis to asthma (De Benedetto, Kubo, & Beck, 2012). Genetic factors influence the epithelium permeability to allergens. These genes include SPINK5, CDSN and FLG. SPINK5 gene encodes a serine peptidase inhibitor and polymorphisms in this gene were associated with atopic dermatitis and serum IgE levels (Kato et al., 2003). Mutations in the CDSN gene coding corneodesmosin, a structural protein of corneodesmosomes, that mediates interconeocyte adhesion in the stratum corneum, have been identified to be associated with chronic dermatitis and bacterial colonization or infection in the skin with atopic

manifestation with allergies (Kubo, Nagao, & Amagai, 2012). A loss-of-function mutation in fillagrin (FLG), a key epidermal protein that regulates the structure and composition of the stratum corneum, was associated with development of allergic sensitization and allergic disorders (Weidinger et al., 2008).

2.3.1.1 Features of allergic skin inflammation

Skin allergy is characterised by two main phases: the early-phase reaction also called sensitization phase and the late-phase reaction, called elicitation phase. A third phase is called the chronic allergic inflammation, which persist, and re-occurs at the site of inflammation when exposed to the same allergen.

The sensitization phase appears very rapidly, within minutes after exposure to the allergen. Allergen-specific IgE will bind on FcεRI on the surface of mast cells and basophils, which will release mediators including histamine, serine protease (tryptases, chymase), prostaglandins and leukotriene B4 (LTB4). These mediators are associated to the symptoms of the sensitization phase (Galli, Tsai, & Piliponsky, 2008). Antigen is presented by APCs to T cells in the lymph node to initiate immune response and the development of T and B cell memory. During elicitation phase, effector and memory T cells (previously exposed to the same allergen) will respond rapidly and induce large amounts of cytokines, accompanied by recruitment and differentiation of eosinophils, neutrophils and basophils. Recent studies have shown that the cytokine TSLP expressed mainly by keratinocytes (KCs) is a key player in the induction of Th2-immune response in allergic skin inflammation (Leyva-Castillo et al., 2013; Li et al., 2009; Li et al., 2006). Moreover, cutaneous exposure to antigen is associated with an expansion of TSLP-elicited basophils in the skin, and both TSLP and basophils were required for consequent antigen-specific Th2 cell responses, antigen-specific IgE production, intestinal mast cell accumulation, and anaphylaxis after oral antigen challenge (figure 5) (Reynolds & Finlay, 2017).

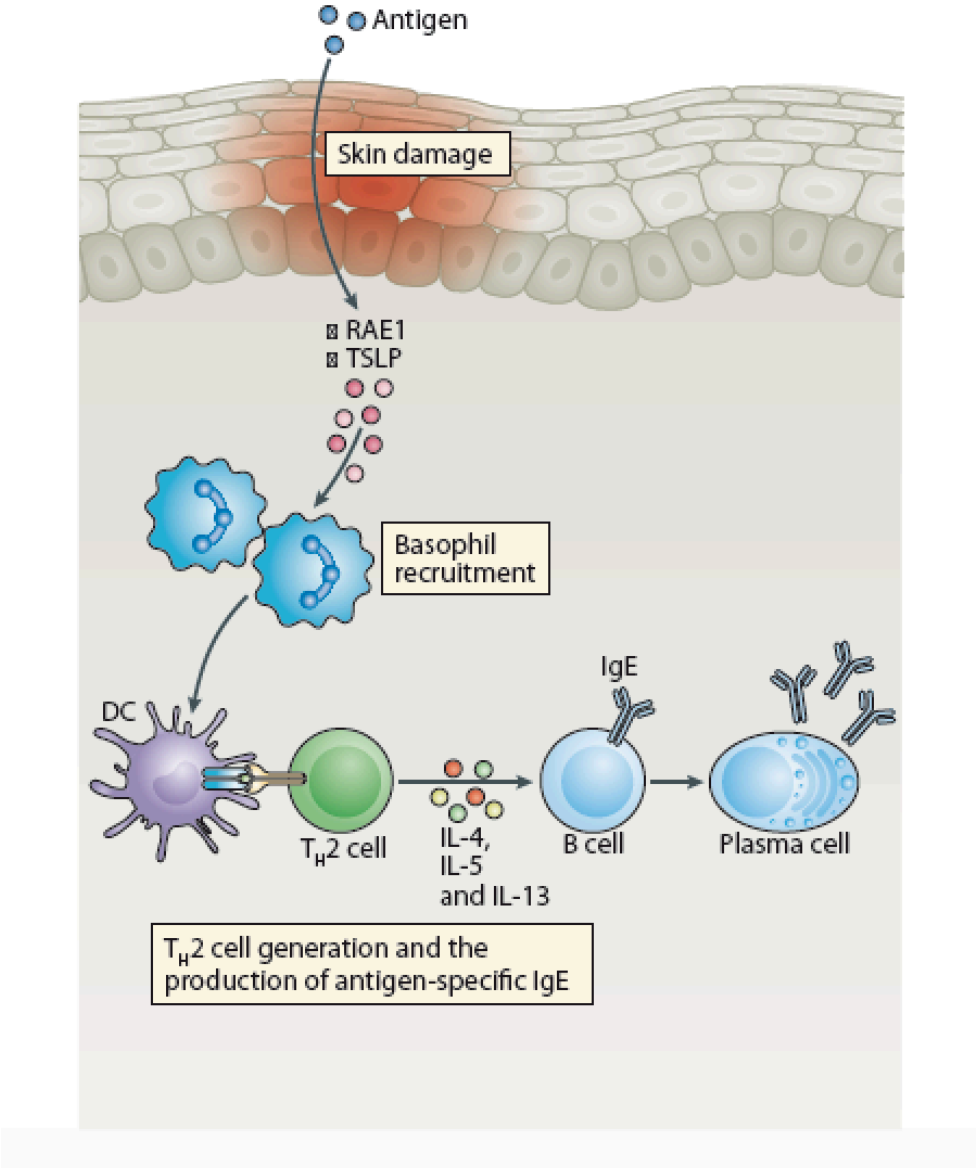

Figure 5: Cutaneous exposure to allergen

Cutaneous antigen exposure causes T helper 2 (TH2) cell- responses, which lead to allergic sensitization. The release of retinoic acid early transcript 1 (RAE1) and thymic stromal lymphopoietin (TSLP) following skin damage results in the recruitment of basophils that promote TH2 cell differentiation — probably through an effect on dendritic cells (DCs) — leading to the release of TH2-type cytokines, which stimulate the production of antigen-specific IgE (Reynolds & Finlay, 2017).

2.3.2 Allergic contact dermatitis

Allergic contact dermatitis (ACD) is a term used to describe an inflammatory reaction in the skin with intensely pruritic erythema and oedema. ACD occurs in response to low-molecular-weight chemical compounds after many repeated exposures. These compounds will enter the skin and bind to endogenous protein and become immunogenic. These common allergens, such as nickel, can be present in cosmetics, jewellery and industrial workplaces. Such compounds include haptens, which are recognized by innate immune cells in the skin such as DCs. For ACD development, there is a cascade of events that starts with the recognition of haptens by DCs. When skin dendritic cells recognize the modified protein, they prime allergen-specific T cells in the skin draining lymph nodes, a phase called “sensitization”. Upon re-exposure to the same antigen (hapten bound to protein), an inflammatory response will develop in the site of inflammation, e.g. the skin, and this phase is called the “challenge” phase (Dudeck et al., 2011).

2.3.3 Psoriasis

Psoriasis is an inflammatory skin disease involving cells from innate and adaptive immune system. Psoriasis vulgaris is the most common type. Its a characterised by red colored dry plaques with well defined borders. Psoriasis can be initiated by different factors like injury and trauma, but also infection, medication and imiquimod (a TLR7 agonist). Studies on mouse have shown that topical imiquimod treatment induce psoriasis mediated by the IL-23/IL-17 axis with activation of DCs. Studies provided evidences that activated T cells have pathogenic actions when they infiltrate the skin lesions of psoriasis vulgaris (Chamian et al., 2007). DCs were reported to secrete IL-12 and IL-23 and thus induce T cell activation, which in their turn will produce IFN-γ, IL-17 and IL-22, known to be Th1, Th17 and Th22 cells respectively. Recently, γδ T cells were also found to secrete IL-17 in psoriasis. These cytokines can cause keratinocyte proliferation and production of chemokines, cytokines and AMP, which is considered as a feed-back loop that act back on DCs, to preserve the inflammatory response in skin (Lowes, Suarez-Farinas, & Krueger, 2014).

2.4. Mouse models of allergic skin inflammation

Our understanding of human allergic skin inflammation has expanded with the use of animal models, because they allow investigation of the pathogenesis in a physiological context, and provide priceless tools for diagnostic and pharmaceutical purposes.

2.4.1 Mouse model with epicutaneous sensitization with allergens

Epicutaneaous exposure to protein allergens (e.g. ovalbumin (OVA)) in mice has to be done by a first step consisting of disrupting the skin barrier, by shaving the mouse and tape stripping the skin to create a barrier defect. Then the patch method is used to administrate the antigen (OVA or saline will be applied on a sterile patch and the patch is placed for a period of time then on the skin), for sensitizing mice to develop a Th2 immune response with high levels of IgE, and produce allergen-specific immunoglobulins. Re-exposure to the same patch will create the “challenge” phase of the allergic immune response. The treated skin of these mice shows lymphocyte infiltration in both dermis and epidermis (L. F. Wang, Lin, Hsieh, & Lin, 1996). Examination of mouse skin lesions revealed increased infiltrate of neutrophils, eosinophils, mononuclear cells, mast cells, CD3+ cells and CD4+ cells, with an increased IL-4, IL-5 and IFNγ expression (Spergel et al., 1998).

A systemic response to the allergen after epicutaneous treatment with OVA is measured by serum OVA-specific IgG1, IgE and IgG2a but also increased levels of IL-4, IL-5 and IL-13. A principal advantage for this model is that it can be generated in any mouse strain. For example using with gene-deficient mice can help to examine the role of various cells and cytokine in the pathophysiology of atopic dermatitis. Mechanical injury is an important step in this model, because application of OVA to the skin of hairless mice does not induce development of an immune response

2.4.2 Mouse models induced by topical application of chemical compounds

Topical application of MC903 (a low-calcemic vitamin D3 analog) induced TSLP expression in epidermal keratinocytes, which results in an atopic dermatitis-like syndrome, characterized by a red, scaly and lesioned skin, accompanied by an epidermal hyperplasia and a dermal infiltration of cD4+ cells, eosinophils, basophils, dendritic cells and mast cells, with an increase in Th2-type cytokines in the skin and elevated IgE and blood eosinophilia (Li et al., 2006). However, this protocol relies on high expression of TSLP, which is the key initiator of the inflammatory process in this context, while no allergen is involved.

Oxazolone is commonly used hapten to induce allergic contact dermatitis with a Th1 dominated response. Haptens are small chemical compounds that can easily penetrate intro the skin and bind to endogenous proteins in the dermis, which makes them antigenic. When skin dendritic cells recognize the modified protein, they prime allergen-specific T cells in the skin draining lymph nodes, a phase called “sensitization”. Upon re-exposure to the same antigen (hapten bound to protein), an inflammatory response will develop in the site of inflammation, e.g. the skin, and this phase is called the “challenge” phase (Dudeck et al., 2011). However, repeated skin exposure to oxazolone in hairless mice was shown to shift from hypersensitivity into a chronic dermatosis with features of AD such as impairment of the skin barrier with decreased expression of structural proiteins such as loricin, involucrin and fillagrin, with immune cell infiltrates of T cells, mast cells and eosinophils as well as increased IgE serum levels (Man et al., 2008)

Fluorescein isothiocyanate (FITC) is used a hapten with potential to induce allergic contact dermatitis (ACD) in mice. It has been shown that FITC combined to its adjuvant dibutyl phthalate (DBP) induces a Th2- type inflammation and increased TSLP levels (Takeshita et al., 2004). An advantage for the use of this model is that it doesn’t need any disruption of the skin, by a simple topical application into mouse skin, we can generate an appropriate Th2 immune response with recruitment of immune cells, like eosinophils, basophils, T cells and mast cells and increased Th2-type cytokines IL-4 and IL-13.

3. Basophils

Basophils were first discovered by Paul Ehlrich in 1879. They represent the least abundant granulocytes (less than 1% of peripheral blood leucocytes) in mammals. They have long been neglected due to their rarity, their short life span, their similarities with mast cells and the lack of tools for their detection and their functional analysis. Recently, antibodies specifically detecting basophils, and reagents /tools for depleting basophils have been developed, thus enabling their functional characterization *in vivo*, which revealed non-redundant and important roles of basophils in immunity.

3.1 Development

Basophils are one type of granulocytes characterized by their basophilic granules in the cytoplasm. In mice, basophils represent a close lineage relationship with mast cells (Metcalf, Ng, Baldwin, Di Rago, & Mifsud, 2013), whereas human basophils seem to be closer to eosinophils (Grundstrom et al., 2012). However, it is important to point out that most of the studies about basophil development derive from mouse models. Basophils originate from the bone marrow from the granulocyte and monocyte progenitor (GMP) and their lineage specification is dependent on the expression of the transcription factor GATA-binding protein 2 (GATA2) and CCAAT/enhancer-binding protein-α (C/EBPα), then they enter the blood circulation under a mature phenotype (H. Iwasaki & Akashi, 2007). Ikaros family zinc finger protein 1 (IKZF1) was shown to negatively regulate basophil development by inhibiting C/ EBPα (Rao, Smuda, Gregory, Min, & Brown, 2013), while the signal transducer and activator of transcription – 5 (STAT 5) was shown to be crucial for basophil development by upregulating GATA2 and C/EBPα expression (X. Qi et al., 2013). Under steady conditions, basophils finish their maturation in the bone marrow then leave it and circulate in the peripheral blood or go to the spleen, but do not further proliferate. Basophils have very short life span of 60 hours in mice (Ohnmacht & Voehringer, 2009).

Other factors have been shown to act on basophil development and differentiation. Thymic stromal lymphopoietin (TSLP) and IL-3 are two cytokines that can promote basophilila by activating STAT5 pathway and reducing apoptosis (Didichenko, Spiegl, Brunner, & Dahinden, 2008). TSLP can mediate the recruitment of basophils to the skin

in a model of atopic dermatitis and can induce basophilia independently of IL-3 (Siracusa et al., 2011). IL-3 was shown to induce basophilia and accumulation of basophils in the lymph node in a model of helminth infection (S. Kim et al., 2010). Progenitors treated with IL-3 were shown to direct GMP differentiation into basophils that were highly involved in IgE-mediated immunity (Ohmori et al., 2009), while TSLP enhanced basophil survival (Siracusa et al., 2011). Moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 which share the same β_c chain with IL-3, were also shown to induce basophil expansion in mice, when IL-3 is not well expressed (Schneider et al., 2009). Overall, IL-3 and TSLP seem to be the major cytokines contributing to basophil development (figure 6).

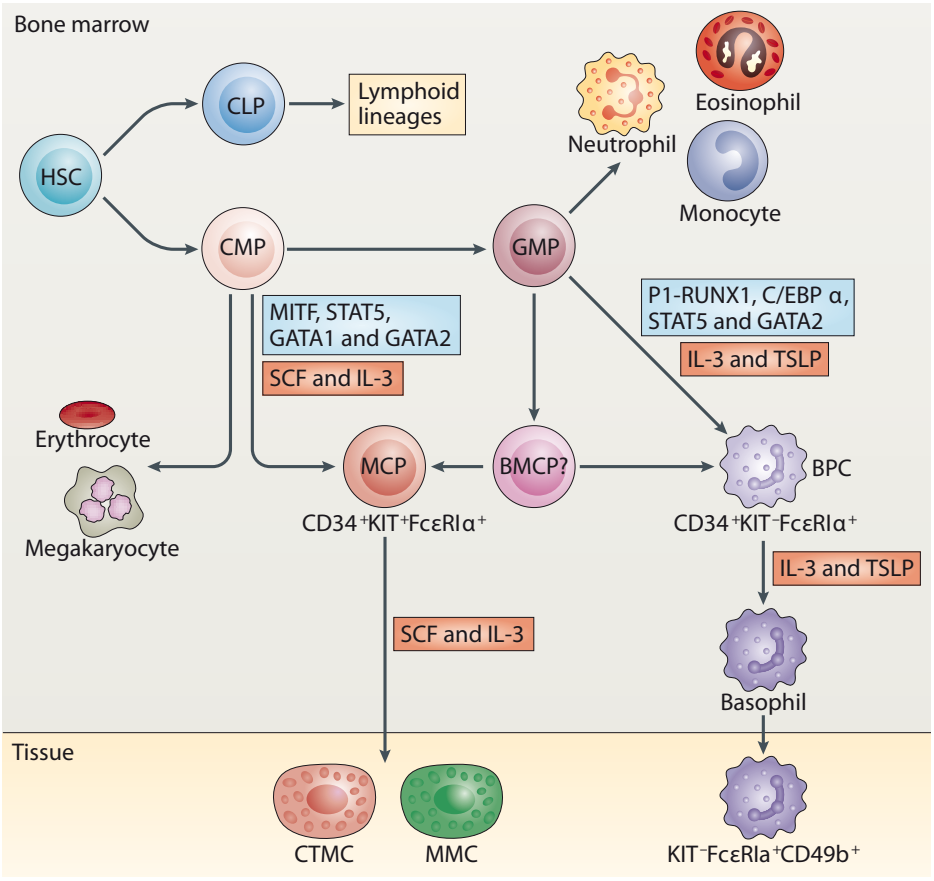

Figure 6: Basophil development in the bone marrow (Voehringer, 2013).

3.2 Activation

Basophils are well known for their effector function marked by the mediator release upon their activation. Different signals can activate basophils. Among these signals, antibodies, cytokines but also proteases and allergens can mediate basophil activation. As a consequence of their activation, basophils will release preformed molecules such as histamine and leukotriene C4, platelet activation factor (PAF), retinoic acid, cytokines including IL-4, IL-13, IL-6, TSLP, but also chemokines and anti-microbial peptides. All of these molecules will be involved in either immediate or late phase reactions of the immune system (Steiner, Huber, Harrer, & Himly, 2016).

3.2.1 Antibody-mediated activation

IgE. Basophils express on their surface the high affinity Fc receptor of IgE, FcεRI. Antigen-specific IgE antibodies generated by humoral responses are captured by circulating basophils through FcεRI (Siracusa, Kim, Spergel, & Artis, 2013). Thus, upon re-exposure to the antigen, in response to IgE cross-linking to the receptor, basophils degranulate and secrete a number of mediators, like histamine, leukotrienes (Schroeder, 2011), lipid mediators, prostaglandines and a variety of cytokines including, IL-3, IL-4, IL-13, IL-9, IL-25, and chemokines like RANTES (Chirumbolo, 2012). IL-4 plays a key role in inducing Th2 cell differentiation from naive CD4+ T cells (Gomez et al., 2014). Furthermore, IL-4, secreted together with IL-6 from basophils was shown to activate B cells in humoral responses by ameliorating their proliferation and immunoglobulin secretion (Denzel et al., 2008). IL-4 secreted by basophils also helps the eosinophil migration by upregulating CCL11 in fibroblasts (Nakashima et al., 2014) and drive the differentiation of inflammatory monocytes into M2 macrophages, in allergic skin inflammation (Egawa et al., 2013).

IgG. It has been shown that basophils can promote anaphylaxis in response to IgG-antigen complexes that bind to FcγRIIIA in mice and FcγRIIA in human (Tsujimura et al., 2008) and thus produce platelet-activating factor (PAF).

IgD. IgD is a class of antibody that is produced in early phase of B cell development and is known to activate basophils, which will result in basophil secretion of antimicrobial peptides and inhibit the growth of bacteria. However, IgD-mediated activation have no

impact on histamine release by basophils but increased the production of IL-4 by basophils (K. Chen et al., 2009).

3.2.2 Cytokines

Recently, studies have shown that basophils can be very heterogeneous, since their development can be influenced by different cytokines, mainly IL-3 and TSLP, although they can generate at a steady state in the absence of both cytokines.

IL-3. IL-3-activated basophils contribute to IgE-dependent (acquired) immune response (Karasuyama & Yamanishi, 2014). IL-3 promotes basophil development both *in vivo* and *in vitro,* in addition, it also promotes basophil activation (Siracusa et al., 2013), and was shown to enhance IL-4 and IL-13 production from human basophils (Schroeder, Chichester, & Bieneman, 2009; Siracusa et al., 2011). In response to helminth infection, IL-3 was reported to be crucial for peripheral basophil expansion (Lantz et al., 2008) and for enhancing the production of IL-4 and IL-13 from basophils when activated by IgE crosslinking (Gibbs et al., 1996). Basophils themselves can produce IL-3, and create thus a positive feed-back loop for their activation (Marone, Borriello, Varricchi, Genovese, & Granata, 2014). IL-3 was also shown to be important for basophil recruitment to the mesenteric lymph node in N.b. helminth infection (S. Kim et al., 2010) and to skin draining lymph node in atopic dermatitis mouse model induced by TSLP-overexpression (Leyva-Castillo et al., 2013). However, the fact that basophils can develop in the absence of IL-3-IL3R signalling shows that other mediators are capable of regulating their development and function (figure 7).

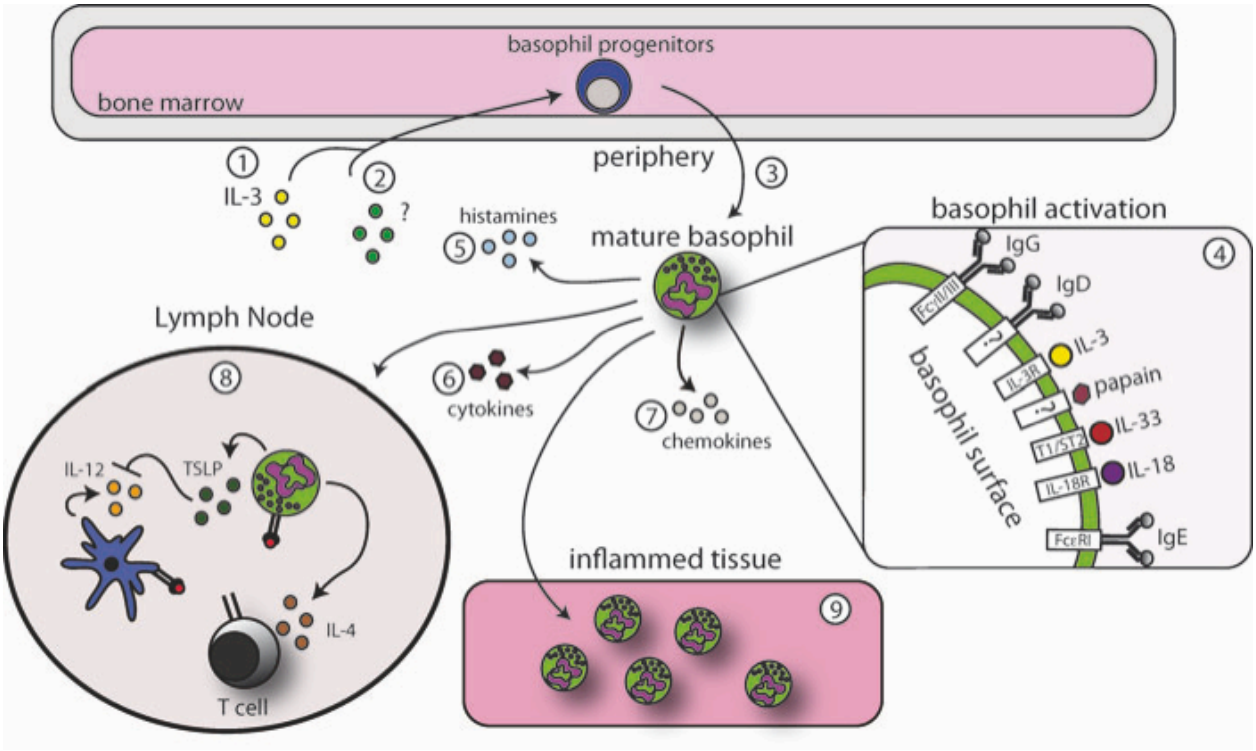

Figure 7: Basophil activation and function

Basophils develop in the bone marrow and then expand in number in response to growth factors. They quit the bone marrow as mature cells and enter the periphery, where they will be activated by an array of signals including those mediated by cytokines (IL-3, IL-33, and IL-18), antibodies (IgG, IgD, and IgE), and antigens. Activated basophils produce histamines, cytokines, and chemokines. Some basophil populations migrate to draining LNs, while others accumulate in inflamed tissues during an ongoing inflammatory response (Siracusa et al., 2011).

TSLP. TSLP (thymic stromal lymphopoietin) is a key epithelium-derived cytokine in allergic inflammation promoting Th2 immune responses (S. Kim et al., 2010; Y. J. Liu, 2006; Ziegler, 2010). TSLP-activated basophils are different from IL-3-induced basophils. TSLP-induced basophils contribute to IgE-independent (innate) immune responses (Karasuyama & Yamanishi, 2014). TSLP-induced basophils were shown to play a role in eosinophilic esophagitis pathogenesis in an IgE-independent manner (Noti et al., 2013). Accordingly, TSLP was also shown to be high in the oesophagus of eosinophilic esophagitis patients. TSLP-basophils contributed to atopic dermatitis pathogenesis (Leyva-Castillo et al., 2013; Siracusa et al., 2013) and food allergy (Muto et al., 2014; Noti et al., 2014). TSLP was reported to promote IL-3–independent basophil hematopoiesis (Siracusa et al., 2011). Following *Trichinella spiralis* infection of mice,

basophil responses in intestinal draining lymph node were critically dependent on TSLP-TSLPR interactions and not on that of IL3-IL3R (Giacomin et al., 2012).

Unpublished studies from my lab recently demonstrated that in an atopic dermatitis (AD) mouse model induced by the overexpression of TSLP, the recruitment of basophils to atopic dermatitis skin involves both IL-3-dependent and independent pathways. How basophils are being recruited to the site of inflammation and how they are activated has been very controversial; moreover, how basophils are being recruited to allergic skin inflammation is far from being clear.

IL25 and IL33. These cytokines are important initiators of Th2 responses. Human basophils express IL17RB and ST2, which are IL-25 and IL-33 receptors respectively. When primed with IL-33, basophils activate the NFkB pathway and secrete IL-4 and IL-13. Moreover, IL-25 was shown to upregulate IL-3Ra and CD203c on basophils surface. (Smithgall et al., 2008; H. Wang et al., 2010).

3.2.3 Protease allergens and glycoproteins

Even though basophils are mostly activated by cytokines and immunoglobulins, studies showed that allergens and parasite antigens could also activate them. Indeed, an active protease in house dust mite antigen Derp1 can induce IL-4, IL-5 and IL-13 production by human basophils, when IgE is absent. Basophils were shown to express mechanisms that can sense the protease activity (Phillips, Coward, Pritchard, & Hewitt, 2003). In addition, it has been demonstrated that the gp120 glycoprotein of the human immunodeficiency virus (HIV), considered as a “super antigen” is interacting with the VH3 region of IgE to induce IL-4 and IL-13 production by human basophils (Bouvet & Marone, 2007). Another super antigen, IPSE/alpa-1, was reported to activate murine basophils and thus their production of IL-4 (Schramm et al., 2007).

3.2.4 Bacteria-derived products

TLR Ligands. TLRs recognize PAMPS from bacteria, viruses, and fungi. Basophils could react to TLR2 stimulated with peptidoglycan and secrete IL-4 and IL-13, but not histamine or LTC4 (Bieneman, Chichester, Chen, & Schroeder, 2005). TLR4, stimulated

by LPS enhance expression of CD63 on basophils and their secretion of histamine of atopic patients (Gyimesi et al., 2013). Flagellin, the ligand of TLR5 stimulated basophils to secrete IL-6 (Jeon et al., 2015). IgE receptor can be stimulated by TLR4 or TLR9 and thus enhance the secretion of IL-4, IL13 and CCL5 from human basophils (Suurmond et al., 2014).

NLR ligands. Recenty, NOD2 was seen in the cytoplasm of basophils (Wong, Chu, Hon, Tsang, & Lam, 2016). NOD2 recognizes muramyl dipeptide, which is a structure of bacterial peptidoglycans.

FPR. The formyl peptide receptors (FRP)-1, 2 and 3 are expressed on basophil surface. FRP-1 binds formyl-methionine-phenylanaline (FMLF) with high affinity, which induces a phosphorylation of the receptor and cause the release of proinflammatory mediators from basophils and trigger chemotaxis (de Paulis et al., 2004).

3.3 Adhesion and migration

For circulating leucocytes to enter a tissue under inflammatory conditions, a cascade of events is required so they can recognize the vascular endothelium. Particularly, leukocyte crossing the barrier of the blood vessel wall involve an interaction between the leukocyte and endothelial cells. The entire process of leucokyte influx to inflamed tissue sites comprises three essential sequential steps: rolling on the vascular endothelium, then adhesion and trans-endothelial migration (TEM). The selectins (P-and E-selectins on the endothelium, and L-selectin on the leukocyte) and their ligands such as P-selectin glycoprotein ligand 1 (PSGL1), regulate the initial leukocyte rolling on the endothelium. Intergins are a complex family of αβ heterodimers, with 18α-subunits and 8 β-subunits, that can assemble in 24 different receptor sin vertebrates (figure 8) (Campbell & Humphries, 2011). The most important and well studies integrins are αLβ2 integrin (lymphocyte function-associated antigen 1; LFA1) and αMβ2 integrin (macrophage antigen 1; MAC1), also α4β1 integrin (very late antigen 4; VLA4). Integrins are activated by a change in their conformation, which alterns from low-affinity to high affinity lingand-binding site (Luo, Carman, & Springer, 2007). The steps occur in a cascade-like manner, mediated by molecules expressed by both leukocytes and endothelium, for example,

binding of P- and E-selectin to PSGL1 induces the signal activation of ITGb2, and promote rolling by interaction with ICAM1 (Vestweber, 2015).

Progress on how leukocytes move from circulation into tissue has been specially studied in neutrophils, T cells and eosinophils. Although basophils are known to be recruited from the circulation to the site of inflammation, the mechanisms by which they adhere to the vascular endothelium and migrate to sites of allergic inflammation, such as in allergic skin, are not understood.

Basophils are initially found in the bone marrow and the spleen but can migrate to the site of inflammation, or to the peripheral lymph nodes upon exposure to allergens or helminth parasites. IL-3 may be a key regulator of basophil recruitment to the lymph nodes, as shown in a murine model of helminth infection (S. Kim et al., 2010). Transendothelial migration (TEM) of basophils was shown to be induced by eotaxin, the ligand of CCR3, expressed on the basophil surface, as well as CCL5, the chemokine ligand of CCR1 and CCR3, *in vitro.* IL-3 stimulated human basophil TEM was shown to be mostly dependent on β_1 integrin and VCAM-1 (Andrews, Kepley, Youssef, Wilson, & Oliver, 2001; Iikura et al., 2004). Basophils could also migrate upons IgE stimulation and FcεRI cross-linking (Suzukawa et al., 2006)

It has been shown that *in vitro* treatment of human basophils with rIL-3 enhances the expression of Itgam (CD11b), which is forming the integrin heterodimer with Itgb2 (CD18), and treatment with mAB directed against ITGAL and ITGB2 significantly inhibited basophil adhesion to endothelial cells (Bochner et al., 1990). Lim et al., showed that *in vitro* stimulation of human endothelium with increasing concentrations of IL-3 significantly induced basophil rolling and adhesion, and pre-incubation of human endothelial cells with IL-3Ra (CD123) inhibited basophil adhesion and rolling. Moreover, using antibodies against ITGB1 or PSGL-1, but not against ITGB2, inhibited basophil rolling and adhesion the vascular endothelium *in vitro* (Lim et al., 2006). Another study from Iikura et al. demonstrated with transwell migration assay, that IL-3-enhanced human basophil transendothelial migration was inhibited when they used antibodies against Itgb1, and Itgb2 presented at the surface of basophils, and adding anti-ICAM-1 to endothelial cells inhibited as well basophil TEM (Iikura et al., 2004). Besides, it has been shown that IL-3R is expressed on human endothelial cells (Brizzi et al., 1993;

Korpelainen, Gamble, Vadas, & Lopez, 1996) thus mediating IL-3/IL-3R signalling. However, to this date, basophil migration studies were all performed *in vitro* on human samples, therefore, there is no evidence from *in vivo* studies to dissect the mechanisms by which IL-3 or other factors are mediating basophil TEM under pathophysiological conditions. My thesis work adds new insights of how IL-3 is acting on integrin expression on basophil surface allowing them to cross the vascular endothelium in the context of allergic skin inflammation.

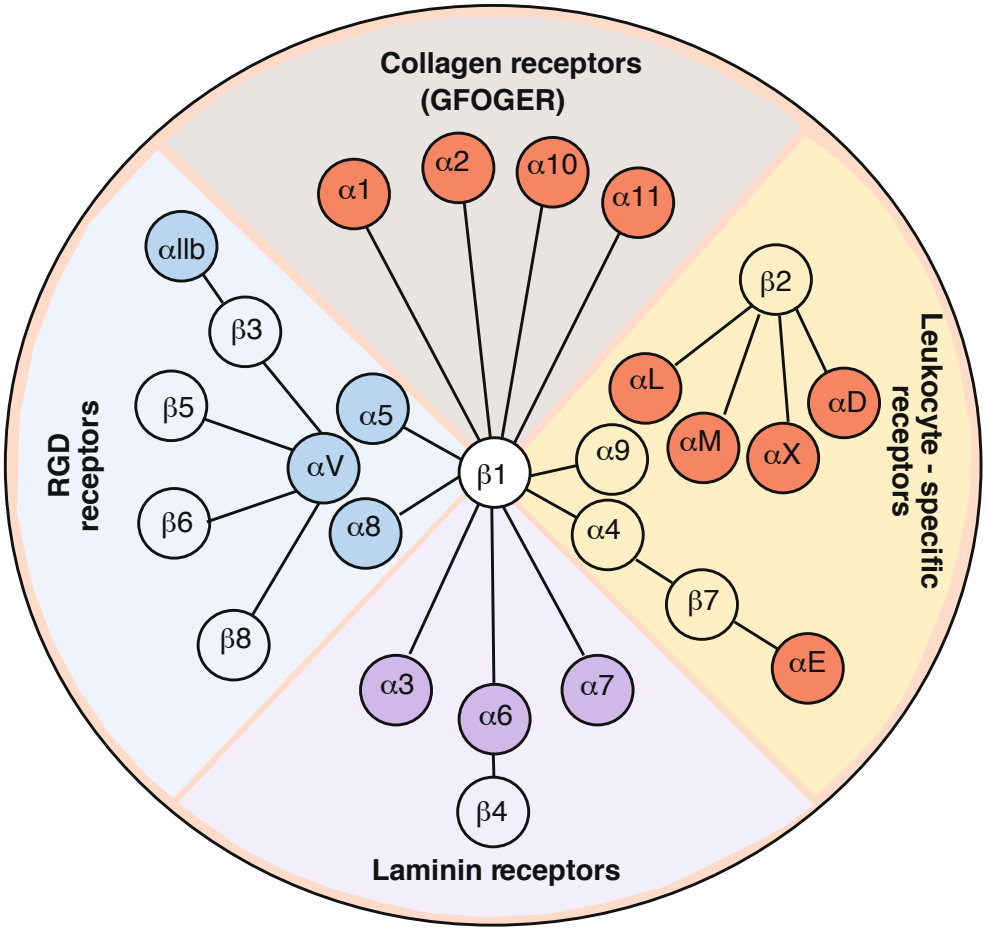

Figure 8: Representation of the integrin family composed of 24 heterodimers (Barczyk, Carracedo, & Gullberg, 2010)

3.4 Do basophils function as antigen presenting cells?

To this date, the most known APCs are dendritic cells, monocytes/macrophages and memory B cells. Although DC contribution to regulating the development of Th1 and

Th17 cell has been well understood, DC function as APCs has been requestioned due to their incapability to secrete IL-4 and the lack of defined mechanisms through which they contribute to Th2 differentiation (Wynn, 2009); in contrast, basophils are competent to secrete IL-4, implicating that these cells as a source of IL-4 could promote Th2-cell differentiation (Min et al., 2004). However, since 2009, it has been a matter of debate whether basophils can act as professional APCs, while DCs may not be essential for the Th2-induced immunity development. Three different studies showed that DCs are not required for $CD4^+$ Th2 responses: Sokol et al. demonstrated that DCs in response to protease allergen papain, were not capable of inducing Th2 responses *in* vitro, unless they are cultured together with basophils. Moreover, basophils were even sufficient alone, to induce proliferation of naive T cells, since they were shown to express MHC class II and capable of endocytosing soluble antigens. This endocytosis was followed by antigen processing and presentation assayed by basophil driven Th2 activation *in vitro* (Sokol et al., 2009). Perrigoue et al. showed that in a model of gastrointestinal nematode parasite *Trichuris muris* infection, DCs were not required for $CD4^+$ Th2 cell development, but IL-4-producing basophils expressing MHC class II are promoting Th2 cell differentiation in vitro, and when they depleted basophils *in vivo* with a monoclonal antibody (Mar-1) specific for the receptor FcεRI, this considerably impaired immunity to *T. muris*, which showed that basophils facilitate the development of protective Th2 immunity (Perrigoue et al., 2009). Both studies confirmed that *in vivo*, selective depletion of $CD11^+$ DCs impaired Th1 response, but Th2 response against papain or *T.muris* was not affected. Yoshimoto *et al.* showed that basophils isolated from the mouse spleens infected with the intestinal nematode *Strongyloides venezuelensis* are MHC class II positive and can secrete IL-4, while IL-4-deficient basophils are not functional, demonstrating that the production of IL-4 by MHC class II-positive basophils is critical for Th2 differentiation. They have also discovered that basophils express the lymph node-homing receptor CD62L, indicating that basophils have the necessary 'machinery' to enter secondary lymphoid tissues where T_H2 responses are initiated (Yoshimoto et al., 2009).

These findings collectively provide evidence that basophils can function as professional APCs, at least for the generation of Th2 responses.

However, the role of basophils as APCs remains uncertain since recent studies contradicted these observations. Hammad et al. showed that in response to house dust mite (HDM) allergen, basophils were incapable of taking up the allergen and present it to T cells or express any antigen presentation gene. When they depleted basophils using MAR-1 antibody (anti- FcεRI), levels of Th2 cytokine were strongly suppressed, however, another antibody depletion method using CD200R2 basophil-specific antibody Ba103 did not result in the same observation. The authors discovered that there was a specific population of inflammatory DCs expressing FcεRI in the lungs, that was affected by the MAR-1 antibody injection, and concluded that DCs were necessary and sufficient for induction of Th2 immunity while basophils were not required (Hammad et al., 2010).
Moreover, recent studies on human basophils showed that they were incapable of playing a role as APCs. Basophils from patients with birch pollen allergy were not inducing allergen-specific T cell responses, while $CD11^{+}$ DCs enhance T cell proliferation *in vitro* (Eckl-Dorna et al., 2012). Even though Charles et al. showed that human basophils express MHC class II molecules and are capable if acting as APCs in systemic lupus erythematosus (SLE) autoimmune disease (Charles, Hardwick, Daugas, Illei, & Rivera, 2010), another study showed that in SLE patient, it was pDCs who were expressing MHC class II molecules and responsible of being the APCs (Dijkstra, Hennig, Witte, & Hansen, 2012). Furthermore, a recent study showed that human basophils do not express HLA-DR or co-stimulatory molecules CD80 and CD86, and when they were stimulated with *Aspergillus fumigatus* allergen *in vitro*, they were still incapable of expressing any APC feature. Instead DCs were promoting Th2 responses when primed with Asp f 1. This study thus confirmed that circulating human basophils do not function as APCs (Sharma et al., 2013). Moreover, in a recent publication of our lab showed that in a murine model of atopic dermatitis, basophils by their production of IL-4 are contributing to Th2 cell differentiation in the lymph node, but were not dispensable, in contrast to DCs, therefore, they are not behaving as APCs (Leyva-Castillo et al., 2013). Thus, studies of basophils acting as APCs have been very contradictory, depending on the model, or the antigen used.

3.5 Basophils in acquired immunity

Studies revealed new functions of basophils in regulating acquired immunity, especially in initiating Th2 immune response and amplifying humoral memory response.

3.5.1 Basophils and IL-4 production

IL-4 drives the differentiation of naive T cells into Th2 cells, implicated in parasitic infections and allergic reactions (Paul & Zhu, 2010). Different cells can secrete IL-4, among these cells, T cells, NKT cells, mast ells, eosinophils and basophils. In human and mice, FcεRI cross-linking with IgE and allergens leads to the generation of large quantities of IL-4 by basophils (Seder et al., 1991). Co-culture of CD4+ T cells with basophils leads to CD4+ T cell differentiation into IL-4-producing Th2 cells (Hida, Tadachi, Saito, & Taki, 2005; Oh, Shen, Le Gros, & Min, 2007). Mice with genetically increased numbers of basophils were shown to accelerate Th2 cell differentiation (Charles et al., 2009). In an atopic dermatitis mouse model, basophils, through a cross talk with DCs and IL-3, were shown to induce Th2 cell differentiation in skin draining lymph nodes (Leyva-Castillo et al., 2013).

3.5.2 Basophil in papain-induced Th2 cell differentiation

Basophils have a key role in detecting cysteine protease antigens, such as papain. After injection of papain, basophils migrate to the lymph node and contributed to Th2 differentiation (Sokol, Barton, Farr, & Medzhitov, 2008). During parasite infections, basophils were also shown to be recruited to the draining lymph nodes, right before Th2 cell differentiation (Perrigoue et al., 2009; Wada et al., 2010). *In vitro*, basophils treated with papain secreted IL-4. Sokol et al. showed that depletion of basophils before papain treatment resulted in abolishment of Th2 cell differentiation in the lymph node, and that this was due to their production of IL-4. Furthermore, TSLP production by lymph node basophils upon papain administration was also critical for Th2 cell induction (Sokol et al., 2008). These recent studies propose that basophils, instead of DCs are important for Th2 responses, however, it is still very controversial and it depends a lot on the model of Th2 immune response studied (figure 9).

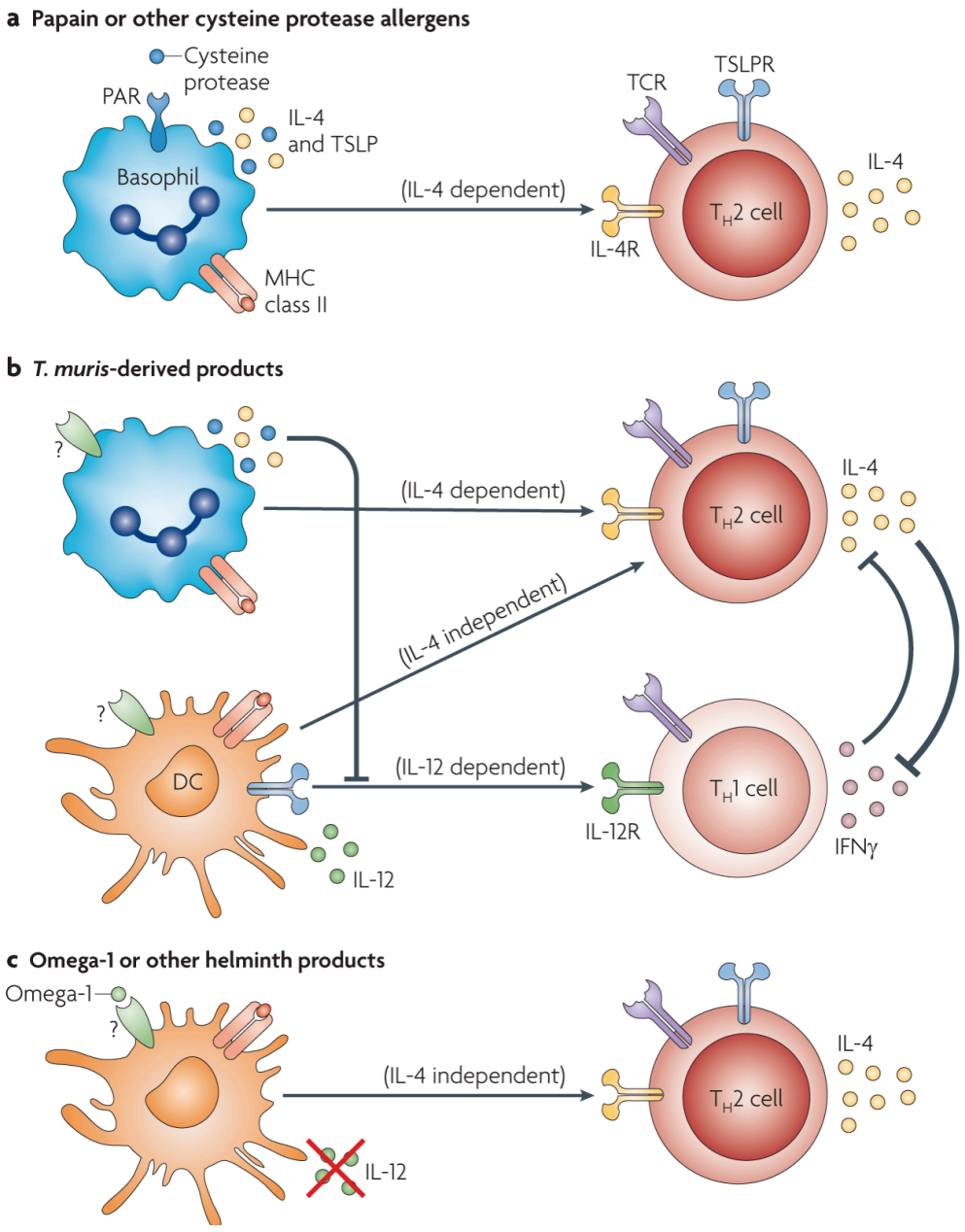

Figure 9: Basophils in Th2 immune responses.

Differential involvement of basophils and dendritic cells, functioning as antigen-presenting cells, in various TH2-type immune responses (Paul & Zhu, 2010).

3.5.3 Basophils and humoral immune responses

Basophils, through their expression of the IgE high affinity receptor FcεRI, can capture antigens through antigen-specific IgE when corss-linked to its receptor (Mack et al., 2005). In response to IgE crosslinking, basophils secrete large quantities of IL-4 (Khodoun, Orekhova, Potter, Morris, & Finkelman, 2004). A recent study showed that basophils, through their secretion of IL-4 and IL-6 could enhance humoral immune response by acting on B cells to secrete more antibodies (Denzel et al., 2008). Moreover, basophil depletion was shown to result in decreased serum levels of antigen-specific IgG1 and IgG2a, and with reduced amount of B cells in the bone marrow and spleen. However, how and where basophils interact with B cell and T cells needs to be identified. In addition to mouse studies, other human in vitro studies showed that human basophils can induce IgE by B cells, through their expression of CD40L and production of IL-4 (Yanagihara et al., 1998) (figure 10). IgD cross-linking on basophils induced production of IL-4 and IL-13, B cell-activating factor (BAFF). In vitro, basophils activated with IgD, stimulated B cells to secrete IgM antibodies. So, in both mouse and human, basophils were shown to act as amplifiers of the humoral immune response.

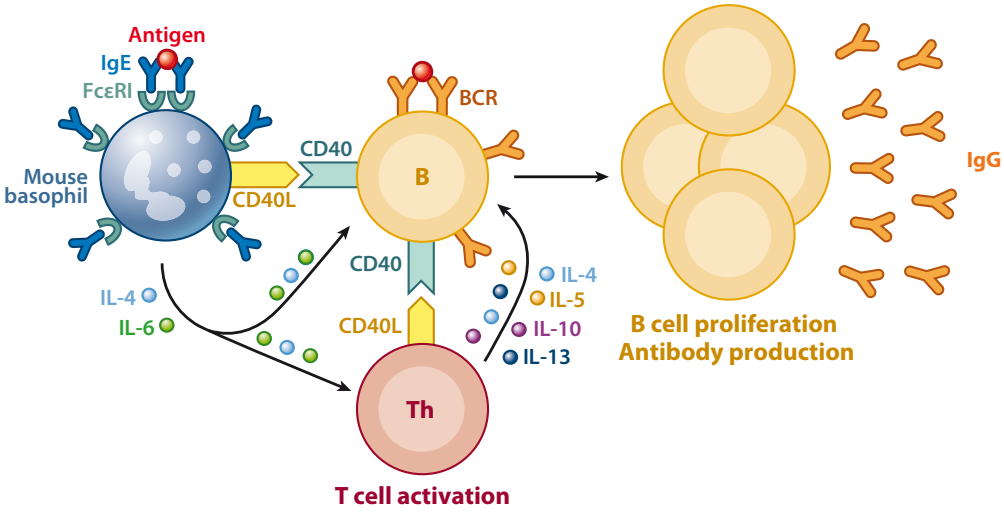

Figure 10: Basophils amplify humoral memory responses

Basophils are activated when antigen-IgE binds to FcεRI expressed on their surface. Following reexposure to the same antigen, basophils capture antigens through IgE-FcεRI complexes, upregulate CD40L expression and secrete IL-4 and IL-6 then they stimulate antigen-specific B and helper T cells. Basophil-stimulated T cells upregulate CD40L expression and secrete Th2 cytokines, like IL-4, while B cells proliferate and produce antigen-specific antibodies (Karasuyama, Mukai, Obata, Tsujimura, & Wada, 2011).

3.6 Basophils in protective immunity

3.6.1 Protection against parasite infection

Helminth, the most common infectious agents, are parasitic worms that induce Th2 immune responses, with increased levels of serum IgE, eosinophils, basophils mast cells and Th2 cells. Basophils were shown to protect the host from *T.muris* infections and N. brasiliensis infection, through their production of Th2 cytokines. (Ohnmacht & Voehringer, 2009; Perrigoue et al., 2009). The immune response is different between primary and secondary infection, basophils contribute to worm expulsion during primary infection, when IL-4 and IL-13 cannot be produced by T cells (Sullivan et al., 2011). Moreover, basophils were shown to have protective immunity against ticks, the blood-feeding ectoparasites that include viruses, bacteria, protozoas and helminths which can cause Lyme borreliosis disease. Tick infestation induces Th2 immune response with high IgE serum levels. Wada et al., demonstrated that basophils are recruited to the site of tick feeding sites during the second infestation and play an important role in acquired immunity against these ectoparasites (Wada et al., 2010). Together, these findings show that basophils are essential for antibody-mediated immunity against parasites, although human data needs to be determined.

3.6.2 Protection against viral and bacterial infection

This area is less studied than parasitic infection when it comes to basophil role. However, some studies showed that IgD cross-linking on basophils, induces antimicrobial peptide production, such as β-defensin and cathelicidin, that help to inhibit the progress of bacteria in vitro (K. Chen et al., 2009). Patients with *Helicobacter pylori* infection were shown to have basophil infiltration. Futhermore, gp120 of HIV-1 virus, through interaction with kappa light chains of IgE, stimulated human basophils *in vitro* to sectrete IL-4 and IL-13 (Genovese et al., 2003).

3.7 Basophils in diseases

3.7.1 Allergy

Basophils have been shown to be involved in allergic reactions through their secretion of histamine, leukotriene C4 and Th2 cytokines. Most of the cases in human allergic diseases, we see basophils recruited to the site of inflammation (Kepley, McFeeley, Oliver, & Lipscomb, 2001; Macfarlane et al., 2000; Nouri-Aria et al., 2001). This area is extensively studied to see whether basophils contribute to the allergic reactions; moreover, mouse studies showed that basophils play important roles in IgG-mediated anaphylaxis as well as in IgE-mediated chronic allergic inflammation (Obata et al., 2007; Tsujimura et al., 2008).

IgG-mediated systemic anaphylaxis. Systemic anaphylaxis is the most severe allergic reaction that can occur after insect venom, drugs or food allergens (Simons & World Allergy, 2010). Patients that were sensitised with an allergen, produce IgE molecules, and after a reexposure the same allergen, IgE will be cross-linked to FcεRI on the surface of mast cells that will end up in releasing their histamines, the main cause of anaphylactic manifestations. However, mice that are lacking mast cells were also shown to develop anaphylaxis (Strait, Morris, Yang, Qu, & Finkelman, 2002). Basophils, rather than mast cells, were recently shown to be implicated in IgG1-mediated systemic anaphylaxis (Tsujimura et al., 2008), where authors showed that depletion of basophils before antigen challenge led to an amelioration of systemic anaphylaxis. On the contrary, basophils were not important in IgE-mediated systemic anaphylaxis. However, a recent study, using *Mcpt8Cre* mice showed no implication of basophils in passive IgG1-mediated anaphylaxis (Ohnmacht & Voehringer, 2010). To sum-up, basophils can induce IgG-mediated anaphylaxis, however, this depends on the context and the settings of the experiment.

IgE-mediated chronic allergic inflammation. Basophils initiate chronic allergic inflammation in response to IgE crosslinking. In a model of IgE- chronic allergic inflammation (CAI), basophils were responsible of the disease induction (Mukai et al., 2005). Basophil infiltration was followed by eosinophils and neutrophils. Two different studies showed that absence of basophils inhibited the elicitation of IgE-CAI (Obata et

al., 2007; Ohnmacht & Voehringer, 2010). Basophils were shown to act as initiators of this disease and not just effector cells and that they could recruit other cells such as neutrophils and eosinophils (figure 11). In human, infiltration of basophils was also present in affected tissue (Kepley et al., 2001; Plager et al., 2006).

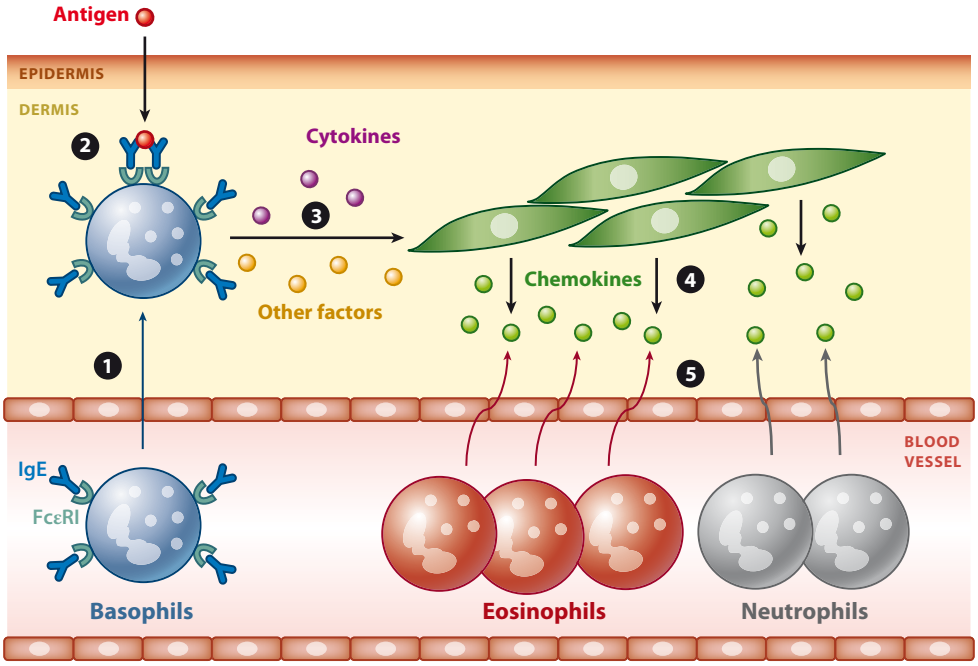

Figure 11: basophils as initiators of skin allergic inflammation

When basophils are armed with antigen-specific IgE bound to FcεRI expressed on their surface, they migrate into the skin lesion, where they get activated to secrete cytokines and other factors, which in turn act on tissue-resident cells, such as fibroblasts to secrete amounts of chemokines which recruit inflammatory cells, such as eosinophils and neutrophils, to the skin (Karasuyama et al., 2011).

Allergic asthma. Asthma is a chronic disease that affects 3000 million people worldwide (Braman, 2006). It's characterized by airway hyper-reactivity, production of mucus, infiltration of eosinophils as well as Th2 cells. Basophil depletion in mice showed a partial reduction of Th2 cell response (Hill et al., 2012). However, in human studies, basophils seem to be important for the late phase of allergic response (Koshino et al., 1993). Moreover, basophils, eosinophils and Th2 cells were found in bronchoalveolar lavage (BAL) fluid of asthmatic patients (M. C. Liu et al., 1991). Another study showed that IL-3 derived from T cells induced amphiregulin from human basophils, a molecule important to airway remodelling (Monticelli et al., 2011).

Atopic dermatitis (AD). AD is a skin disease that occurs in early infancy and which is associated in a later stage with asthma, allergic rhinitis and food allergies. It is typically associated with high serum levels of IgE and Th2 cytokine responses. A recent study showed that antigen-specific IgE activated basophils in the blood of AD patients (Zeller et al., 2009). In mice, basophils were found to be important for chronic IgE- dependent AD pathogenesis, where depletion of basophils caused a significant decrease of eosinophils and neutrophils, as well as skin swelling (Ohnmacht & Voehringer, 2010). TSLP-triggered basophilia was observed in lesional AD-skin (Siracusa et al., 2011), although their precise role needs to be well defined. To this date, the best treatment available consists of topical steroids. But, these treatments have been associated with many side effects, especially in children (Gelbard & Hebert, 2008).

3.7.2 Autoimmune diseases

Mouse model of systemic lupus erythematosus (SLE) showed that basophils were responsible of autoantibody production. In fact, basophils, through their secretion of IL-4, in an IgE-dependent manner can act on plasma cells in the spleen which result in amplification of the humoral response and the production of autoantibodies leading to SLE pathogenesis (Charles et al., 2010). Those basophils express MCH II in the lymph node, which suggested that they mediated the activation of B and T cells. Importantly, basophils were as well detected in patients with SLE and were associated with the increased disease activity. These studies suggest that basophils may have a role in the production of autoantibodies in autoimmune diseases.

3.7.3 Skin disorders

The group of Karasuyama were the first to demonstrate that basophils have a key role in allergic inflammation in mice (Mukai et al., 2005). Basophil recruitment and activation are hallmarks of allergic reaction development (Ito et al., 2011). The crucial step in allergic skin reaction is the development of Th2 immune response, and basophils have been shown to play a key role in Th2 cell differentiation in mice (Leyva-Castillo et al., 2013; Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). Moreover, in a model of papain injection, basophils acted as antigen-presenting cells when they migrate into the draining lymph nodes from the skin site (Sokol et al., 2009). Thus, basophils are

supposed to induce Th2 cell development, without the need of dendritic cells, under certain circumstances. On the other side, using a model of house dust mite, basophils were recruited to the mediastinal lymph nodes in mice, secreted IL-4 and contributed to Th2 immune response in the lungs, but in that case, they didn't act as antigen presenting cells (Hammad et al., 2010). In that model, dendritic cells were considered as sufficient to induce Th2 immunity, without the help of basophils. In a model of IgE-mediated chronic allergic inflammation, basophil depletion mice showed resistance to the development of the disease (Mukai et al., 2005), and the role of basophils was further confirmed by mouse models of basophil deficiency (Egawa et al., 2013; Ohnmacht & Voehringer, 2010; Sawaguchi et al., 2012). Basophils were as well suggested to have an immunoregulatory role in the skin, in fact, after allergen challenge, monocytes recruited to the skin were polarized to an anti-inflammatory M2-Like phenotype by basophils-derived IL-4 (Egawa et al., 2013). However, the exact mechanisms are still poorly understood. In response to hapten challenge, basophils induced a Th2-type cutaneous inflammation (Otsuka et al., 2013). In a mouse model of atopic dermatitis (AD), induced by over-expression of TSLP by topical treatment with the vitamin D analog MC903 (Leyva-Castillo et al., 2013; Li et al., 2009; Li et al., 2006), basophils were recruited to the skin in a TSLP-dependent, IL3-independent manner (Siracusa et al., 2011). However, how basophils are contributing to MC903-induced AD is still unknown.

Basophils were as well detected in human skin disorders, such as chronic idiopathic urticaria and allergic AD (Dvorak & Mihm, 1972; Ito et al., 2011; Ying, Kikuchi, Meng, Kay, & Kaplan, 2002). In human, basophil infiltration in the skin was correlated with that of eosinophils. Basophil infiltration was also prominent in patch-tested sites for house-dust mite allergen in AD patients (Ito et al., 2011). Furthermore, protein A of *Staphylococcus aureus*, which is commonly causing kin infections in AD patients, is an activator of human basophils (Kuo, Yoshida, De Benedetto, & Beck, 2013). Recent studies showed that IL-33 could enhance CD11b expression on human basophils and induce their transmigration into inflamed skin (Pecaric-Petkovic, Didichenko, Kaempfer, Spiegl, & Dahinden, 2009; Suzukawa et al., 2008). Basophils were found in other human diseases, such as bullous pemphigoid, eosinophilic pustular folliculitis, Henoch-

Schnonlein purpura and in tick-infested patients (Ito et al., 2011; Otsuka et al., 2013). Taking into consideration the difference between mouse and human basophils, It remains a critical challenge to translate the relevance of mouse model to human diseases (table 2).

		References
Human skin diseases		
CIU	Basophils have been observed in skin lesions of CIU. Basopenia is a common feature of CIU and is inversely correlated with urticaria severity. CIU patients can be classified as ''responders'' or ''non-responders'' (respectively CIU-R and CIU-NR) on the basis of histamine release in response to anti-IgE-mediated activation of basophils. Basopenia and suppressed CIU basophil anti-IgE-mediated histamine release improve in spontaneous remission and in response to therapy	Ying et al., 2002; Saini, 2009; Grattan et al., 2003; Kern and Lichtenstein, 1976; Eckman et al., 2008; Gober et al., 2008
AD	Marked basophil infiltration has been observed in patch-tested sites for house-dust mite allergen in sensitized AD patients. Basophils infiltrate chronic skin lesions of approximately 60% of AD patients	Mitchell et al., 1982; Ito et al., 2011
ACD	Basophils infiltrate skin lesions of some patients with ACD	Dvorak and Mihm, 1972
Lepromatous leprosy, bullous pemphigoid, scabies, insect bites (Cimex lectularius), Henoch-Schó nlein purpura, eosinophilic pustular folliculitis	Basophil infiltration (often together with eosinophil infiltration) has been detected in lesions of these human skin disorders. However, basophil density and the ratio of tissue basophils/eosinophils can be variable. The specific contribution of human basophils to the pathophysiology of these conditions remains to be established	Ito et al., 2011; Otsuka et al., 2013b; Nakahigashi et al., 2013
SLE	Basophils have been implicated in the development and progression of lupus-like nephritis in Lyn $^{-/-}$ mice. However, basophils have not been detected in SLE skin lesions, and their involvement in SLE pathogenesis is still debated	Charles et al., 2010; Ito et al., 2011; Dijkstra et al., 2012
HIV infection	Several HIV products can modulate human basophil functions in vitro . Patients with AIDS have HIV-1-infected basophil/mast cell precursors in their peripheral blood	Marone et al., 2001; Li et al., 2001
Mouse models of skin diseases		
IgE-CAI	Basophils have a fundamental role as initiator cells of IgE-CAI. Furthermore, they can promote the resolution of IgE-CAI through the induction of alternative activation of monocytes/macrophages	Mukai et al., 2005; Obata et al., 2007; Egawa et al., 2013; Ohnmacht et al., 2010; Sawaguchi et al., 2012
Hapten-induced skin inflammation	Basophil depletion in DT-treated Bas-TRECK mice led to a reduced hapten-induced skin Th2-type response	Otsuka et al., 2013a
Acquired anti-tick immunity	Basophils infiltrate the skin surrounding tick mouthparts during the second tick infestation. Basophils have a fundamental role in the development of tick-acquired resistance	Wada et al., 2010
TSLP-dependent atopic dermatitis	Basophils infiltrate skin lesions in a TLSP-dependent mouse model of AD. The contribution of basophils to the pathophysiology of this model remains to be established	Li et al., 2006; Li et al., 2009; Leyva-Castillo et al., 2013; Siracusa et al., 2011

Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; CIU, chronic idiopathic urticaria; DT, diphtheria toxin; IgE-CAI, IgE–mediated chronic allergic inflammation; SLE, systemic lupus erythematosus; TSLP, thymic stromal lymphopoietin.

Table 2: Basophils in human and mouse models of skin diseases (Borriello, Granata, & Marone, 2014).

3.8 New tools for the functional analysis of basophils

Studies on basophils have long been hard due to their rarity and the lack of appropriate analytical tools for their identification and functional analyses. Since basophils exhibit numerous characteristics shared by tissue-resident mast cells, they remained considered as minor for a long time after they were discovered. Fortunately, during the past two decades, basophil research crossed a new level due to the newly developed tools for their functional analysis, from basophil-depleting antibodies to genetically engineered mice deficient only in basophils. This progress powered basophil research and permitted new important discoveries on previously unrecognized roles of basophils.

Human basophils are identified by flow cytometry using surface markers such as: FSc^{lo}, SSc^{lo},$Fc\varepsilon RI^{+}$, $CD203c^{+}$, $CD117^{-}$, and can be detected by histology using the monoclonal antibody against basogranulin (BB1), a cytoplasmic protein (2D7) and the pro-major basic protein 1 (J75-7D4) (Kepley et al., 2001; McEuen, Buckley, Compton, & Walls, 1999; Plager et al., 2006). CD63 marker can detect upregulation of anaphylactic degranulation by flow cytometry and exocytosis of intracellular granules (Knol, Mul, Jansen, Calafat, & Roos, 1991; MacGlashan, 2010; H. Qi, 2016).

Mouse basophils are identified by flow cytometry by a panel of surface markers such as: FSc^{lo}, SSc^{lo}, $Fc\varepsilon RI^{+}$, $CD49b^{+}$, $CD69^{+}$, $CD123^{+}$, $CD200R^{+}$, $CD117^{-}$, $CD19^{-}$, $CD14^{-}$, $CD122^{-}$, $Gr1^{-}$,... (Siracusa, Wojno, & Artis, 2012). In histological sections, mouse basophils can be detected by a monoclonal antibody directed against mouse mast cell protease 8 (mMCP-8), a basophil-specific granzyme B-like protease (Ugajin et al., 2009).

3.8.1 Basophil-depleting monoclonal antibodies

To date, two monoclonal antibodies are used to deplete basophils *in vivo:* CD200R3, known as Ba103 (Obata et al., 2007), and FcεRIα, known as MAR-1(Denzel et al., 2008). Ba103 recognizes the CD200 receptor-like molecule CD200R3 that is related to DAP12, an activating adaptor (Kojima et al., 2007). One injection of Ba103 was shown to reduce peripheral basophils to 10-20% for 10 days, without having impact on mast cells (Obata et al., 2007). In addition, MAR-1 antibody was also shown to deplete

efficiently basophils in vivo, although it can also react with mast cells (Sokol et al., 2008). Furthermore, another antibody, anti-Thy-1, was generated to deplete basophils, but only in the absence of T cells (Ohnmacht & Voehringer, 2009). A key problem using those monoclonal antibodies is their side effects on mast cells, such as activation or partial depletion. Depending on the dose administrated intravenously, Ba103 and Mar-1 can stimulate mast cells to induce anaphylaxis (Obata et al., 2007; Ohnmacht & Voehringer, 2010). Intraperitoneal injection of MAR-1 reduced drastically mast cell population in the peritonea (Denzel et al., 2008). Moreover, in a model of asthma using house dust mite, MAR-1 injection led to the depletion of a subset of dendritic cells in the lungs expressing FcεRIα (Hammad et al., 2010) (figure12).

3.8.2 Engineered mice for specific depletion of basophils

Imperfections of antibody-mediated basophil depletion pushed the basophil research field to overcome these limitations. For that, newly engineered mice, allowing specific depletion of basophils were generated (figure 12).

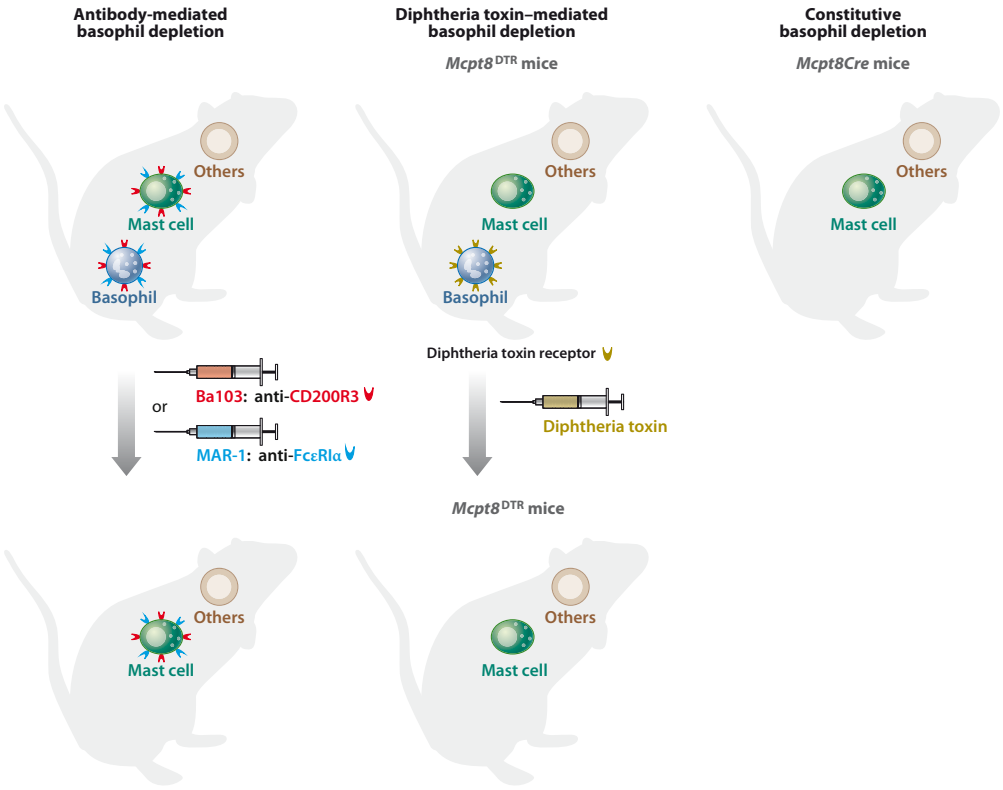

Figure 12: Mouse models of basophil depletion (Karasuyama et al., 2011)

MCPT8^{DTR} Mice. The lab of Karasuyama recently generated a powerful mouse tool, MCPT8DTR mice, that express the human diphtheria toxin receptor (DTR) specifically in basophils. Thus, injection of diphtheria toxin allows the specific depletion of basophils (Wada et al., 2010). These mice contain a cDNA-encoding DTR fused to GFP and an internal ribosome entry site, inserted into the 3' untranslated region of the *Mcpt8* gene. Mcpt8 gene is specifically expressed by basophils (Poorafshar, Helmby, Troye-Blomberg, & Hellman, 2000; Ugajin et al., 2009). In these mice, GFP was only seen expressed in basophils and not in other mature cell types (neutrophils, eosinophils, mast cells). 3 days after injection of DT in *Mcpt8^{DTR}* mice, basophils were transiently depleted from the blood, spleen and bone marrow. No systemic anaphylaxis was caused in these mice. Recent studies used efficiently *Mcpt8^{DTR}* system, and reported important roles of basophils in IgE-chronic allergic inflammation, where DT was injected intravenously (Egawa et al., 2013); in a model of OVA-induce eosinophilic esophagitis, DT injection showed that basophils were important for eosinophil recruitement (Venturelli et al., 2016); *Mcpt8^{DTR}* were efficiently used in a study of malaria infection where authors reported a new marker for basophil detection, CD41 (Bakocevic et al., 2014); Moreover, in a model of parasite infection, IL-3 dependent basophil recruitment was shown to be dispensable for TH2 immune responses (S. Kim et al., 2013), and in a peanut-induced anaphylaxis mouse model, basophils were efficiently depleted using *Mcpt8^{DTR}* system, without affecting any other cell type (Reber et al., 2013).

Bas-TRECK mice. These mice were established by the team of Kubo. It is as well a DT-based conditional deletion system, but using Il4 enhancer elements previously shown to be important for IL-4 production by basophils (Sawaguchi et al., 2012; Yagi, Tanaka, Motomura, & Kubo, 2007). Bas-TRECK mice express the human DTR under the control of 3' untranslated region elements in the Il4 gene locus. Injection of DT showed efficient depletion of basophils without affecting any other cell type (Sawaguchi et al., 2012). Using BAS-TRECK mice, in a irritant contact dermatitis model, basophils were shown to be important for recruitment of eosinophils to the skin, in a mechanism that involved fibroblasts (Nakashima et al., 2014). Basophils were shown to be important of IL-4 and IL-5 production, Th2 cell differentiation in the lymph node and induction of cutaneaous

Th2 responses against protein antigens and hapten-induced akin allergic inflammation (Giacomin et al., 2012; Hill et al., 2012; Otsuka et al., 2013; Siracusa et al., 2011). Depletion of basophils was also associated with a decrease in oesophageal eosinophilia and Th2 cytokine responses, reduction of skin inflammation, and reduction in eosinophil infiltration in lungs and airways (B. S. Kim et al., 2014; Motomura et al., 2014; Noti et al., 2014; Noti et al., 2013).

Mcpt8Cre mice. *Mcpt8Cre* trangenic mice were generated by Ohnmacht et al. for constitutive depletion of basophils. Mice expressing the Cre recombinase under control of regulatory elements for the Mcpt8 gene were crossed to ROSAtdRFP mice that contain a Cre-inducible conditional allele of the red fluorescent protein (RFP) in the locus of ROSA26, ubiquitously expressed. Depletion of basophils in these mice was 90% efficient, and did not affect other cells (Ohnmacht & Voehringer, 2010). Studies using these mice showed an impaired memory IgE response in a model of *N. brasiliensis* infection (Turqueti-Neves et al., 2015). However, it was difficult to draw clear conclusions from these mice, since the depletion was not 100% efficient.

Baso MCPT8-Rosa-DTa mice. These constitutive-basophil-depleting mice were originally generated to track and deplete basophils with high specificity. Mcpt8 encoding gene was replaced by a reporter cassette containing a sequence encoding YFP, followed by an IRES and a sequence encoding humanized Cre recominase (Basoph8 mice). Then, to delete basophils, Basoph8 mice were crossed with Rosa-DTa mice, that express the gene encoding diphtheria toxin alpha chain inserted intro the ubiquitous Rosa26 locus downstream of a loxP-flanked transcriptional stop site. The Basoph8 x Rosa-DTa mice were depleted efficiently of basophils with around 96% efficiency after *N. brasiliensis* infection (Sullivan et al., 2011). Using these mice, Cheng et al. recently demonstrated in a model of IgE-dependent eosinophilic skin inflammation a role of basophils in eosinophil recruitment to the skin, involving endothelial cells (Cheng et al., 2015).

4. Objectives

Allergy is a state in which the host overreacts to otherwise innocuous antigens (allergens) by inducing T helper type 2 (Th2) inflammation. The prevalence of allergic diseases has dramatically increased, with nearly 30 % of world population affected by one or more allergies, posing an important health and financial burden. A number of cellular and molecular players have been implicated in the generation of allergic skin inflammation, but how they act and crosstalk in the inflammatory network remains largely unknown. This greatly hampers the understanding of allergy pathogenesis and the development of effective therapeutic and prevention approaches.

One focus of my lab is to better understand inflammatory networks in allergic skin. Basophils, one type of circulating granulocytes that account for 1 % of blood leukocytes, represent one characteristic cellular component in allergic skin inflammation. Basophils have been recognized for their effector functions in allergy and recently, new antibodies specifically detecting basophils, and reagents/tools for depleting basophils in mice have been developed, which are casting new light on this cell type, and revealing previously unappreciated and non-redundant roles of basophils in immunological disorders including parasite infections and allergies. Despite these progresses, how basophils are recruited to the inflamed tissue, get activated and crosstalk with other cells in the local inflammatory microenvironment remain still incompletely understood.

The **objective** of my PhD study is to investigate the recruitment, activation and function of basophils in the inflammatory network of allergic skin. Using experimental mouse model for allergic contact dermatitis, combined with mouse genetic tools, immunology and cellular/molecular biology approaches, the study of my thesis has discovered:

1) IL-3 plays a crucial role in basophil extravasation to mouse skin with allergic contact dermatitis (Part 1, manuscript in preparation).

2) Depletion of basophils in MCPT8DTR mice leads to a systemic reduction of eosinophils and neutrophils (Part 2, manuscript in preparation).

RESULTS

RESULTS

PART 1

IL-3 plays a crucial role in basophil extravasation to mouse skin with allergic contact dermatitis

Carole El Hachem et al. (manuscript in preparation).

Abstract

Basophils have been recognized as a characterized cellular player for Th2 immune responses implicated in allergic diseases, however mechanisms responsible for basophil recruitment and activation in allergic skin remain not well understood. Using a hapten fluorescein isothiocyanate (FITC)-induced allergic contact dermatitis (ACD) mouse model, we showed that basophil recruitment in ACD skin is dependent on adaptive immunity. We found that IL-3 but not thymic stromal lymphopoietin (TSLP) plays a crucial role for basophil recruitment to skin and for their production of IL-4 and IL-13. Interestingly, our study revealed that basophils in FITC-treated *Il3*$^{-/-}$ mice were defective in crossing the vascular endothelium to enter the inflamed skin. Moreover, by generating mice in which IL-3 is selectively ablated in T cells, we showed that IL-3 produced by T cells mediated basophil extravasation to skin. Furthermore, we found that basophils sorted from FITC-treated *Il3*$^{-/-}$ mice exhibited a decreased expression of PSGL-1 and a number of integrins including ITGAM, ITGB2, ITGA2B and ITGB7, which could be potentially implicated in rolling and adhesion of basophils on endothelial cell surface during their extravasation process. These results thus provide in vivo evidence on the crucial role of IL-3 in basophil extravasation to the inflamed skin, and further raise a hypothesis for exploring the underlying mechanisms.

INTRODUCTION

Basophils, one type of circulating granulocytes that account only for 1% of blood leukocytes, represent a characteristic cellular component in parasite infection and allergic skin inflammation. Basophils complete their maturation in the bone marrow, circulate in the blood and migrate to tissue under inflammatory conditions. They have been shown to infiltrate skin lesions in certain skin disorders such as allergic contact dermatitis, acute atopic dermatitis, prurigo, urticaria and bullous pemphigoid, but are absent in other skin disorders like psoriasis vulgaris (Dvorak & Mihm, 1972; Ito et al., 2011).

Despite that basophils are the least abundant circulating leukocytes, increasing evidence has suggested that basophils play important roles in physiological and pathological contexts (Karasuyama et al., 2011). Basophils are well known for the presence of basophilic granules in their cytoplasm that release mediators upon activation. This activation can be mediated by different molecules like immunoglobulins, cytokines/chemokines, factors of the complement system, growth factors, bacteria-derived ligands, and proteases interacting with surface receptors. Activated basophils can release preformed molecules such as histamine and leukotriene C4 (LTC4), chemotactic factors, and cytokines including IL-4, IL-13 or thymic stromal lymphopoietin TSLP, that are involved in immediate and late-phase reactions of the immune system (Steiner et al., 2016). For example, basophils have been proposed to contribute to Th2 differentiation and responses through their secretion of IL-4, either during parasite infection or allergic inflammation (Leyva-Castillo et al., 2013; Min et al., 2004; Sokol et al., 2008; Sullivan et al., 2011; Voehringer, Shinkai, & Locksley, 2004). In addition, basophils were recently reported to crosstalk with other inflammatory cells, for example to mediate eosinophil recruitment to allergic skin (Cheng et al., 2015; Nakashima et al., 2014) or to confer an M2-like phenotype on macrophages (Egawa et al., 2013).

Although our knowledge on basophil function is being rapidly expanded, how these cells infiltrate to inflammatory sites such as the allergic skin remains still incompletely understood. Chemokines such as CCL11 and CCL24 were previously shown to be related with basophil recruitment (Forssmann et al., 1997; Menzies-Gow et

al., 2002). Moreover, IL-3 and TSLP have both emerged to be implicated in basophil recruitment to tissue, but results remain controversial. IL-3 was shown to play an important role for basophil survival *in vitro* (Voehringer, 2012), to regulate their expansion in blood in a mouse model with *Nippostrongylus brasilensis (N.b.)* parasite infection, their production from the bone marrow and their survival (Lantz et al., 2008; Shen et al., 2008). It was also reported to play a role for basophil recruitment to the mesenteric lymph nodes in *N.b.* infection (S. Kim et al., 2010) and to skin draining lymph nodes in an atopic dermatitis (AD) mouse model (Leyva-Castillo et al., 2013). On the other hand, TSLP, a pro-Th2 cytokine (Li et al., 2005; Zhou et al., 2005), was reported to promote IL-3-independent basophil hematopoiesis (Siracusa et al., 2011), and to mediate basophil responses upon *Trichinella spirali*s independently of IL-3 signalling (Giacomin et al., 2012). My lab recently showed that in an AD mouse model induced by the overexpression of TSLP (Leyva-Castillo et al., 2013), the recruitment of basophils to the inflamed AD skin involves both IL-3-dependent and independent pathways (Leyva-Castillo et al., unpublished data). These “controversial” reports on IL-3 and TSLP in basophil tissue recruitment possibly reflect different mechanisms under different contexts; nevertheless, it remains unclear whether TSLP or IL-3, or both, are crucial for basophil recruitment in allergic skin inflammation, and moreover, what could be the underlying cellular and molecular mechanisms. Exploration of these questions is important for a better understanding of basophil-related inflammatory pathogenesis, and for developing efficient strategies for treating the related inflammatory disorders.

Tissue inflammatory immune response develops upon the extravasation of leukocytes into the tissue by crossing blood vessels. For circulating leukocytes to enter a tissue under inflammatory conditions, a cascade of events is required so they can recognize the vascular endothelium (Vestweber, 2015). Particularly, leukocyte crossing the barrier of the blood vessel wall involves an interaction between the leukocyte and endothelial cells (ECs). The entire process of leukocyte influx to inflamed tissue sites comprises several essential sequential steps: chemo-attraction, rolling, adhesion to the blood vessel wall and trans-endothelial migration (TEM). First, Chemokines that are generated by ECs or from inflammatory cells are presented on the EC surface, which triggers the activation of leukocyte rolling and adhesion (Alon & Feigelson, 2012).

Second, the binding of selectins (P-and E-selectins on the endothelium) to their ligands such as P-selectin glycoprotein ligand 1 (PSGL-1) expressed by leukocytes, critically regulate the leukocyte rolling on the endothelium. Third, adhesion of leukocytes to blood vessels is mediated by intergins expressed on leukocyte surface to bind to their ligands expressed on ECs (e.g. ICAM-1, VCAM-1, MADCAM1, fibronectin...). Integrins are composed of a complex family of αβ heterodimers, with 18 α-subunits and 8 β-subunits, that can assemble in 24 different receptors in vertebrates (Campbell & Humphries, 2011). Finally, TEM is the last step where leukocytes cross ECs lining the blood vessels by paracellular way (taken place at the endothelial borders) or transcellular way (through the endothelial cell body(Muller, 2013; Vestweber, 2015).

Studies have identified molecules implicated in leukocyte extravasation, particularly integrins. For example ITGAL/ITGB2 integrin (lymphocyte function-associated antigen 1; LFA-1) and ITGAM/ITGB2 integrin (macrophage antigen 1; MAC-1) were well shown to be involved in neutrophil extravasation (Choi, Santoso, & Chavakis, 2009) and ITGA4/ITGB7 was reported to be important for T cell migration (Shulman et al., 2009). As to basophil extravasation, *in vitro* studies showed that IL-3 receptor complex was expressed in ECs or basophils (Brizzi et al., 1993; Korpelainen et al., 1996), and treating ECs (Lim et al., 2006) or basophils (Bochner et al., 1990; Iikura et al., 2004) with IL-3 enhanced basophil rolling, adhesion and TEM. Moreover, using antibodies against PSGL-1, P-selectin, ITGAM, ITGB2 or ITGB1 was shown to inhibit basophil adhesion and migration to ECs (Bochner et al., 1990; Iikura et al., 2004; Lim et al., 2006). However, all of these studies were performed *in vitro* and there is no *in vivo* study to explore the mechanisms of basophil extravasation to inflamed tissues.

In order to investigate basophil recruitment and activation in allergic skin, we took use of a hapten FITC-induced ACD mouse model. As small chemical compounds, haptens can easily penetrate into the skin and bind to endogenous proteins in the dermis, which makes them antigenic. When skin dendritic cells recognize the modified protein, they prime allergen-specific T cells in the skin draining lymph nodes, a phase called “sensitization”. Upon re-exposure to the same antigen (hapten bound to protein), an inflammatory response will develop in the site of inflammation, e.g. the skin, and this phase is called “challenge” (Dudeck et al., 2011). It has been shown that FITC, when

combined to its adjuvant dibutyl phthalate (DBP), induces a Th2- type ACD inflammation (Takeshita, Yamasaki, Akira, Gantner, & Bacon, 2004). Using this model, we showed that basophil infiltrate was a characterized feature of ACD skin. We then dissected the role of TSLP and IL-3 in basophil recruitment. Of importance, we provided evidence that IL-3 plays a crucial role for basophil extravasation to the inflamed skin, and furthermore, we showed that PSGL-1 and a few integrins could be potentially implicated in this process and raised a model for further investigating the underlying mechanisms.

MATERIALS AND METHODS

Mice

Wild-type BALB/c mice and *Rag1*$^{-/-}$ mice were purchased from Charles River Laboratories. *Tslp*$^{-/-}$ mice have been previously described (Li et al., 2009). *Il3*$^{-/-}$ mice (Mach et al., 1998) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. 4C13R dual reporter mice (Roediger et al., 2013) were kindly provided by Dr. William E Paul (NIH, USA). All of these mouse lines were backcrossed to Balb/c genetic background (>99.5%). CD4-Cre mice (Lee et al., 2001) were purchased from the Jackson laboratory and were backcrossed into Balb/c background (>93%). *Il3*$^{L2/L2}$ mice (in Balb/c background) were generated by us (Marschall et al., unpublished data). Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the Animal Care and Use Committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) and Institut Clinique de la Souris (ICS).

FITC treatment

Fluorescein isothiocyanate (FITC, ≥97.5% (HPLC) (Sigma) was first dissolved in acetone (to a concentration of 2%), then mixed with equal volume of dibutyl phthalate (DBP, Sigma) to get a final concentration of 1% FITC (in 1:1 DBP/acetone). Mice were sensitized with 25µl of FITC (in 1:1 DBP/acetone) on the left ear (LE) followed by the challenge on the right ear (RE) with 25µl of FITC (in 1:1 DBP/acetone), as indicated in experimental schemes in figures.

MC903 topical application

MC903 (calcipotriol; Leo Pharma) was dissolved in ethanol (ETOH) and topically applied on mouse ears (1 nmol in 25 µl per ear).

All-trans RA treatment

All trans-RA (at-RA; MP Biomedicals) was first dissolved in ETOH for a stock solution (5mg/ml; 16mM). For topical treatment, at-RA was diluted in ETOH to a final concentration of 40µM and topically applied on mouse ears (25 µl per ear); for intraperitoneal (i.p.) injection, 0.1 ml of RA (5mg/ml in ETOH) was mixed with 4.9 ml of sunflower oil; vortexed and sonicated to make a solution with final concentration of 0.1 mg/ml for injection (10 µl/g mouse).

ELISA

Mouse ear was chopped and homogenized with a Mixer Mill MM301 (Retsch, Dusseldorf) in lysis buffer (25 mmol/L Tris pH 7.8, 2 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche). Protein concentrations of skin extract were quantified by using the Bio-Rad Protein Assay (Bio-Rad Laboratories). TSLP levels in skin extracts were determined with the DuoSet ELISA Development Kits (R&D Systems).

Cell preparation for FACS analyses

For preparation of dermal cells, ears were split into two halves, floated on a solution of Dispase (4mg/ml in PBS, Gibco) with epidermis side up, and incubated at 37°C for 1h. Dermis was then separated from epidermis and was further incubated on an agitator at 37°C for 1h in a solution containing 1 mg/ml collagenase D (Roche), 0.25 mg/mL DNaseI (Sigma) and 2.5% of foetal calf serum (FCS) (ThermoFisher) in PBS, then passed through a cell strainer (EASYstrainer 70µm, Greiner bio-one). Cells were then centrifuged at 1200 rpm, 4°C for 5 min, resuspended in FACS buffer (1% of FCS + 2mM EDTA in PBS), counted and used for FACS staining ($2x10^6$ cells) or for sorting.

For preparation of blood cells, 400 µl of blood was collected from mice by retro-orbital bleeding in EDTA-coated tubes, mixed with the same volume of Dextran (2% in PBS, Sigma-Aldrich) and incubated for 30 min at 37°C. The upper phase was

transferred into new tubes, 600 µl of FACS buffer was added, then centrifuged at 4000 rpm for 4 min at 4°C. The pellet was resuspended in 0.3 ml of ACK lysis buffer (Ammonium-Chloride-Potassium: NH4Cl 0.15M; KHC03 1mM; Na_2EDTA 0.1mM), incubated for 2 minutes at room temperature (RT), and then added 1ml of FACS buffer and centrifuged 4000 rpm for 4 min at 4°C. The pellet was resuspended in FACS buffer and used for FACS staining.

Antibody staining and FACS analyses

Cells were first incubated with CD16/CD23 (mouse Fc block) (Table 1) for 10 min on ice, then washed and stained with the surface Abs (see Table 1), starting with biotinylated Abs in 25µl of FACS buffer for 10 min on ice, then washed and stained with streptavidin mixed with other surface Abs in 25µl of FACS buffer for 10 min on ice (except for CD34 Ab which was incubated for 90 min on ice). Cells were then washed with FACS buffer, incubated for 3 min with DAPI (final concentration: 1µg/ml) for exclusion of dead cells before passing on LSRII (BD).

For intracellular staining, dermal cells were cultured in RMPI medium w/o HEPES, + 10% FCS +1% P/S and 2mM Glutamin, in presence or absence of GolgiSTOP (BD) and Cell Stimulation Cocktail (eBioscience) at 37°C for 2h. Cells were then washed with FACS buffer then incubated with CD16/CD23 (mouse Fc block) for 10 min on ice, then washed with FACS buffer and stained with the surface Abs (see Table 1) as described above. Cells were then washed and resuspended with 100 µl of Fixation/Permeabilization solution (BD Cytofix/Cytoperm kit) for 20 min on ice, then washed twice with the wash buffer (BD Cytofix/Cytoperm kit). IL-3 Ab (see table 1) was added and incubated on ice for 30 min. After washing, cells were finally resuspended with FACS buffer and passed on LSRII analyser

Table 1. Antibodies used for Flow cytometry

Name	Fluorophore	Clone	Company	Dilution
CD16/CD32 (Fc block)		93	eBioscience	0.5:25
CD49b-biotin		DX5	eBioscience	0.5:25

IgE-biotin		R35-72	BD Biosciences	0.5:25
Streptavidin	BV605		Invitrogen	0.5:25
CD45	APC-eFluo780	30-F11	eBioscience	0.05:25
TCR-beta	PerCP-Cy5.5	H57-597	eBioscience	1:25
Siglec-F	Alexa Fluor647	E50-2440	BD Biosciences	1:25
Gr1	PE	RB6-8C5	eBioscience	0.02:25
CD34	eFluor 700	RAM34	eBioscience	4:25
ESAM-1	APC	1G8/ESAM	Biolegend	1.25:25
CD19	PerCP-Cy5.5	eBio1D3	eBioscience	1:25
CD3	FITC	145-2C11	eBioscience	1:25
CD45R/B220	PE-Cy7	RA3-6B2	Biolegend	1:25
IL-3	PE	MP2-8F8	Biolegend	1.25:50
FcεRIα	Alexa Fluor 647	Fc23cpg	eBioscience	1:25

RNA extraction of cells sorted from ears

Ear dermal cells were prepared as described above. Staining was done at a concentration of $100x10^6$ cells/500µl of FACS buffer (two ears were combined for each sorting; the number of sorted cells is shown in table 2). Cells were sorted in FACS buffer, then centrifuged at 2000 rpm, 4°C for 6 min, resuspended and vortexed in lysis buffer from NucleoSpin RNA XS kit (Macherey- Nagel) then stocked at -80°C. RNA was extracted from these cells following the manufacturer's instruction.

Table 2. Number of sorted cells

Cell type	**Number of sorted cells aprrox (from 2 challenged-ears)**
Endothelial cells ($CD45^-CD34^+ESAM\text{-}1^+$)	30000
Hematopoietic cells ($CD45^+$)	100000
Basophils ($CD45^{lo}$)	40000 (CT); 20000 (IL3-/-)
Neutrophils ($CD45^+TCR\beta^+Gr1^{hi}$) Eosinophils ($CD45^+TCR\beta^+Siglec\text{-}F^+$)	100000
TCRβ cells ($CD45^+TCR\beta^+$)	50000

Histology

Mouse ears were fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) were stained with haematoxylin and eosin.

Immunohistochemistry staining

For immunohistochemistry (IHC) staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), paraffin sections were treated with 0.6% H_2O_2 (in PBS) to block endogenous peroxidase activity before antigen retrieval with either Pepsin (Invitrogen) incubated for 10 min at 37°C (for IHC of MBP), or with citric buffer (9mL 0,1M citric acid + 41mL 0,1M sodium citrate + 450mL H2O to make pH=6) boiled in the microwave (700W, 70% for 5 min) (for IHC of MCPT8). Slides were then blocked with 1.5% of normal rabbit serum (Vector Laboratories) and incubated with primary antibody overnight at 4°C, followed by incubation with biotinylated rabbit anti-rat IgG (Vector Laboratories) for 1h at RT (room temperature) and treatment of AB complex (Vector Laboratories) for 30 min at RT. Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

Table 3. Antibodies used for IHC

Antibody	Vendor	Clone
MBP (Dilution: 1/2000)	Provided by Dr James J. Lee, Mayo Clinic, Rochester	Rat anti-mouse mAb
MCPT8 (Dilution: 1/500)	BioLegend	Rat anti-mouse TUG8

Quantitative RT-PCR

RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of PCR primers are shown below

Table 4: Primer sequences

Gene name	Sequence 5' to 3'	Oligo number (Lab)
HPRT	TGGATACAGGCCAGACTTTG GATTCAACTTGCGCTCATCTTA	BBP 138 BBN 251
IL-3	TGAAGGACCCTCTCTGAGGA CGCAGATCATTCGCAGAT	LIM 132 LIM 133
IL-4	GGCATTTTGAACGAGGTCAC AAATATGCGAAGCACCTTGG	AHU 218 AHU 219
IL-5	AGCACAGTGGTGAAAGAGACCTT TCCAATGCATAGCTGGTGATTT	AEM 155 AEM 156
IL-13	GGAGCTGAGCAACATCACACA GGTCCTGTAGATGGCATTGCA	AEM 162 AEM 163
IL-17a	CCAGGGAGAGCTTCATCTGT ACGTGGAACGGTTGAGGRTAG	LIM 040 LIM 041
IFNγ	AACGCTACACACTGCATCTTGG GACTTCAAAGAGTCTGAGG	ACY 102 ACY 103
CCL5	CCCTCACCATCATCCTCACT CCTTCGAGTGACAAACACGA	AER 115 AER 116
CCL11	ATGCACCCTGAAAGCCATAG ATTCCCTCAGAGCACGTCTT	AGJ 45 AGJ 46
CCL24	GTGCCTGACCTCCAGAACAT TCTTATGGCCCTTCTTGGTG	BAQ 116 BAQ 117
CCR3	TAAAGGACTTAGCAAAATTCACCA TGACCCCAGCTCTTTGATTC	BAQ 114 BAQ 115
MCPT8	GTGGGAAATCCCAGTGAGAA TCCGAATCCAAGGCATAAAG	BDS 202 BDS 203
P-selectin (SELP)	AAAAGGTTCCTGGACGCCAA GACGTCATTGAGGTGAGCGA	LIM 378 LIM 379
E-selectin (SELE)	ACGGATAGAGAGAAGCAGGAGC TCATGAGCTCACTGGAGGCA	LIM 384 LIM 385
PSGL1 (SELPLG)	GGGACCACAAGTGTCTGGCA TCCTTGGTTTCATGCCCCCA	LIM 390 LIM 391
ICAM-1	GCTCAGTATCTCCTCCCCA GCTGTGCTTTGAGAACTGTG	LIM 442 LIM443
VCAM-1	CCCAAACAGAGGCAGAGTGT CAGGACTGCCCTCCTCTAGT	LIM 274 LIM 275
ITGB1	GCTGGGTTTCACTTTGCTGG TGTGCCCACTGCTGACTTAG	LIM 382 LIM 383
ITGB2	CAACAACGTCAAGAAGCTGGG GCCTTCTCCTTGTTGGGACA	LIM 435 LIM 436
ITGB3	GTGTGGGCCTCAAGATTGGA	LIM 431

	AGGCACAGTCACAGTCGAAG	LIM 432
ITGB7	GACGACTTGGAACGTGTGCG TGGGTGGTGAAGCTTGGAGG	LIM 433 LIM 434
ITGAM	AAACAAGGATGCTGGGGAGG GTCTCATCAAAGAAGGCACGG	LIM 427 LIM 428
ITGAL	CTGGACCTGCGTGAAGACC GGTACCGTGGGGCTCCTG	LIM 425 LIM 426
ITGA2b	AGACACCAGTCAGCTGCTTC CCTGACGGGGCTTCTGTAAG	
ITGA4	TAGCGAATCTTGGCGACATT ACCAACGGCTACATCAACAT	LIM 454 LIM 455
ITGA5	ATGCCCTGAAGCCAAGTGTT TATTCCCGCTGCAAGAAGGT	
ITGAE	AGCCGGGACATTAACGCCTC ACCACCATGACCTTCAATGCTT	LIM 423 LIM 424

RNA-seq data analysis

Basophils were FACS sorted from MC903-treated ears. RNA was extracted using RNeasy Micro Kit (Qiagen). RNA-seq was performed in IGBMC high-throughput mRNA sequencing facility. Reads were mapped onto the mm10 assembly of mouse genome using Tophat v2.0.10 (D. Kim et al., 2013) and the bowtie2 v2.1.0 aligner (Langmead & Salzberg, 2012). Read counts were normalized across libraries with the method proposed by Anders and Huber (Anders & Huber, 2010). Comparisons of interest were performed using the method proposed by Love et al. (Love, Huber, & Anders, 2014) implemented in the DESeq2 Bioconductor library (DESeq2 v1.0.19). Resulting p-values were further adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini, 1995). Quantification of gene expression was performed using HTSeq v0.5.4p3 (Anders, Pyl, & Huber, 2015) using gene annotations from Ensembl release 77.

Statistical analysis

Data were analysed using SigmaStat (Systat Software, Point Richmond, CA, USA) by Student's t-test or Mann–Whitney rank sum non- parametric test depending on results from the Kolmogorov–Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance.

RESULTS

Basophil recruitment in FITC-induced ACD skin is dependent on adaptive immunity

To induce allergic contact dermatitis (ACD) in mice, we employed an experimental protocol (Takeshita et al., 2004), in which Balb/c mice were first sensitized on one ear (left ear, LE) at Day (D) 0, 1 and 2, with fluorescein isothiocyanate (FITC, a hapten with potential to induce ACD with Th2 features when combined to dibutyl phthalate DBP), and challenged with the same solution on the other ear (right ear, RE) at D6 (Fig. 1A). Hematoxylin and eosin (H&E) staining of REs at D7 showed that the FITC treatment induced an inflammatory response with an epidermal hyperplasia and an immune infiltrate in dermis. Immunohistochemistry (IHC) analyses using an antibody against MCPT8 (mast cell protease 8, a basophil specific marker) (Ugajin et al., 2009) revealed basophil recruitment in the dermis of FITC-treated WT mice (Fig. 1B). In addition, IHC analyses using an antibody against MBP (major basic protein, an eosinophil specific marker (Denzler, Levin, Quiram, Lee, & Lee, 1997) showed that eosinophils, another characteristic cell type in allergic skin inflammation, were recruited in the dermis of FITC-treated WT mice (Fig. 1B). We then performed FACS analyses to further characterize immune cells in the dermis. Results showed an increase of $CD45^+$ hematopoietic cells in FITC-treated WT ears compared to untreated ears. These include basophils (identified as $TCR\beta^-Gr1^-SiglecF^-CD45^{lo}CD49b^+$), eosinophils (identified as $CD45^{hi}TCR\beta^-SiglecF^+$), $TCR\beta^+$ T cells, neutrophils ($CD45^{hi}TCR\beta^-Gr1^{hi}$) and mast cells (identified as $TCR\beta^-Gr1^-SiglecF^-CD45^{hi}CD49b^+$) (Fig. 1C, compare untreated WT and WT+FITC).

Studies using *Nippostrongylus brasiliensis* (Nb) parasite infection mouse models reported that basophil expansion and accumulation in tissues were adaptive immunity dependent (Min et al., 2004; Sullivan et al., 2011; Voehringer et al., 2004), or independent (Giacomin et al., 2012; Siracusa et al., 2011). Moreover, our previous study using an atopic dermatitis (AD) mouse model induced by the overexpression of TSLP (Leyva-Castillo et al., 2013), showed that basophil recruitment to the skin involves both innate and adaptive immune mechanisms ((Li et al., 2006) and Leyva-Castillo et al.,

unpublished data). To examine whether basophil recruitment in FITC-induced ACD is dependent on adaptive immunity, *Rag1*$^{-/-}$ mice which lack mature T- and B-lymphocytes were subjected to FITC treatment. As shown in Fig. 1B, FITC-treated *Rag1*$^{-/-}$ mice did not exhibit dermal infiltrate (H&E saining), and presented very few basophil or eosinophil recruitments (IHC stainings). FACS analyses confirmed that there was no increase of basophils, eosinophils, neutrophils, or mast cells in *Rag1*$^{-/-}$ compared to WT REs upon FITC treatment (Fig. 1C).

We further examined cytokine and chemokine expression of REs by RT-qPCR. We observed an increase in Th2-type cytokines (IL-3, IL-4 and IL-13), MCPT8 (expressed by basophils) and CCR3 (expressed by eosinophils and basophils) in REs from FITC-treated WT compared to untreated WT mice. In addition, an increase in IL-17a (Th17-type cytokine) and IFN-γ (Th1-type cytokine), as well as chemokines related to eosinophil / basophil recruitment (CCL5, CCL11 and CCL24) was also seen. The increase of these genes was all abolished in *Rag1*$^{-/-}$ mice (Fig. 1D).

Taken together, these results indicate that skin recruitment of basophils, as well as of other immune cells in FITC-induced ACD, is dependent on adaptive immunity.

TSLP plays a minimal role in basophil recruitment in FITC-induced ACD

It has been reported that FITC/DBP or DBP alone induced the expression of TSLP (Shigeno, Katakuse, Fujita, Mukoyama, & Watanabe, 2009), a pro-Th2 cytokine produced mainly by keratinocytes. In agreement with this, our analyses showed that upon one treatment of DBP or FITC/DBP at D0, increased TSLP protein levels were detected by ELISA at D1, D2 D3 or D4 although TSLP RNA levels were not clearly increased (Fig. 2A). As TSLP has been previously shown to promote basophil differentiation (Siracusa et al., 2011), basophil responses induced by infection (Giacomin et al., 2012), and basophil recruitment to the skin (our unpublished data using TSLP overexpressing AD model), we examined the role of TSLP in basophil recruitment to FITC-induced ACD skin. *Tslp*$^{-/-}$ mice (Li et al., 2009) were subjected to the FITC sensitization and challenge as described in Fig. 1A. H&E staining showed a less dermal cell infiltrate in *Tslp*$^{-/-}$ mice compared to WT mice upon FITC treatment, with less basophils and eosinophils (Fig. 2B). FACS analyses showed a significant decrease

of frequency and number of $CD45^+$ hematopoietic cells, including $TCR\beta^+$ T cells and neutrophils in FITC-treated *Tslp*$^{-/-}$ skin (Fig. 2C). A tendency of decrease (but not significant) was observed for the total cell number of basophils, eosinophils and mast cells in the dermis of FITC-treated *Tslp*$^{-/-}$ mice (Fig. 2C). RT-qPCR analyses showed a decrease for IL-13 in FITC-treated *Tslp*$^{-/-}$ mice, while only a tendency of decrease (not significant) was observed for IL-3, IL-4, MCPT8, CCR3 and CCL24 (Fig. 2D). Taken together, these data suggest that TSLP seems to be important for T cell and neutrophil recruitment to FITC-treated skin, but only plays a minimal role in basophil recruitment.

IL-3 is crucial for basophil extravasation to FITC-induced ACD skin

It has been shown that in mouse models with *Nippostrongylus brasilensis (N.b.)* parasite infection, IL-3 plays an important role for the expansion of basophils in the blood, their production from bone marrow and their survival (Lantz et al., 2008; Shen et al., 2008). IL-3 was also shown to mediate basophil recruitment to mesenteric lymph nodes in *N.b.* helminth infection (S. Kim et al., 2010) or to skin draining lymph nodes in AD mouse model induced by TSLP overexpression (Leyva-Castillo et al., 2013). However, the role of IL-3 for basophil recruitment in allergic skin inflammation has not been well defined.

We had observed that IL-3 mRNA levels were highly induced in FITC-induced ACD skin (Fig. 1D). To examine whether IL-3 is crucial for basophil recruitment to ACD skin, *Il3*$^{-/-}$ and their wildtype control (CT) littermate mice were subjected to the FITC treatment as described in Fig. 1A. FACS analyses showed that the number of basophils was highly reduced in REs from FITC-treated *Il3*$^{-/-}$ mice compared to that from FITC-treated CT mice (Fig. 3A). In contrast, no decrease was observed in the number of $TCR\beta^+$ T cells, neutrophils, eosinophils or mast cells (Fig. 3A), suggesting a specialized role of IL-3 in basophil recruitment and accumulation in ACD skin. To validate these results, we further performed MCPT8 IHC staining. Interestingly, we observed that in addition to the decrease of basophil number, there was another phenomenon, that is, all of the detected basophils were restricted inside blood vessels in REs of FITC-treated *Il3*$^{-/-}$ mice (Fig. 3B), suggesting a defect of basophils in crossing the vascular endothelium. In

contrast to basophils, MBP IHC staining showed that there was no difference for eosinophil recruitment to skin between FITC-treated *Il3*$^{-/-}$ and CT mice (Fig. 3B).

To examine whether what we observed could reflect a delay of basophil recruitment in *Il3*$^{-/-}$ mice, we performed FITC treatment and analysed RE at later time points (D8, D9 and D10) (Fig. 3C). A similar phenotype was observed as at D7, showing that basophils in FITC-treated *Il3*$^{-/-}$ mice were not able to cross vascular endothelium at any of these time points (Fig. 3C), indicating that basophil extravasation is not delayed but defective in FITC-treated *Il3*$^{-/-}$ mice.

Next, we performed blood analyses of basophils by FACS. The frequency of basophils in the blood was comparable between untreated CT and *Il3*$^{-/-}$ mice (Fig. 3D), indicating that IL-3 is not necessary for the development of baseline levels of basophils in the mouse, in agreement with previous reports (Lantz et al., 1998; Min, Brown, & Legros, 2012; Shen et al., 2008). Upon FITC treatment, frequency of basophils in the blood of CT mice was decreased (Fig. 3D, compare CT+ FITC with untreated CT), possibly due to the extravasation of basophils to skin. This was different from previous reports showing an increase of blood basophil frequency in mice with parasite infection (Lantz et al., 2008; Voehringer et al., 2004). Interestingly, the decrease of basophil frequency in the blood was abolished in *Il3*$^{-/-}$ mice upon FITC treatment (Fig. 3D, compare *Il3*$^{-/-}$ + FITC with untreated *Il3*$^{-/-}$), fitting with the above observation of the defect of basophil in crossing vascular endothelium in these mice. In contrast to basophils, no difference was observed for frequency of eosinophils, neutrophils, T cells and B cells in the blood (Fig. 3D and data not shown). Altogether, these data suggest that basophil extravasation to inflamed ACD skin is defective in mice lacking IL-3.

IL-3 is crucial for IL-4 and IL-13 expression by basophils but not T cells in FITC-induced ACD

IL-4 and IL-13 are characteristic cytokines of Th2-type allergic inflammation. RT-qPCR analyses showed that in addition to MCPT8 that was highly reduced in FITC-treated *Il3*$^{-/-}$ skin, a significant decrease of IL-4 and IL-13 was also observed (Fig. 4A). As IL-4 and IL-13 have been reported to be produced by various cell types including Th2 cells and basophils (Karasuyama et al., 2011), we further examined in which cells IL-4 and IL-13

expression could be reduced in FITC-treated *Il3*$^{-/-}$ skin. To this aim, *Il3*$^{-/-}$ mice were crossed with *4C13R* dual reporter mice, which have transgenic expression of the cyan fluorescent protein AmCyan under the control of Il4 regulatory elements and the red fluorescent protein dsRed under the control of Il13 regulatory elements (Roediger et al., 2013). FACS analyses of REs were performed to compare AmCyan (IL-4) and dsRed (IL-13) between FITC-treated CT/4C13R$^{Tg/0}$ and *Il3*$^{-/-}$/4C13R$^{Tg/0}$ mice. Results showed that first, IL-4 (AmCyan) and IL-13 (dsRed) were expressed by both TCRβ^{+} T cells and basophils in FITC-treated CT/4C13R$^{Tg/0}$ mice; second, while AmCyan (IL-4) and dsRed (IL-13) expression in TCRβ^{+} T cells was comparable between FITC-treated CT/4C13R$^{Tg/0}$ and *Il3*$^{-/-}$/4C13R$^{Tg/0}$ skin (Fig. 4B), their expression in basophils was largely diminished in FITC-treated *Il3*$^{-/-}$/4C13R$^{Tg/0}$ skin (Fig. 4C). Noting that basophils detected by FACS of *Il3*$^{-/-}$/4C13R$^{Tg/0}$ mice were those remained inside blood vessels in skin (see Fig. 3B). These results thus indicate that IL-3 is critical for the expression of IL-4 and IL-13 by basophils but not TCRβ^{+} T cells in FITC-induced ACD, and suggest that basophils may acquire their higher expression of IL-4 and IL-13 upon extravasating into the ACD skin.

IL-3 does not act in synergy with TSLP for basophil recruitment in ACD skin

To determine whether there could be any synergy between IL-3 and TSLP in basophil recruitment in FITC-induced ACD, we generated *Il3*$^{-/-}$/*Tslp*$^{-/-}$ mice, which were subjected to FITC treatment as described in Fig. 1A. FACS analyses showed that the reduction of basophils in RE dermis of *Il3*$^{-/-}$/*Tslp*$^{-/-}$ mice was comparable to that seen in *Il3*$^{-/-}$ mice (compare Fig. 5A with Fig. 3A). IHC staining showed that MCPT8^{+} signals were restricted to the blood vessel of FITC-treated *Il3*$^{-/-}$/*Tslp*$^{-/-}$ mice (Fig. 5B), as seen in FITC-treated *Il3*$^{-/-}$ mice (Fig. 3B). Moreover, blood cell analyses showed a higher basophil frequency in *Il3*$^{-/-}$/*Tslp*$^{-/-}$ compared to CT mice (Fig. 5C), again as observed in *Il3*$^{-/-}$ mice (Fig. 3D). On the other hand, TCRβ^{+} T cells, neutrophils, eosinophils and mast cells all exhibited a similar decrease in REs from *Il3*$^{-/-}$/*Tslp*$^{-/-}$ mice as *Tslp*$^{-/-}$ mice, when compared with WT mice (compare Fig. 5A & B with Fig. 2B & C; the same group of WT mice was used in both cases). In addition, RT-qPCR analyses of REs from FITC-treated *Il3*$^{-/-}$/*Tslp*$^{-/-}$ mice showed that the decrease observed for MCPT8 and IL-4 was

comparable to that observed in *Il3*$^{-/-}$ mice (Fig. 5D and Fig. 4A), whereas the decrease of IL-13 was comparable to that observed in *Tslp*$^{-/-}$ mice (Fig. 5D and Fig. 2D). Therefore, it does not appear to have any synergy between IL-3 and TSLP on basophil recruitment. Rather, IL-3 plays a central role for basophil recruitment to FITC-induced ACD, while the minimal effect of TSLP could be mediated through IL-3, which is reflected by the fact that IL-3 expression was slightly decreased in FITC-treated *Tslp*$^{-/-}$ skin compared to WT skin (Fig. 2D).

IL-3 produced by T cells mediates basophil extravasation to FITC-induced ACD skin

By performing intracellular staining, we showed that IL-3 was produced by both basophils and TCRβ$^+$ T cells but not mast cells in FITC-treated WT skin (Fig. 6A). To investigate whether IL-3 produced by T cells could mediate basophil recruitment to ACD skin, we generated mice in which IL-3 is ablated selectively in both CD4$^+$TCRβ$^+$ and CD8$^+$TCRβ$^+$ T cells, by breeding Il3$^{L2/L2}$ (Marschall et al., our unpublished data) with *CD4-Cre*$^{Tg/0}$ mice (Lee et al., 2001). Our preliminary experiment using one *CD4-Cre*$^{Tg/0}$*/Il3*$^{L2/L2}$ and one *CD4-Cre*$^{0/0}$*/Il3*$^{L2/L2}$ CT mouse found that FITC-treated *CD4-Cre*$^{Tg/0}$*/Il3*$^{L2/L2}$ exhibited a similar phenotype as *Il3*$^{-/-}$ mice: MCPT8 IHC showed that basophils could not cross blood vessels (Fig. 6B, compare with Fig. 3B); FACS analysis of RE dermis showed a decreased number of basophils, while no decrease was observed in TCRβ$^+$ T cells, eosinophils, neutrophils or mast cells (Fig. 6C compare with Fig. 3A); and moreover, a higher frequency of basophils in blood was seen (Fig. 6D, compare with Fig. 3D). These preliminary results, which need to be validated with more mice, suggest that IL-3 produced by T cells is crucial for basophil extravasation in ACD skin.

Decreased expression of PSGL-1 and integrins in basophils from FITC-treated *Il3*$^{-/-}$ skin

Next we sought to explore the mechanisms underlying the role of IL-3 in basophil extravasation in FITC-induced ACD skin. It has been recognized that leukocyte extravasation is regulated by a concerted action between leukocytes and ECs, which

comprises multisteps including rolling, adhesion and TEM (Vestweber, 2015). IL-3R was shown to be expressed by both human ECs (Brizzi et al., 1993; Korpelainen et al., 1996) and human / mouse basophils (Ochensberger, Tassera, Bifrare, Rihs, & Dahinden, 1999; Schroeder et al., 2009). In vitro studies suggested that either basophils or ECs could respond to IL-3 signalling: IL-3 stimulation of human basophils enhances their adhesiveness to ECs (Bochner et al., 1990) and their transendothelial migration (Iikura et al., 2004); on the other hand, stimulation of human ECs by IL-3 induced the expression of P-selectin and selective basophil accumulation (Khew-Goodall et al., 1996; Lim et al., 2006).

We then sorted ECs and basophils by FACS from FITC-challenged REs and analysed by RT-qPCR the expression of molecules potentially implicated in basophil-EC interaction. As shown in Fig. 7B, P-selectin, E-selectin, ICAM-1 and VCAM-1 were all well detected in ECs (as expected, their levels were much higher compared to $CD45^+$ hematopoietic cells). No decrease (rather an increase) of these molecules was observed in ECs from *Il3*$^{-/-}$ compared to CT mice. Interestingly, analyses of the sorted basophils (noting that basophils sorted from the FITC-treated *Il3*$^{-/-}$ REs were those stuck inside blood vessels) revealed that, while there was no decrease observed for ITGA4, ITGA5, ITGAE, ITGB1, ITGAL and ITGB3, a significant decrease was seen in PSGL-1, ITGAM, ITGB2, ITGA2B and ITGB7 from FITC-treated *Il3*$^{-/-}$ compared to CT mice. Importantly, such a decrease was specific for basophils, as no change was observed in neutrophils, eosinophils or $TCR\beta^+$ T cells which were sorted with basophils at the same time (Fig. 7C). It is also notable that among these genes, PSGL-1, ITGAM, ITGB2 and ITGB7 were all well expressed in basophils and particularly, ITGA2B showed a specific expression by basophils (Fig. 7C). These data suggest a possible implication of PSGL-1, ITGAM, ITGB2, ITGA2B and ITGB7 in the defective extravasation of basophils from *Il3*$^{-/-}$ mice.

PSGL-1 and integrin expression in basophils from MC903-treated *Il3*$^{-/-}$ skin

We have previously shown that in an AD mouse model, in which keratinocytic TSLP overexpression is induced by topical application of MC903 (Li et al., 2009; Li et al., 2006), basophil recruitment to the skin was partially dependent on adaptive immunity (Li

et al., 2006) and IL-3 produced by CD4 T cells (Leyva-Castillo et al., unpublished data). Upon MC903 treatment (Fig. 8A), *Il3*$^{-/-}$ mice exhibited less basophil recruitment to the skin compared to CT mice (Fig. 8B). However, different from what was observed in the skin of FITC-treated *Il3*$^{-/-}$ mice, basophils detected by MCPT8 IHC were not restricted inside blood vessels (Fig. 8B). These results suggest that beside IL-3, there should be other factor(s) (e.g. TSLP) mediating the extravasation of basophils in MC903-induced AD skin.

We had previously sorted basophils from skin of MC903-treated WT and *Il3*$^{-/-}$ mice and performed mRNA-seq analyses (Hener, EL Hachem et al., unpublished data). Mining these data, we found with interest that among integrin gene family, one group exhibited a tendency of decreased expression in basophils from MC903-treated *Il3*$^{-/-}$ mice, including PSGL-1, ITGAM, ITGB2, ITGA2B and ITGB7 and a few others (ITGA1, ITGB1, ITGAE and ITGB3) (Fig. 8C). Moreover, PSGL-1 (P-selectin glycoprotein ligand 1, also known as Selplg) expression was also decreased. When validating these results with RT-qPCR, we observed that PSGL-1, ITGAM, ITGB2 and ITGB7 exhibited a tendency of decrease (however not significant), while ITGA2B did not show any decrease (Fig. 8D). These results were in agreement with the fact that basophil extravasation was not totally defective in MC903-treated *Il3*$^{-/-}$ skin (different with FITC-treated *Il3*$^{-/-}$ skin, where basophil extravasation was completely blocked), and add evidence that the expression of these genes could be implicated in basophil extravasation to ACD skin.

DISCUSSION

Employing a FITC-induced ACD mouse model, we explored in this study the basophil recruitment to allergic skin. Our results demonstrated that basophil recruitment to ACD skin is dependent on adaptive immunity. We found that IL-3 but not TSLP plays a crucial role for basophil recruitment to skin and for their production of IL-4 and IL-13. Interestingly, we revealed that basophils in FITC-treated *Il3*$^{-/-}$ mice were defective in crossing the vascular endothelium to enter the inflamed skin. Moreover, by generating *CD4-Cre*$^{Tg/0}$/*Il3*$^{L2/L2}$ mice in which IL-3 is selectively ablated in T cells, we showed that IL-3 produced by T cells mediated basophil extravasation to skin. Furthermore, we found that basophils sorted from FITC-treated *Il3*$^{-/-}$ mice exhibited a decreased expression of PSGL-1 and a number of integrins including ITGAM, ITGB2, ITGA2B and ITGB7, which could be potentially implicated in rolling and adhesion of basophils on endothelial cell surface during their extravasation process.

Our results demonstrate that the adaptive immunity is required for basophil recruitment to FITC-induced ACD skin, and show that IL-3 produced by T cells plays a crucial role in basophil extravasation. In our previous study of an AD mouse model, in which keratinocytic TSLP overexpression is induced by topical application of MC903, we showed that TSLP triggered basophil recruitment through both innate and adaptive mechanisms. Although it remains to be further studied, the innate recruitment of basophils could be mediated by TSLP, while the adaptive recruitment of basophils was shown to be mediated through IL-3 (Leyva-Castillo et al., our unpublished data obtained through the adoptive transfer of CD4^{+} cells isolated from CT or *Il3*$^{-/-}$ mice). It is clear that basophil number is decreased in MC903-treated *Il3*$^{-/-}$ skin (Fig. 8B), indicating that IL-3 is indeed implicated in basophil recruitment in MC903-induced AD skin, although basophil extravasation is not completely blocked. However, in FITC-induced ACD model, basophil extravasation was totally dependent on RAG-1 and IL-3. Why is there such a difference between FITC and MC903 models? One possibility is that the level of TSLP induced is much higher in MC903 model than in FITC model (i.e. 50000 pg TSLP/mg protein in MC903-treated skin, compared with 250 pg TSLP/mg protein in FITC-treated skin, measured by ELISA), therefore the highly expressed TSLP may drive the IL-3-

independent recruitment of basophils. Another difference between the two models is the presence of an exogenous allergen (hapten) in the FITC model but not in the MC903 model.

Our results showed that in FITC-treated *Il3*$^{-/-}$ or *CD4-Cre*$^{Tg/0}$/*Il3*$^{L2/L2}$ mice, basophils were stuck inside the blood vessels, while the extravasation of neutrophils, eosinophils and T cells was not affected, thus providing for the first time the *in vivo* evidence that IL-3 is crucial for basophil extravasation to allergic skin. Moreover, our further exploration found that basophils in FITC-treated *Il3*$^{-/-}$ mice presented a decreased expression of PSGL-1, ITGAM, ITGB2, ITGA2B and ITGB7, which we suspect could be responsible for the defective extravasation of basophils. The regulation of integrin expression by IL-3 is supported by a previous study of human basophils showing that IL-3 increases the expression of ITGAM (CD11b) in basophils and enhances their adhesiveness to ECs (Bochner et al., 1990). In addition, mouse basophils stimulated *in vitro* with IL-3 (or with TSLP) showed a higher expression of ITGAM (Siracusa et al., 2011). Three *in vitro* studies (Bochner et al., 1990; Iikura et al., 2004; Lim et al., 2006) suggested that IL-3 could stimulate either human basophils or human ECs to induce basophil rolling and adhesion to ECs, and blocking Abs against PSGL-1, ITGB1, ITGB2, ITGAM and ITGAL (expressed by basophils), or P-selectin (expressed by ECs) inhibited basophil rolling and adhesion to ECs. Therefore, it appears that PSGL-1 and ITGAM/ITGB2 heterodimer could be implicated in both human and basophils' rolling and adhesion in the extravasation process. Moreover, our data suggest that their RNA expression is regulated by IL-3 signalling. In addition, our data indicate that the expression of ITGB7 and ITGA2B is also under the control of IL-3. Considering also their expression in basophils is high compared to neutrophils, eosinophils and T cells (Fig. 7C), we suspect that they could be new players implicated in basophil extravasation. ITGB7 together with its heterodimer pair ITGA4 has been found to be implicated in T cell homing to the intestine (Agace, 2006) and in eosinophil adhesion (Walsh, Symon, Lazarovils, & Wardlaw, 1996). As to ITGA2B, it is highly specific for basophils (not expressed by neutrophils, eosinophils or T cells). Indeed, a recent study reported ITGA2B (CD41) as a reliable identification and activation marker for murine basophils (Bakocevic et al., 2014). Notably, its heterodimer partner ITGB3

also showed a tendency of decrease in basophils from *Il3*$^{-/-}$ mice (Fig. 7C). Therefore, it will be particularly interesting to explore whether ITGA2B/ITGB3 plays an important role in basophil extravasation.

In MC903-induced AD model where, IL-3 is partially required for basophil recruitment, and basophil extravasation is not totally blocked in *Il3*$^{-/-}$ skin. Interestingly, we observed that the expression of PSGL-1, ITGB7, ITGAM, and ITGB2 had a decreasing tendency in basophils from MC903-treated *Il3*$^{-/-}$ mice, but such a decrease was much less striking than that observed with FITC model. This could be due to the fact that other factors, like TSLP, may also regulate the expression of these molecules, thereby mediating the IL-3-independent basophil extravasation in MC903-treated skin. For example, besides IL-3, TSLP was also shown to induce ITGAM (CD11b) expression by murine basophils *in vitro* (Siracusa et al., 2011). Of note, ITGA2B expression was not decreased in basophils from MC903-treated *Il3*$^{-/-}$ mice (Fig. 8C), whereas its level was highly reduced in basophils from FITC-treated *Il3*$^{-/-}$ mice (Fig. 7C), suggesting again that it could be a new player in basophil extravasation.

Our next question is how IL-3 signalling could regulate the expression of PSGL-1 and integrins. Interestingly, it was previously reported that in a co-culture of human basophils with mast cells, mast cell-derived IL-3 induced the expression of the retinaldehyde dehydrogenease ALDH1A2 (also called RALDH2), an enzyme that catalyses the last oxidative step of the cascade leading retinol to produce retinoic acid (RA) (Spiegl, Didichenko, McCaffery, Langen, & Dahinden, 2008). The authors proposed that RA produced by basophils promotes the expression of ITGA4/ITGB7 heterodimer on T cells in a paracrine manner, thus influencing T cell polarisation (Spiegl et al., 2008). Another study also showed that *in vitro* culture of human basophil with IL-3 induced the expression of ALDH1A2 gene (MacGlashan, 2012). In addition, a recent study in mouse showed that upon IL-33 and IgE stimulation, basophils expressed ALDH1A3 (also called RALDH3) gene (Chhiba, Hsu, Berdnikovs, & Bryce, 2017). We then examined the expression of ALDH1A1, ALDH1A2 or ALDH1A3, all of which are known to be implicated in RA synthesis, in basophils, eosinophils, neutrophils and T cells sorted by FACS from FITC-treated WT and *Il3*$^{-/-}$ REs. RNA levels of ALDH1A1 and ALDH1A2 appeared to be very low as they could not be well detected by RT-qPCR in

any of the samples (Cross Point Cp >35), however, ALDH1A3 was expressed and its RNA level was decreased in basophils from FITC-treated *Il3*$^{-/-}$ compared to CT mice (Fig. 9A). In addition, ALDH1A3 expression tended to decrease in basophils from MC903-treated *Il3*$^{-/-}$ compared to CT mice (Fig. 9B). Therefore, it seems that IL-3 regulates ALDH1A3 in mouse basophils, while it regulates ALDH1A2 expression in human basophils. Whether ALDH1A3 expression could be also regulated by IL-3 in human basophils is unknown.

We then further tested whether RA may indeed act on basophil extravasation. *Il3*$^{-/-}$ and CT mice were treated with FITC as described in Fig. 1A, and all-trans RA (at-RA) was either topically applied to RE 2h before the FITC-challenge, or i.p. injected 24h before the FITC-challenge. Our preliminary analysis with MCPT8 IHC showed that upon at-RA topical treatment, more basophils were extravasated to FITC-treated CT skin (Fig. 9A, compare CT +FITC w/o at-RA and CT +FITC + topical at-RA); moreover, while FITC-treated *Il3*$^{-/-}$ (w/o at-RA) mice showed that basophils were stuck inside blood vessels, at-RA topical treatment of FITC-treated *Il3*$^{-/-}$ mice revealed MCPT8^{+} signals outside blood vessels (Fig. 9C), suggesting that at-RA promotes basophil extravasation in *Il3*$^{-/-}$ skin. The i.p. injection of at-RA showed a less effect on basophil extravasation (Fig. 9C). These data thus suggest that the addition of at-RA may overcome the defect of basophil extravasation in *Il3*$^{-/-}$ mice.

Based on these observations, we propose a model (Fig. 10), in which upon FITC-treatment, CD4^{+} T cells secrete IL-3, which binds to IL-3 receptor complex on basophils and induces expression of ALDH1A2 or ALDH1A3, allowing the production of RA by basophils. In turn, RA secreted by basophils activates RAR/RXR receptor heterodimer in an autocrine manner, which stimulates the expression of PSGL-1, ITGAM, ITGB2, ITGA2B, ITGB7 and others by basophils. This results in the interaction between basophils and endothelial cells, leading to the extravasation of basophils to ACD skin through rolling, adhesion and TEM (Fig. 10).

PERSPECTIVES

To test our model, I propose the following experiments:

1) To validate whether RA promotes basophil extravasation, at-RA topical treatment can be optimized with different doses at various time points in FITC-treated *Il3*$^{-/-}$ mice. To test whether RA mediates basophil extravasation, antagonists of RARs (e.g. BMS 453, RARα and RARγ antagonist) can be administrated to see whether it can block basophil extravasation in FITC-treated WT mice.

2) To check whether basophils can respond to RA and if RA induced the expression of PSGL-1, ITGAM, ITGB2, ITGA2B, ITGB7 in basophils, three strategies could be considered: a) sort basophils from mouse spleen, culture them *in vitro* in presence or absence of at-RA, then check by RT-qPCR and FACS the expression of these genes; also, we could sort ECs and culture them with basophil in transwell inserts: basophils will be cultured in the upper wells in the presence or absence of at-RA or BMS 453 (RARα and RARγ antagonist), then examine the migration of basophils to the lower chamber (Muller & Luscinskas, 2008) ; b) as RXRβ (but not RXRα or RXRγ) is expressed by basophils (Immgen database), we could use RXRβ$^{-/-}$ mice and subject them to FITC treatment to examine whether basophil extravasation is blocked; c) we could also use RARE-*lacZ* reporter mice (Rossant, Zirngibl, Cado, Shago, & Giguere, 1991) and check if FITC treatment can induce the expression of *lacZ* reporter in basophils.

3) To demonstrate whether ALDH1A3 is important for RA synthesis by basophils during the extravasation process, we could generate mice in which ALDH1A3 is conditionally knocked out in basophils (crossing Aldh1a3$^{L2/L2}$ mice (Dupe et al., 2003) with Mcpt8^{Cre} (Sullivan et al., 2011)), and test if basophil extravasation will be blocked.

4) To explore the potential role of ITGA2B/ITGB3 in basophil adhesion during the extravasation process, neutralizing antibodies could be injected to examine if basophil extravasation is blocked in FITC-treated WT mice. This could also be tested in vitro with sorted basophils and ECs using the TEM assay (Muller & Luscinskas, 2008).

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Figure 1. FITC-induced basophil recruitment to ACD skin is dependent on adaptive immunity.

(A) Experimental protocol. Eight to twelve-week-old female mice were sensitized with 1% FITC (in DPB:acetone) on left ear (LE) at Day (D)0, D1 and D2. Right ears (RE) were then challenged at D6 with 1% FITC (in DPB:acetone) and sampled for analyses at D7. **(B)** Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining of RE sections from *Rag1*$^{-/-}$ and Balb/c wildtype (WT) mice, either untreated or treated with FITC (+FITC). Arrow points to one of the positive cells of IHC staining. Objective used: 20x. **(C)** FACS analyses of dermal cells of REs from untreated WT, FITC-treated WT and *Rag1*$^{-/-}$ mice for CD45^{+} (hematopoietic cells), CD45^{+}TCRβ^{+} T cells, CD45^{low}CD49b^{+} (basophils), CD45^{hi}CD49b^{+} (mast cells), CD45^{+}TCRβ^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD45^{+}TCRβ^{-}Gr1^{hi} (neutrophils). **(D)** RT-qPCR analyses of cytokines and chemokines in REs from untreated WT, FITC-treated WT and *Rag1*$^{-/-}$ mice. *P≤0.05 **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

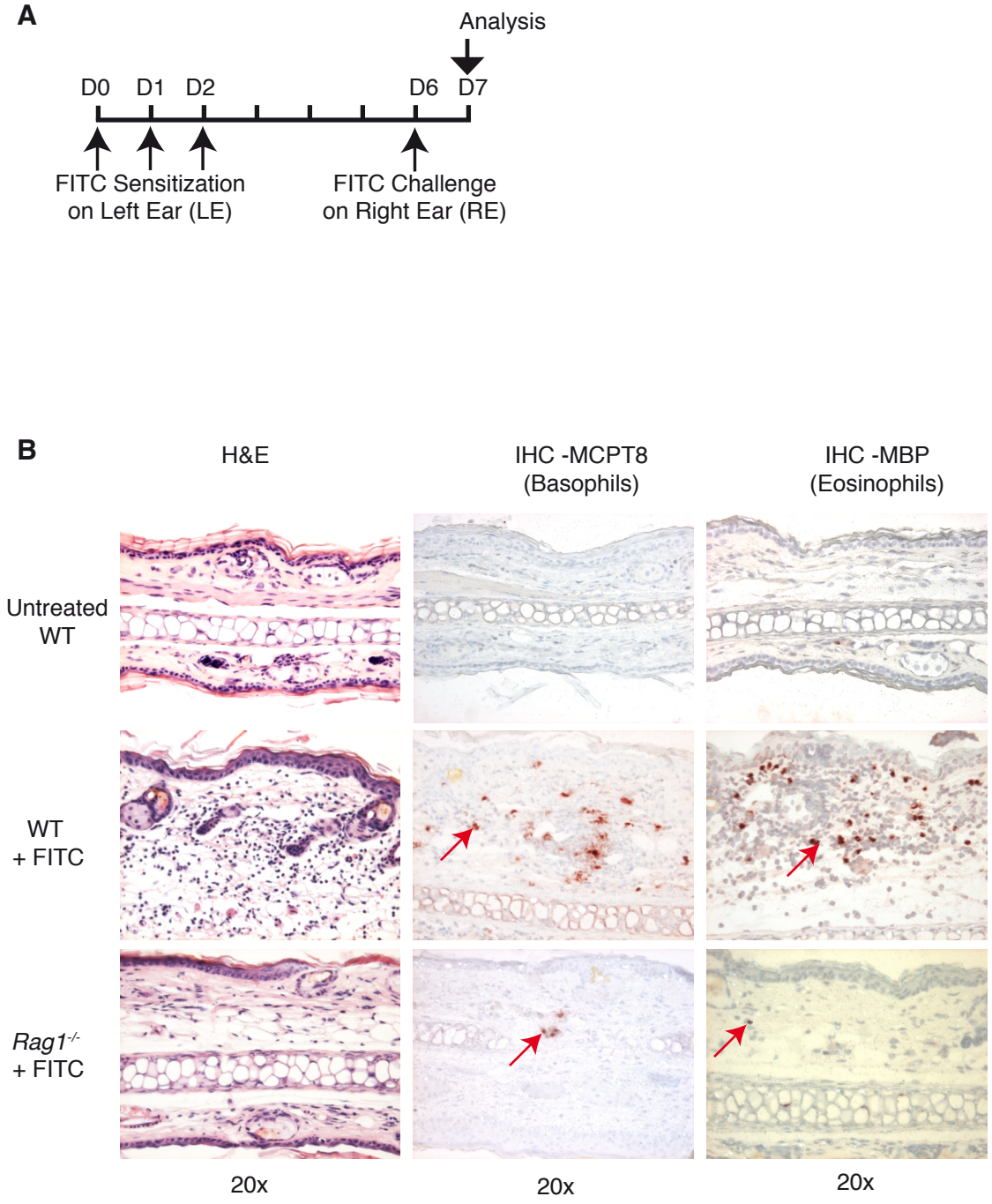
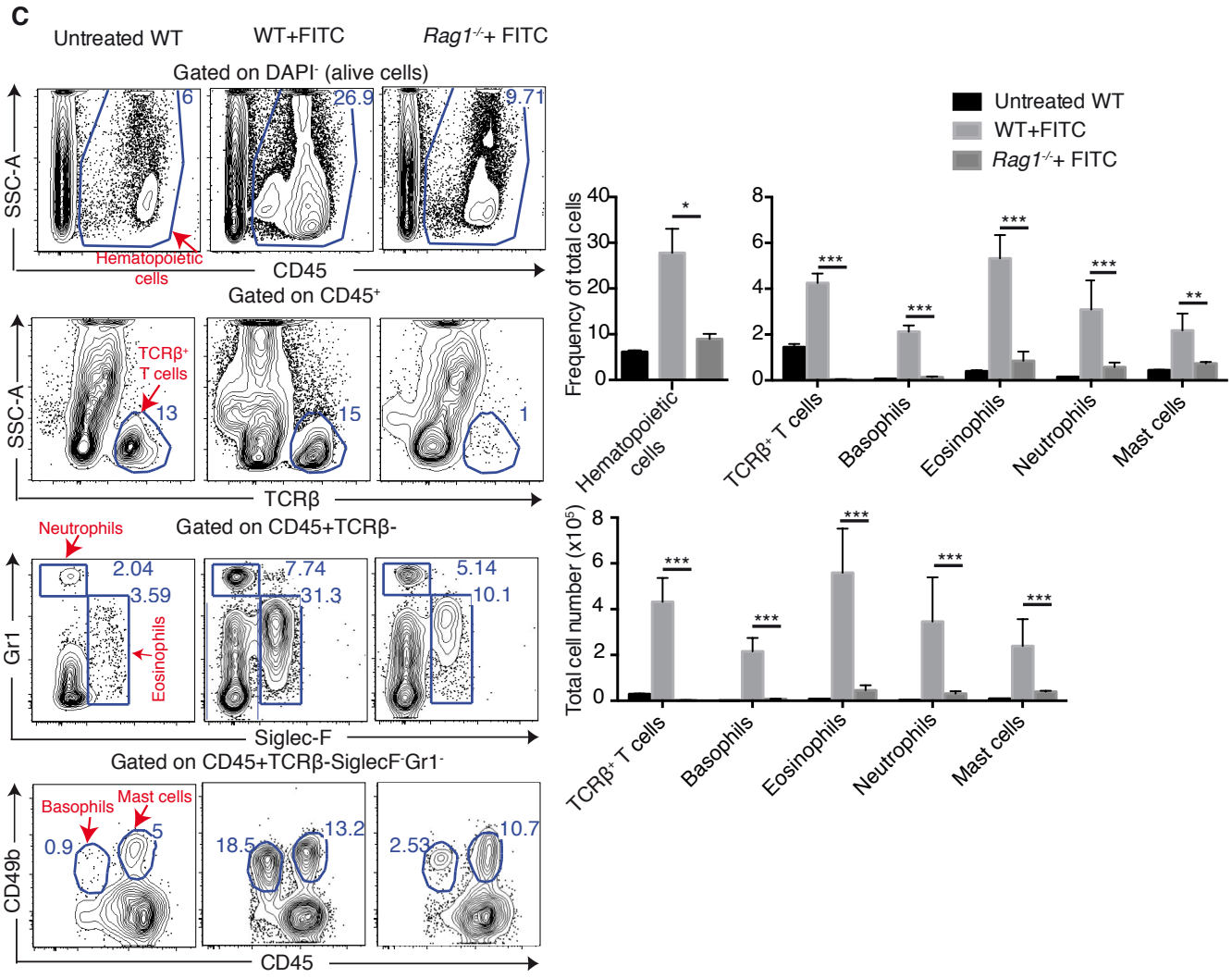

A
Analysis
D0
D1
D2
D6
D7
FITC Sensitization
on Left Ear (LE)
FITC Challenge
on Right Ear (RE)
B
H&E
IHC -MCPT8
(Basophils)
IHC -MBP
(Eosinophils)
Untreated
WT
WT
+ FITC
Rag1-/-
+ FITC
20x
20x
20x

Figure 1


C
Untreated WT
WT+FITC
Rag1-/-+ FITC
Gated on DAPI- (alive cells)
SSC-A
CD45
Hematopoietic cells
16
26.9
9.71
Gated on CD45+
TCRβ+ T cells
13
15
1
TCRβ
Neutrophils
Gated on CD45+TCRβ-
2.04
3.59
Eosinophils
7.74
31.3
5.14
10.1
Gr1
Siglec-F
Gated on CD45+TCRβ-SiglecF-Gr1-
Basophils
Mast cells
0.9
5
18.5
13.2
2.53
10.7
CD49b
CD45
Frequency of total cells
Hematopoietic cells
TCRβ+ T cells
Basophils
Eosinophils
Neutrophils
Mast cells
Total cell number (x10^5)

EAR

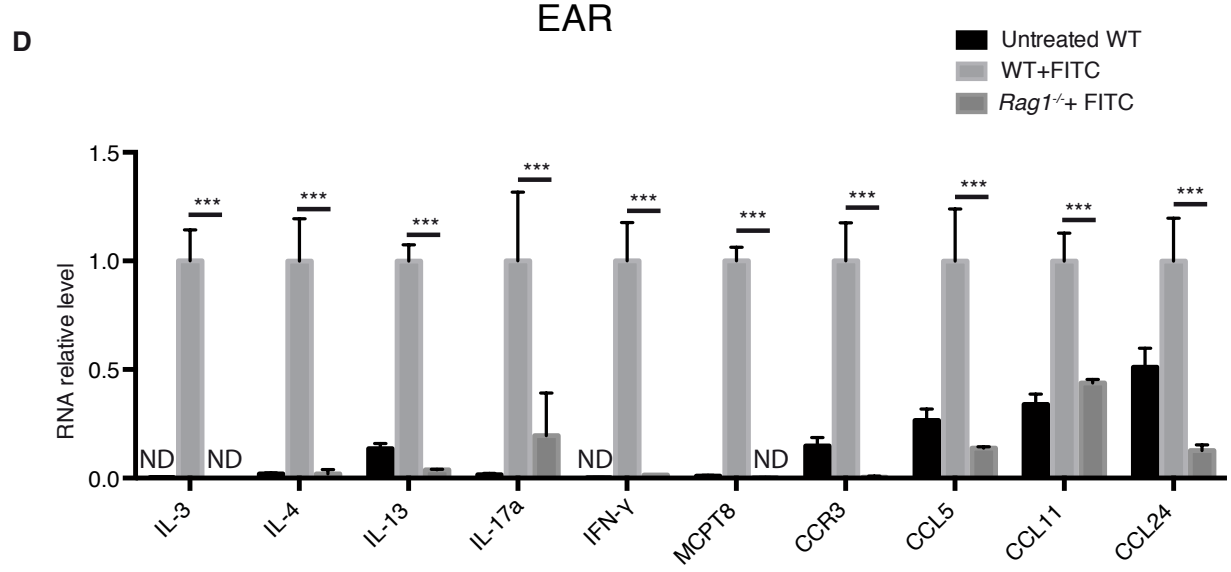

D
Untreated WT
WT+FITC
Rag1-/-+ FITC
RNA relative level
ND
IL-3
IL-4
IL-13
IL-17a
IFN-γ
MCPT8
CCR3
CCL5
CCL11
CCL24

Figure 1

Figure 2. A minimal role of TSLP in basophil recruitment to FITC-induced ACD skin.

(A) TSLP expression is induced upon FITC/DBP treatment. Balb/c wildtype (WT) ears were treated with DBP or FITC (in DPB:acetone) at D0. Ears were sampled at D1, D2, D3 and D4. TSLP protein levels were measured by ELISA, and TSLP RNA levels were assessed by RT-qPCR. **(B-D)** WT and *Tslp*$^{-/-}$ mice were subjected to FITC-sensitization and challenge as in Fig. 1A, and inflammatory responses of the RE were analysed. **(B)** H&E, IHC staining of RE sections. Arrow points to one of the positive cells of IHC staining. Objective used: 20x. Cell counts for infiltrating basophils and eosinophils, obtained by calculating the average number of positively stained cells per microscopic field (at 10x magnification; n=5 fields). **(C)** FACS analyses of dermal cells of REs from FITC-treated WT and *Tslp*$^{-/-}$ mice for CD45^{+} (hematopoietic cells), CD45^{+}TCRβ^{+} T cells, CD45^{low}CD49b^{+} (basophils), CD45^{hi}CD49b^{+} (mast cells), CD45^{+}TCRβ^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD45^{+}TCRβ^{-}Gr1^{hi} (neutrophils). **(D)** RT-qPCR analyses of cytokines and chemokines in REs from FITC-treated WT and *Tslp*$^{-/-}$ mice. *P≤0.05 **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

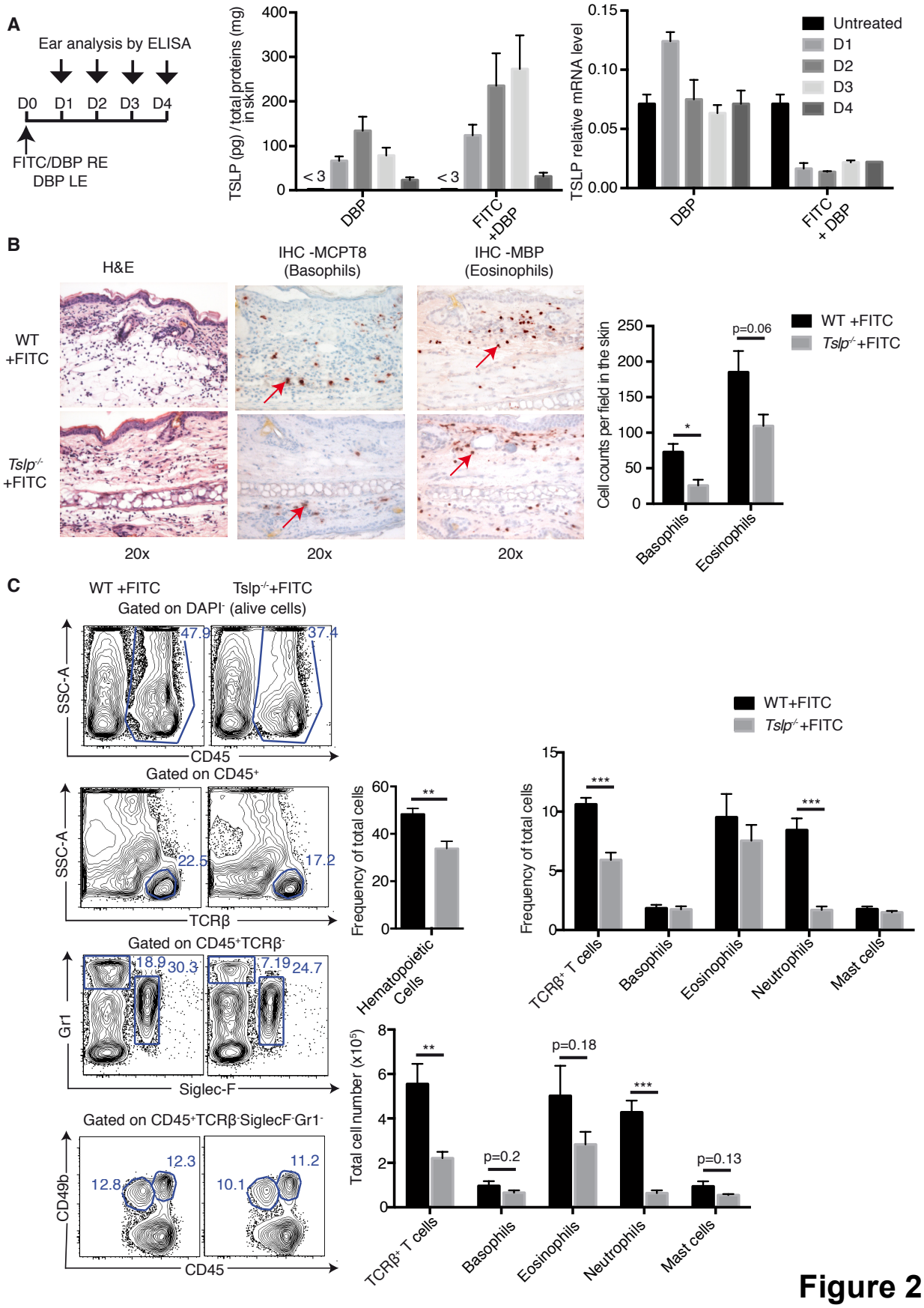

A
Ear analysis by ELISA
D0 D1 D2 D3 D4
FITC/DBP RE
DBP LE
TSLP (pg) / total proteins (mg) in skin
< 3
< 3
DBP
FITC +DBP
TSLP relative mRNA level
Untreated
D1
D2
D3
D4
DBP
FITC + DBP
B
H&E
IHC -MCPT8 (Basophils)
IHC -MBP (Eosinophils)
WT +FITC
Tslp-/- +FITC
20x
20x
20x
Cell counts per field in the skin
WT +FITC
Tslp-/- +FITC
p=0.06
*
Basophils
Eosinophils
C
WT +FITC
Tslp-/-+FITC
Gated on DAPI- (alive cells)
SSC-A
CD45
47.9
37.4
Gated on CD45+
SSC-A
TCRβ
22.5
17.2
Gated on CD45+TCRβ-
Gr1
Siglec-F
18.9
30.3
7.19
24.7
Gated on CD45+TCRβ-SiglecF-Gr1-
CD49b
CD45
12.8
12.3
10.1
11.2
Frequency of total cells
**
Hematopoietic Cells
WT+FITC
Tslp-/- +FITC
Frequency of total cells
***
***
TCRβ+ T cells
Basophils
Eosinophils
Neutrophils
Mast cells
Total cell number (x10^5)
**
p=0.2
p=0.18
***
p=0.13
TCRβ+ T cells
Basophils
Eosinophils
Neutrophils
Mast cells

Figure 2

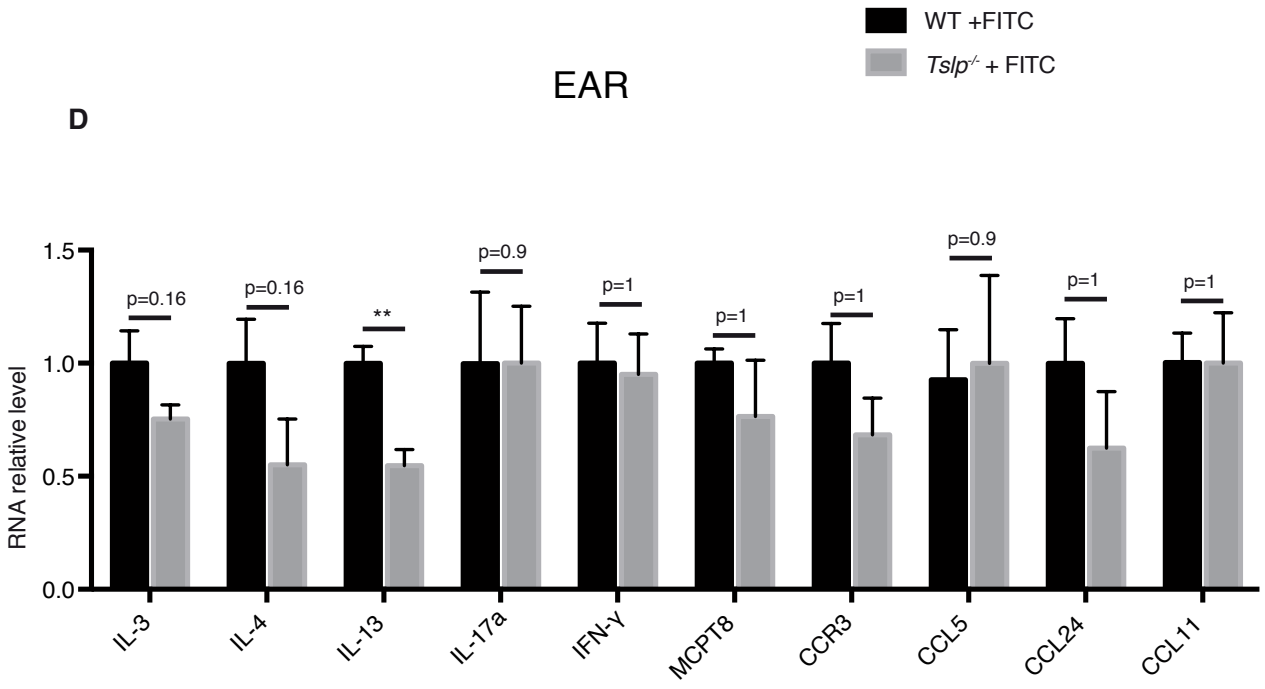
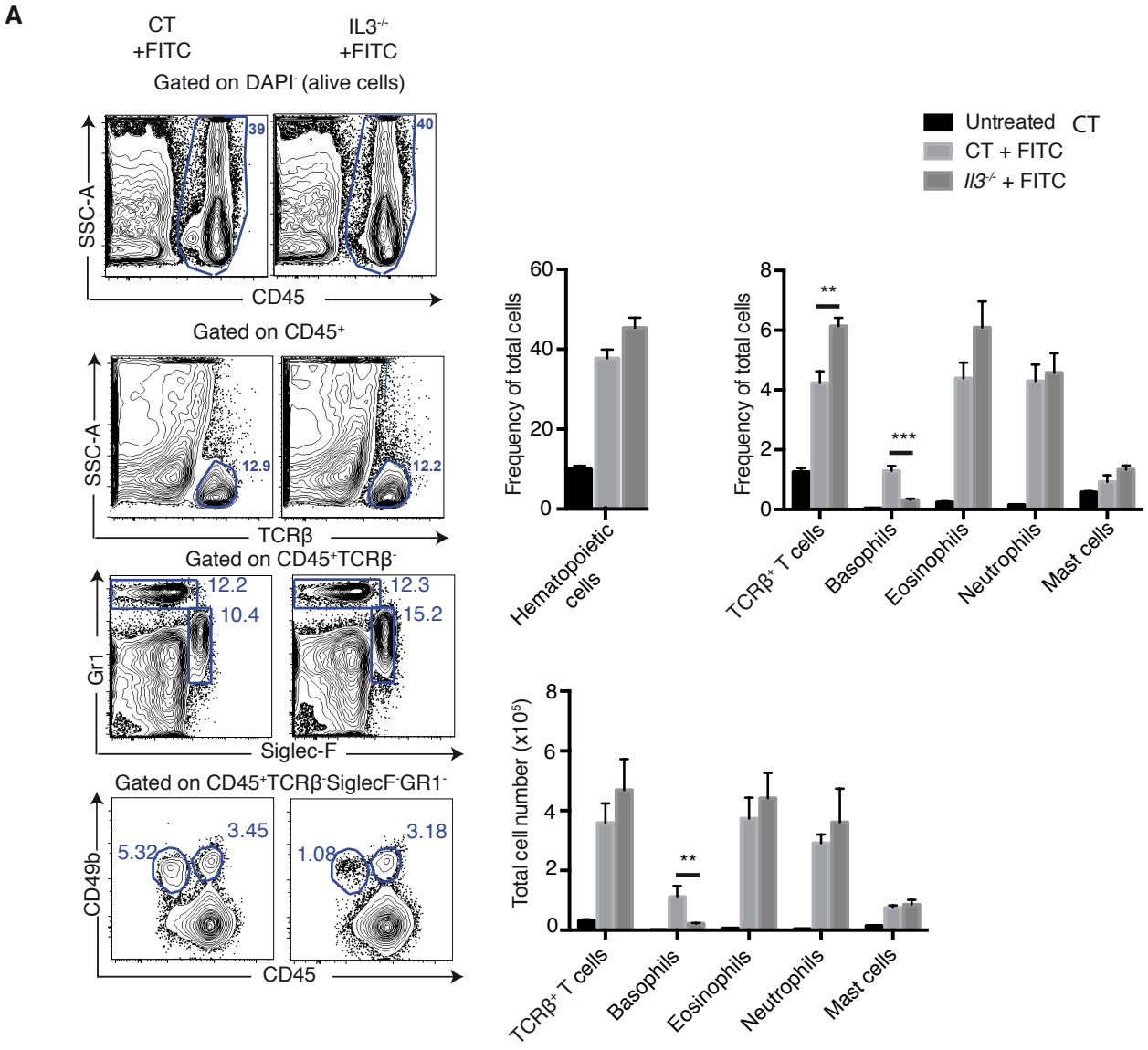

WT +FITC
Tslp-/- + FITC
EAR
D
RNA relative level
1.5
1.0
0.5
0.0
p=0.16
p=0.16
**
p=0.9
p=1
p=1
p=1
p=0.9
p=1
p=1
IL-3
IL-4
IL-13
IL-17a
IFN-Y
MCPT8
CCR3
CCL5
CCL24
CCL11

Figure 2

Figure 3. IL-3 plays a crucial role for basophil recruitment to FITC-induced ACD skin.

(**A**) FACS analyses of dermal cells of REs from FITC-treated *Il3*$^{-/-}$ and *Il3*$^{+/+}$ control (CT) littermate mice, for CD45^{+} hematopoietic cells, CD45^{+}TCRβ^{+} T cells, CD45^{low}CD49b^{+} (basophils), CD45^{hi}CD49b^{+} (mast cells), CD45^{+}TCRβ^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD45^{+}TCRβ^{-}Gr1^{hi} (neutrophils). **(B)** H&E and IHC staining of RE sections. Arrow points to one of the positive cells of IHC staining. Objectives used: 20x or 40x. Dashed circles outline blood vessels. **(C)** *Il3*$^{-/-}$ and CT mice were sensitized at D0, D1 and D2 on LEs and challenged at D6 on REs, which were analysed at D8, D9 and D10 for MCPT8 IHC staining. Arrow points to one of the positive cells. Objective used: 20x. Dashed circles outline blood vessels. **(D)** FACS analyses of blood cells from untreated CT, untreated *Il3*$^{-/-}$, FITC-treated CT and FITC-treated *Il3*$^{-/-}$ mice for CD19^{-}CD3^{-} Gr1^{lowneg}SiglecF^{-}IgE^{+} (basophils), CD19^{-}CD3^{-}Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD19^{-} CD3^{-}Gr1^{hi} (neutrophils). *P≤0.05 **P≤0.01, ***P≤0.001 (Student's t-test). Values are mean ± SEM (n≥3 mice per group).


A
CT
+FITC
IL3-/-
+FITC
Gated on DAPI- (alive cells)
39
40
SSC-A
CD45
Gated on CD45+
12.9
12.2
TCRβ
Gated on CD45+TCRβ-
12.2
10.4
12.3
15.2
Gr1
Siglec-F
Gated on CD45+TCRβ-SiglecF-GR1-
5.32
3.45
1.08
3.18
CD49b
CD45
Untreated CT
CT + FITC
Il3-/- + FITC
Frequency of total cells
Hematopoietic cells
TCRβ+ T cells
Basophils
Eosinophils
Neutrophils
Mast cells
**
***
Total cell number (x10^5)

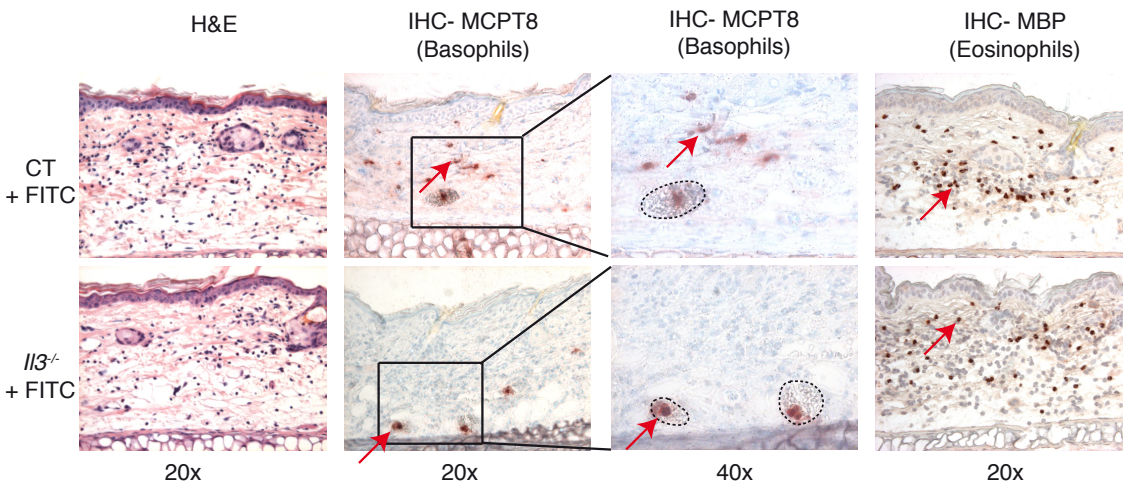

B
H&E
IHC- MCPT8
(Basophils)
IHC- MCPT8
(Basophils)
IHC- MBP
(Eosinophils)
CT
+ FITC
Il3-/-
+ FITC
20x
20x
40x
20x

Figure 3

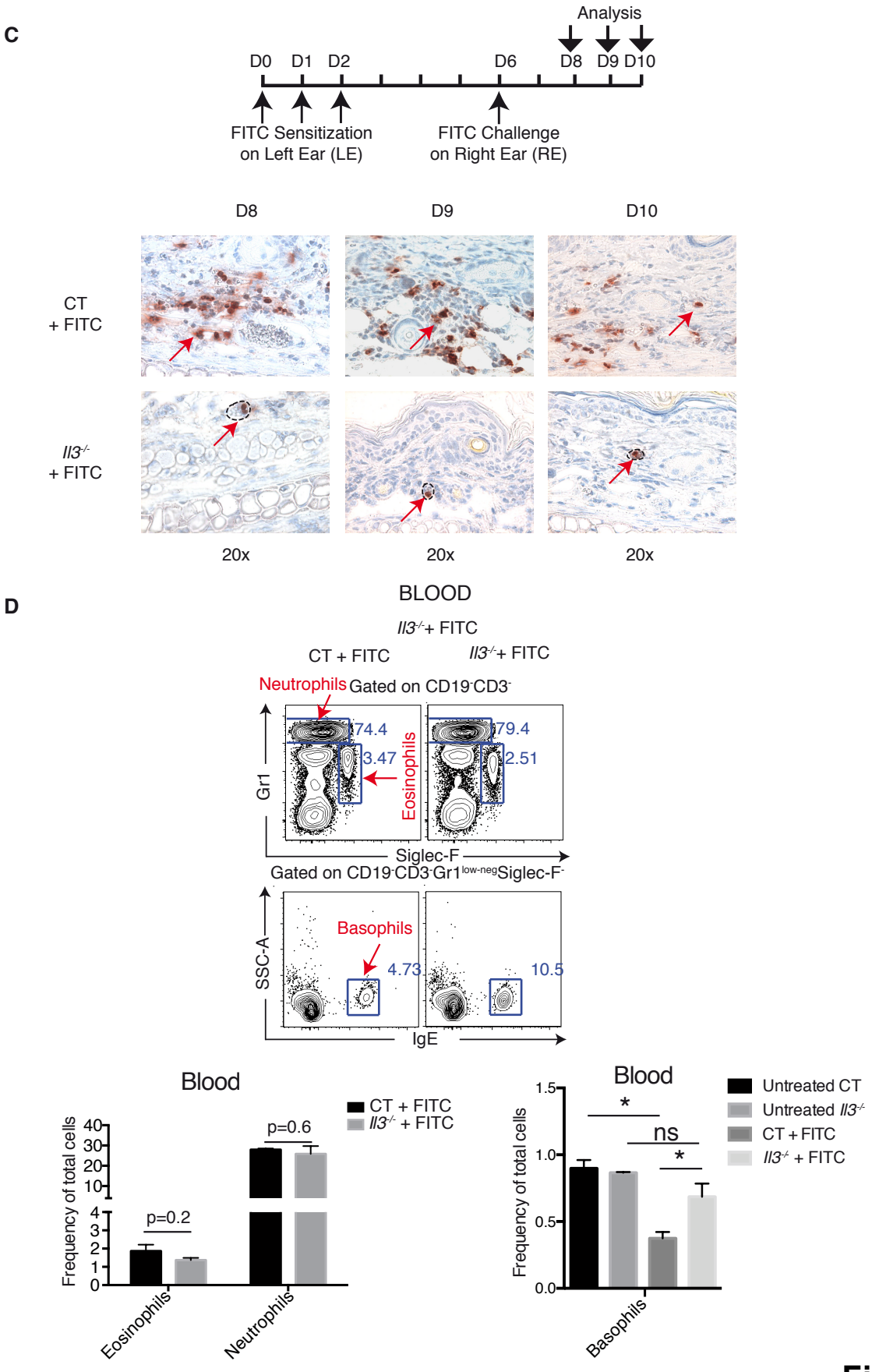

C
Analysis
D0 D1 D2 D6 D8 D9 D10
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
D8 D9 D10
CT + FITC
Il3-/- + FITC
20x 20x 20x
D
BLOOD
Il3-/- + FITC
CT + FITC
Il3-/- + FITC
Neutrophils
Gated on CD19-CD3-
74.4
3.47
79.4
2.51
Eosinophils
Gr1
Siglec-F
Gated on CD19-CD3-Gr1low-negSiglec-F-
Basophils
4.73
10.5
SSC-A
IgE
Blood
CT + FITC
Il3-/- + FITC
Frequency of total cells
p=0.6
p=0.2
Eosinophils
Neutrophils
Blood
Untreated CT
Untreated Il3-/-
CT + FITC
Il3-/- + FITC
*
ns
*
Frequency of total cells
Basophils

Figure 3

Figure 4. IL-3 is crucial for IL-4 and IL-13 expression by basophils but not T cells in FITC-induced ACD skin.

(A) RT-qPCR analyses of cytokines and chemokines in REs from untreated *Il3*$^{-/-}$, FITC-treated CT and FITC-treated *Il3*$^{-/-}$. (**B-C**) FACS analyses of AmCyan (IL-4) and dsRed (IL-13) expression by CD45^{+}TCRβ^{+} T cells (**B**) and CD45^{low}CD49b^{+} basophils (**C**) in the dermis of FITC-treated *Il3*$^{-/-}$/4C13R$^{Tg/0}$ and CT/4C13R$^{Tg/0}$ mice. Dermal cells from FITC-treated CT/4C13R$^{0/0}$ mice were included for setting the gates for Amcyan and dsRed. *P≤0.05, **P≤0.01, **P≤0.001 (Student's t-test). Values are mean ± SEM (n≥3 mice per group)

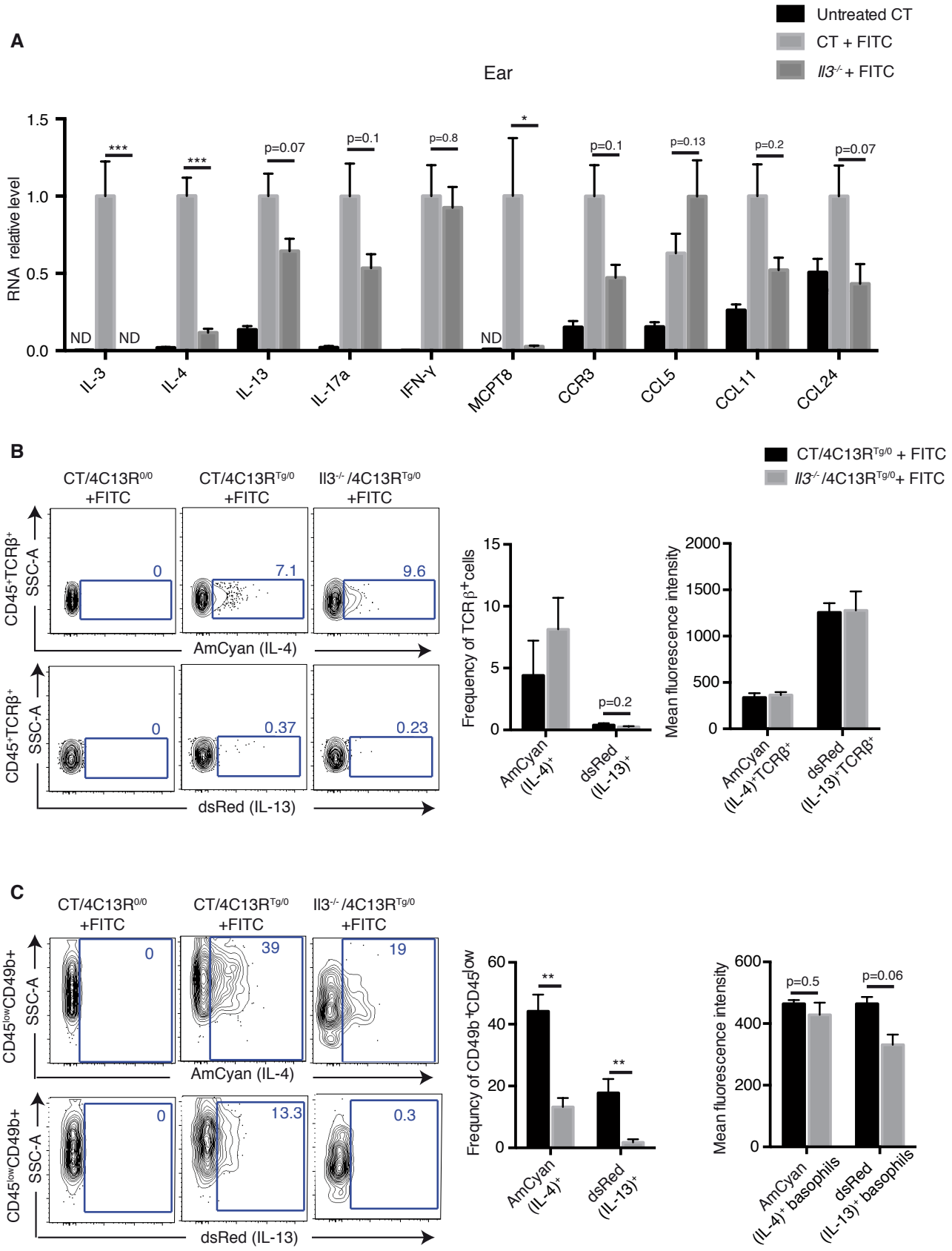

A
Untreated CT
CT + FITC
Il3-/- + FITC
Ear
RNA relative level
1.5
1.0
0.5
0.0
***
***
p=0.07
p=0.1
p=0.8
*
p=0.1
p=0.13
p=0.2
p=0.07
ND
ND
ND
IL-3
IL-4
IL-13
IL-17a
IFN-γ
MCPT8
CCR3
CCL5
CCL11
CCL24
B
CT/4C13R0/0 +FITC
CT/4C13RTg/0 +FITC
Il3-/- /4C13RTg/0 +FITC
CT/4C13RTg/0 + FITC
Il3-/- /4C13RTg/0+ FITC
CD45+TCRβ+ SSC-A
AmCyan (IL-4)
dsRed (IL-13)
0
7.1
9.6
0
0.37
0.23
Frequency of TCR β+cells
15
10
5
0
p=0.2
AmCyan (IL-4)+
dsRed (IL-13)+
Mean fluorescence intensity
2000
1500
1000
500
0
AmCyan (IL-4)+TCRβ+
dsRed (IL-13)+TCRβ+
C
CT/4C13R0/0 +FITC
CT/4C13RTg/0 +FITC
Il3-/- /4C13RTg/0 +FITC
CD45lowCD49b+ SSC-A
CD45lowCD49b+ SSC-A
AmCyan (IL-4)
dsRed (IL-13)
0
39
19
0
13.3
0.3
Frequency of CD49b+CD45low
60
40
20
0
**
**
AmCyan (IL-4)+
dsRed (IL-13)+
Mean fluorescence intensity
600
400
200
0
p=0.5
p=0.06
AmCyan (IL-4)+ basophils
dsRed (IL-13)+ basophils

Figure 4

Figure 5. No synergy between IL-3 and TSLP for basophil recruitment to ACD skin. **(A)** FACS analyses of dermal cells of REs from FITC-treated WT and FITC-treated *Tslp*$^{-/-}$/*Il3*$^{-/-}$ mice, for CD45^{+} hematopoietic cells, CD45^{+}TCRβ^{+} T cells, CD45^{low}CD49b^{+} (basophils), CD45^{hi}CD49b^{+} (mast cells), CD45^{+}TCRβ^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD45^{+}TCRβ^{-}Gr1^{hi} (neutrophils). (**B**) IHC staining of RE sections. Arrow points to one of the positive cells of IHC staining. Objective used: 20x. Cell counts for infiltrating basophils and eosinophils. Results were obtained by calculating the average number of positively stained cells per microscopic field (at 10x magnification; n=5 fields). Dashed circles outline blood vessels. **(C)** Frequency of basophils (CD19^{-}CD3^{-}Gr1^{lowneg}SiglecF^{-} CD45^{low}CD49b^{+}) in blood cells from FITC-treated WT and *Tslp*$^{-/-}$/*Il3*$^{-/-}$ mice. **(D)** RT-qPCR analyses of cytokines and chemokines in REs from FITC-treated WT and *Tslp*$^{-/-}$/*Il3*$^{-/-}$ mice. *P≤0.05 **P≤0.01, ***P≤0.001 (Student's t-test). Values are mean ± SEM (n≥3 mice per group).

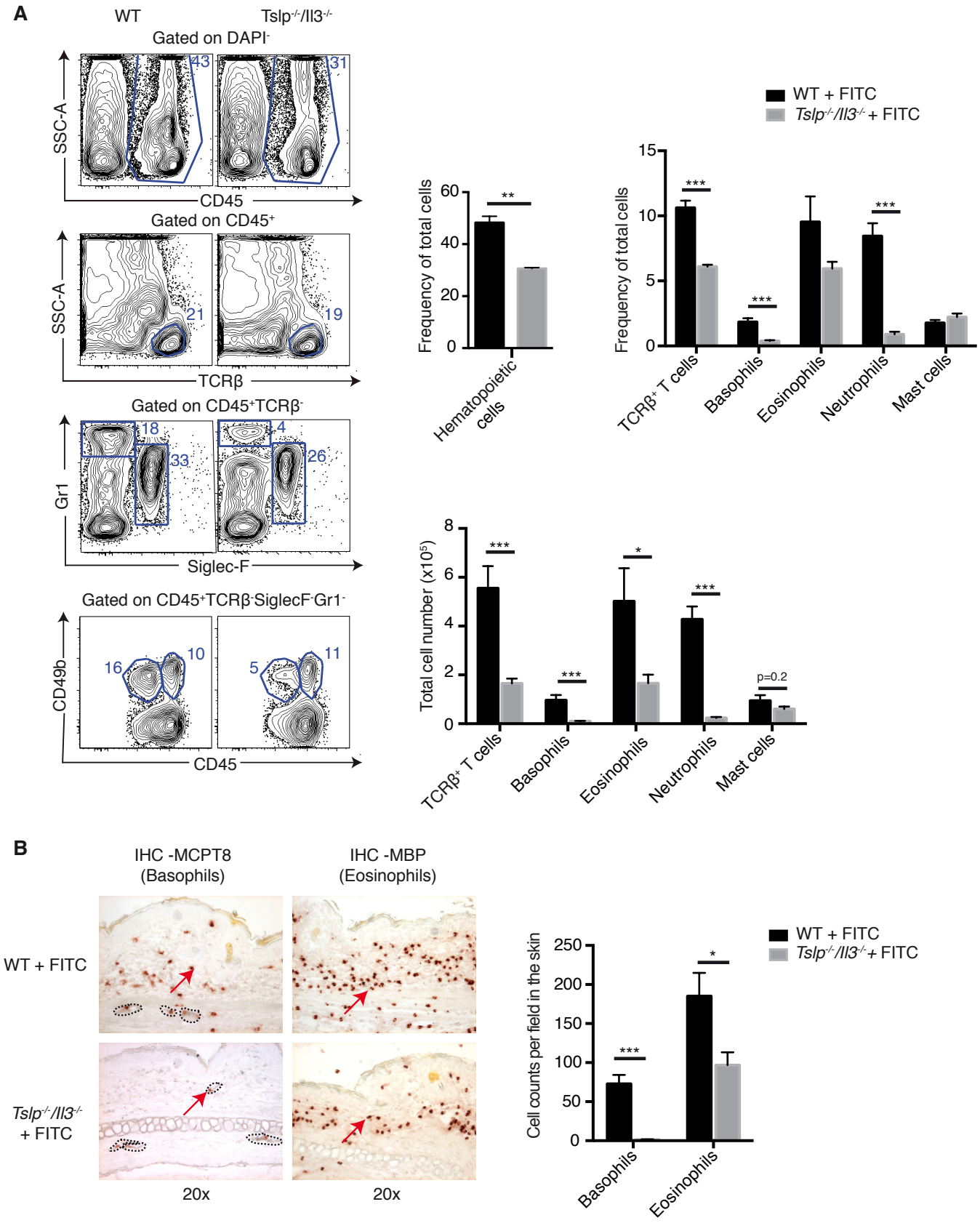

A
WT
Tslp-/-/Il3-/-
Gated on DAPI-
SSC-A
CD45
43
31
Gated on CD45+
21
19
TCRβ
Gated on CD45+TCRβ-
Gr1
Siglec-F
18
33
4
26
Gated on CD45+TCRβ-SiglecF-Gr1-
CD49b
16
10
5
11
WT + FITC
Tslp-/-/Il3-/- + FITC
Frequency of total cells
Hematopoietic cells
TCRβ+ T cells
Basophils
Eosinophils
Neutrophils
Mast cells
Total cell number (x10^5)
p=0.2
B
IHC -MCPT8 (Basophils)
IHC -MBP (Eosinophils)
20x
Cell counts per field in the skin

Figure 5

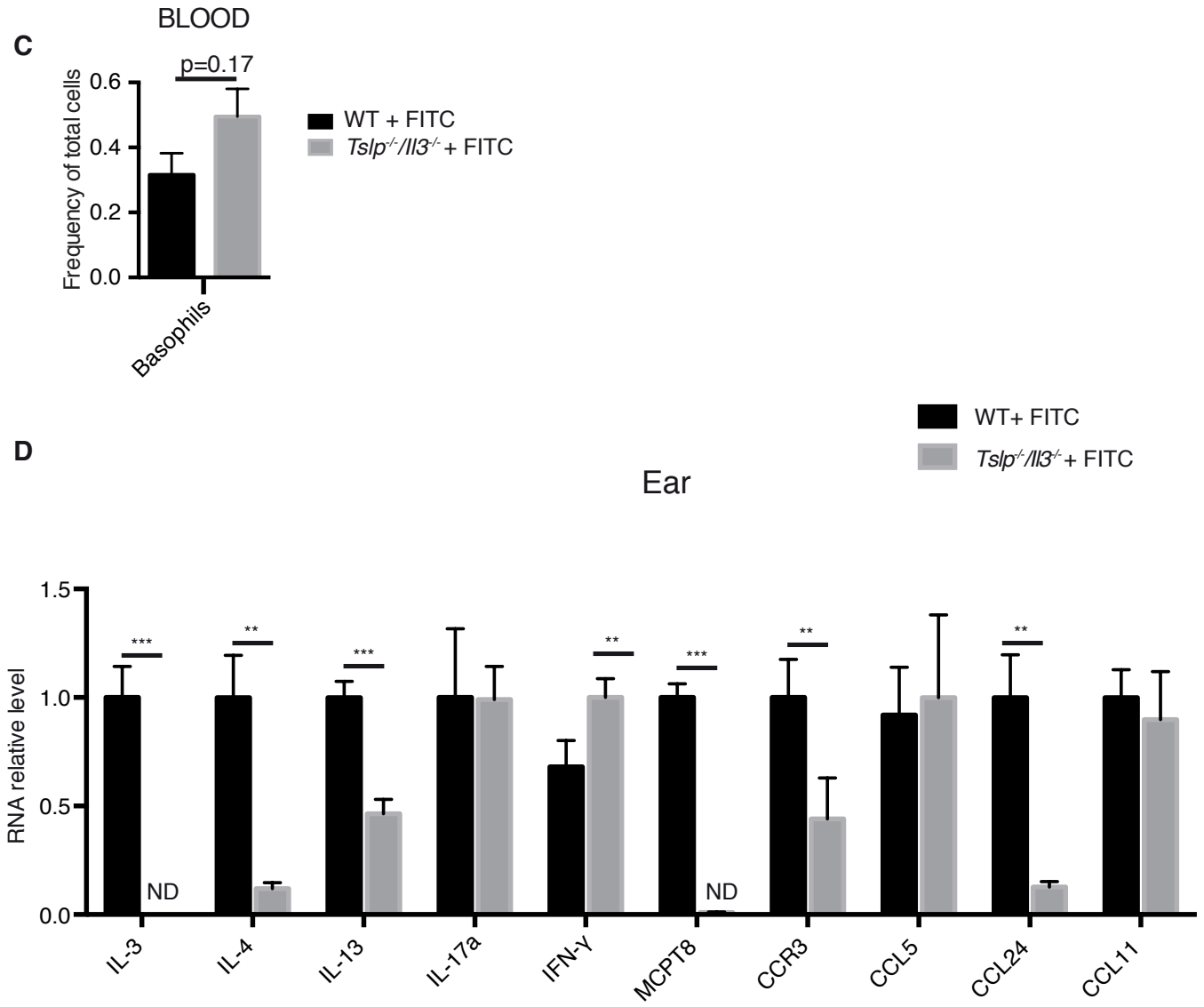

C
BLOOD
p=0.17
Frequency of total cells
0.6
0.4
0.2
0.0
Basophils
WT + FITC
Tslp-/-/Il3-/- + FITC
D
WT+ FITC
Tslp-/-/Il3-/- + FITC
Ear
RNA relative level
1.5
1.0
0.5
0.0
***
**
***
**
***
**
**
ND
ND
IL-3
IL-4
IL-13
IL-17a
IFN-γ
MCPT8
CCR3
CCL5
CCL24
CCL11

Figure 5

Figure 6. IL-3 produced by T cells mediates basophil recruitment to FITC-induced ACD skin.

(**A**) Intracellular staining of IL-3 in dermal cells of REs from untreated or FITC-treated Balb/c WT mice, with or without (w/o) stimulation of PMA and ionomycin. (**B**) IHC staining with MCPT8 antibody in RE of FITC-treated *CD4-Cre*$^{0/0}$*/Il3*$^{L2/L2}$ (CT) and *CD4-Cre*$^{Tg/0}$*/Il3*$^{L2/L2}$ mice. Arrow points to one of the positive cells of IHC staining. Objective used: 20x. Dashed circles outline blood vessels (**C**) FACS analyses of dermal cells of REs from FITC-treated *CD4-Cre*$^{Tg/0}$*/Il3*$^{L2/L2}$ and CT mice, for CD45^{+} hematopoietic cells, CD45^{+}TCRβ^{+} T cells, CD45^{low}CD49b^{+} (basophils), CD45^{hi}CD49b^{+} (mast cells), CD45^{+}TCRβ^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD45^{+}TCRβ^{-}Gr1^{hi} (neutrophils). (**D**) FACS analyses of blood cells from FITC-treated *CD4-Cre*$^{Tg/0}$*/Il3*$^{L2/L2}$ and CT mice for CD19^{-}CD3^{-}Gr1^{lowneg}SiglecF^{-}IgE^{+} (basophils), CD19^{-}CD3^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ (eosinophils), and CD19^{-}CD3^{-}Gr1^{hi} (neutrophils).

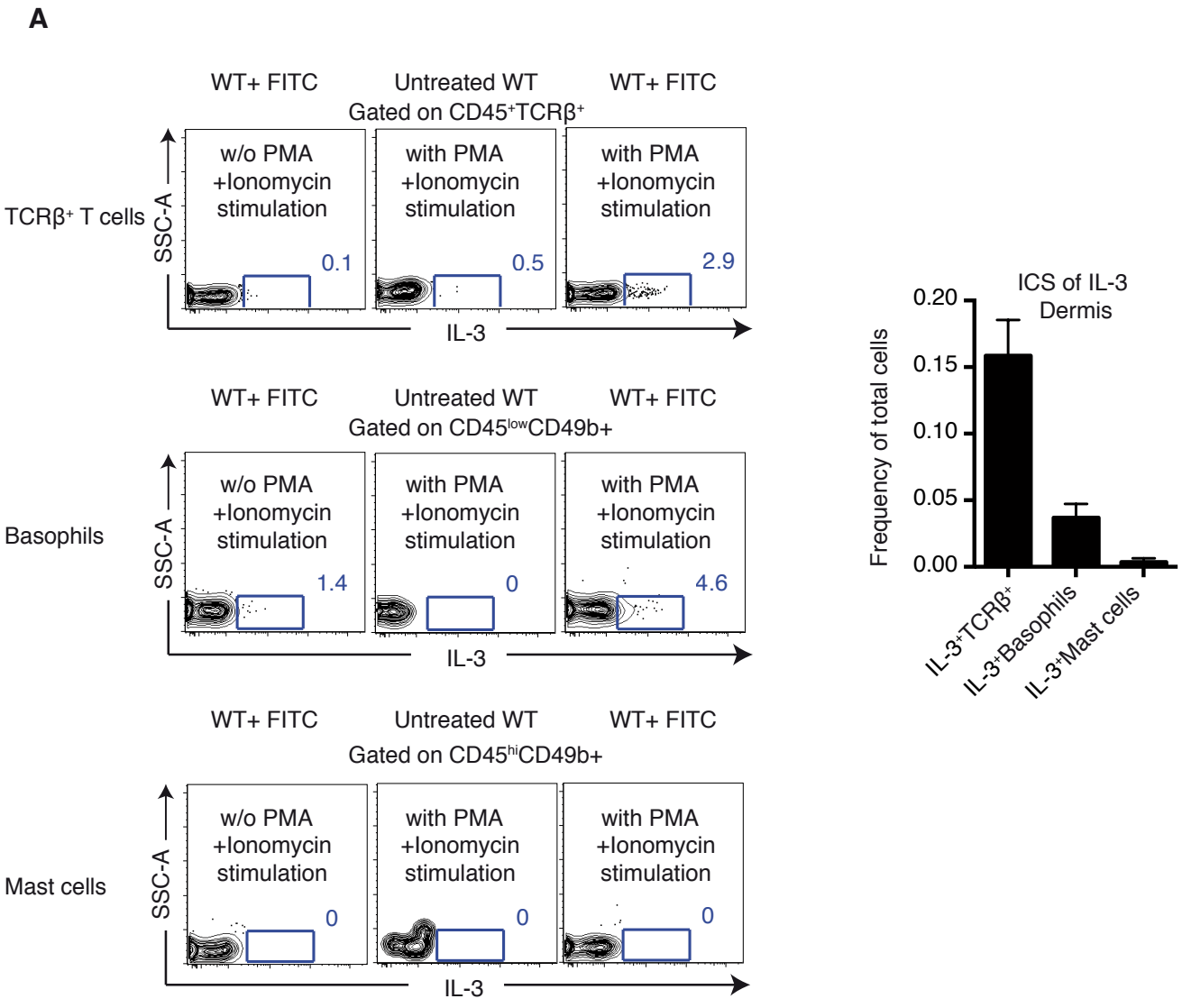

A
WT+ FITC
Untreated WT
WT+ FITC
Gated on CD45+TCRβ+
TCRβ+ T cells
SSC-A
w/o PMA +Ionomycin stimulation
with PMA +Ionomycin stimulation
with PMA +Ionomycin stimulation
0.1
0.5
2.9
IL-3
Gated on CD45lowCD49b+
Basophils
1.4
0
4.6
Gated on CD45hiCD49b+
Mast cells
0
0
0
ICS of IL-3
Dermis
Frequency of total cells
0.20
0.15
0.10
0.05
0.00
IL-3+TCRβ+
IL-3+Basophils
IL-3+Mast cells

Figure 6

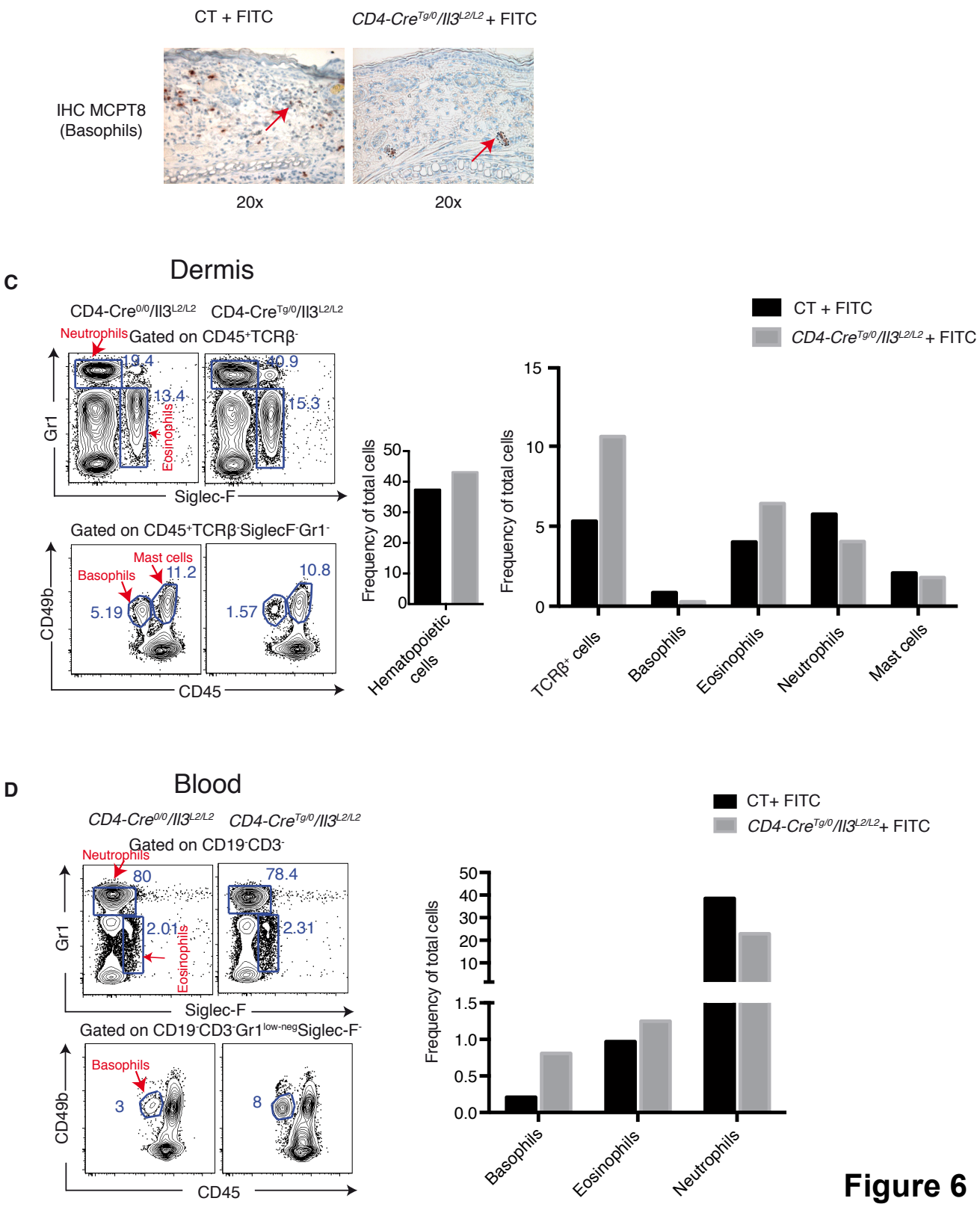
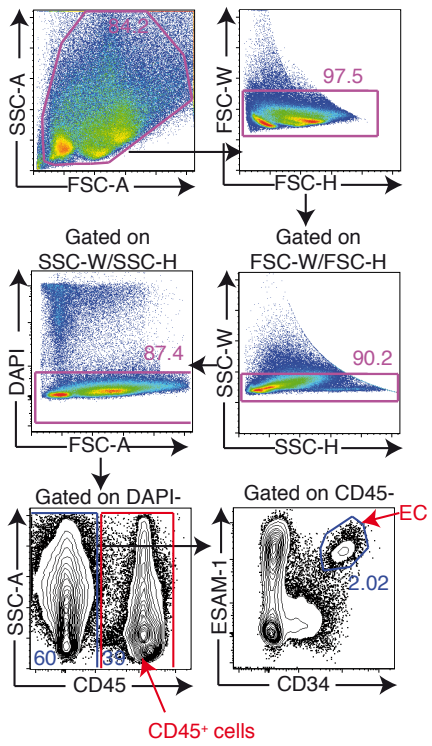

B
CT + FITC
CD4-Cre^Tg/0/Il3^L2/L2 + FITC
IHC MCPT8 (Basophils)
20x
20x
C
Dermis
CD4-Cre^0/0/Il3^L2/L2
CD4-Cre^Tg/0/Il3^L2/L2
Neutrophils
Gated on CD45+TCRβ-
19.4
13.4
Eosinophils
40.9
15.3
Gr1
Siglec-F
Gated on CD45+TCRβ-SiglecF-Gr1-
Mast cells
Basophils
11.2
5.19
10.8
1.57
CD49b
CD45
CT + FITC
CD4-Cre^Tg/0/Il3^L2/L2 + FITC
Frequency of total cells
Hematopoietic cells
TCRβ+ cells
Basophils
Eosinophils
Neutrophils
Mast cells
D
Blood
CD4-Cre^0/0/Il3^L2/L2
CD4-Cre^Tg/0/Il3^L2/L2
Gated on CD19-CD3-
Neutrophils
80
2.01
Eosinophils
78.4
2.31
Gr1
Siglec-F
Gated on CD19-CD3-Gr1low-negSiglec-F-
Basophils
3
8
CD49b
CD45
CT+ FITC
CD4-Cre^Tg/0/Il3^L2/L2+ FITC
Frequency of total cells
Basophils
Eosinophils
Neutrophils

Figure 6

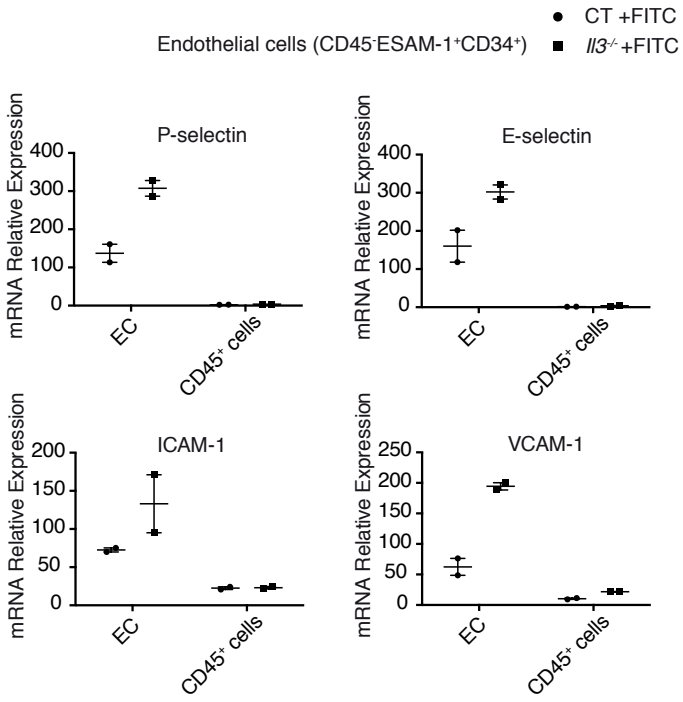
Figure 7. Decreased expression of integrins by basophils sorted from FITC-treated *Il3*$^{-/-}$ skin.

(**A**) Gating strategy for sorting of CD45^{-}ESAM^{-}1+CD34^{+} endothelial cells (ECs) and CD45^{+} hematopoietic cells from RE dermis of FITC-treated CT and *Il3*$^{-/-}$ mice. (**B**) RT-qPCR analyses of the sorted ECs and CD45^{+} cells. (**C**) RT-qPCR analyses of the sorted basophils, neutrophils, eosinophils and TCRβ^{+} T cells. *P≤0.05 **P≤0.01 (Student's t-test). Values are mean ± SEM (n≥2 mice per group).

A



B



C

Alpha chain	Beta chain	Heterodimer	Ligand
ItgαL (CD11a)	Itgβ2 (CD18)	αL/β2 (CD11a/CD18) Also called : LFA-1 Lymphocyte function-associated antigen-1	ICAM-1 ICAM-2 ICAM-3
Itgαm (CD11b)	Itgβ2 (CD18)	αM/β2, (CD11b/CD18) Also called : MAC-1 Macrophage antigen-1	ICAM-1 ICAM-2 ICAM-3
Itgα2b	Itgβ3	α2b/β3	fibronectin, fibrinogen, plasminogen, prothrombin, thrombospondin and vitronectin
Itgα4 (CD49d)	Itgβ7	α4/β7, (CD49d/ β7) Also called : LPAM-1	VCAM-1, MAdCAM-1
IgαE (CD103)	Itgβ7	αE/β7, (CD103/ β7)	E-cadherin
Itgα5	Itgβ1 (CD29)	α5/β1, (α5/CD29)	Fibronectin and fibrinogen
Itgα4 (CD49d)	Itgβ1 (CD29)	α4/β1, (CD49d/CD29) Also called : VLA-4 Very late antigen-4	VCAM-1
Itgα2	Itgβ1 (CD29)	α2/β1, (α2/CD29)	Collagen, laminin, fibronectin E-cadherin
Itgα1	Itgβ1 (CD29)	α1/β1, (α1/CD29)	Laminin, collagen
ItgαV	Itgβ5	αV/β5	Fibronectin

Figure 7

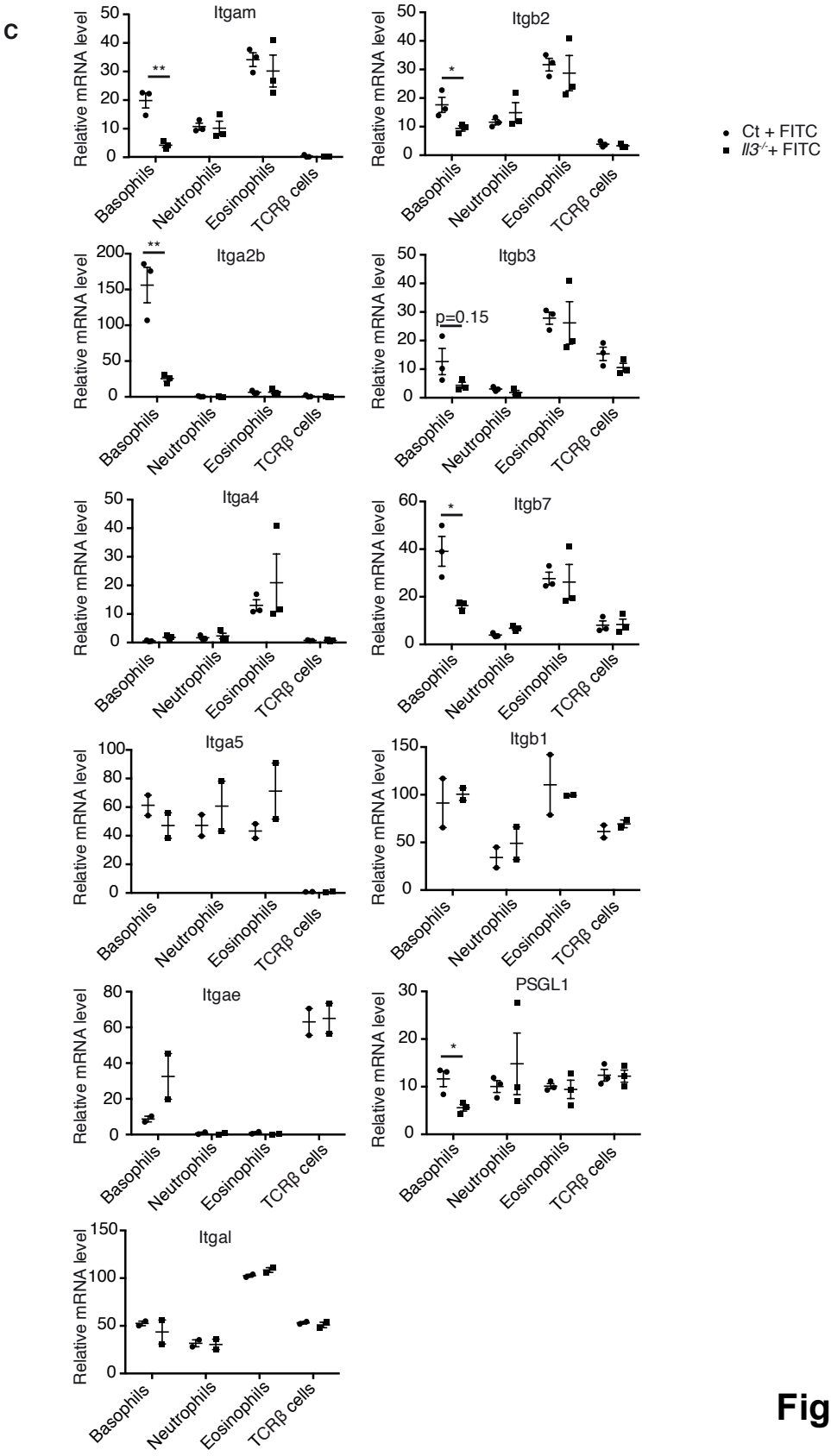
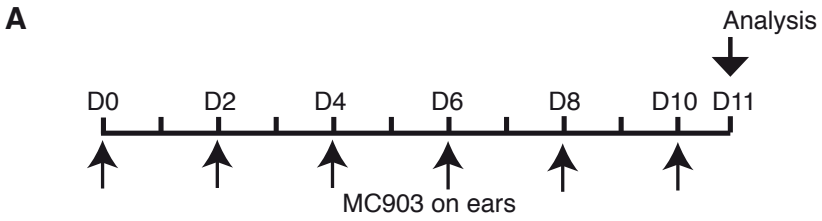
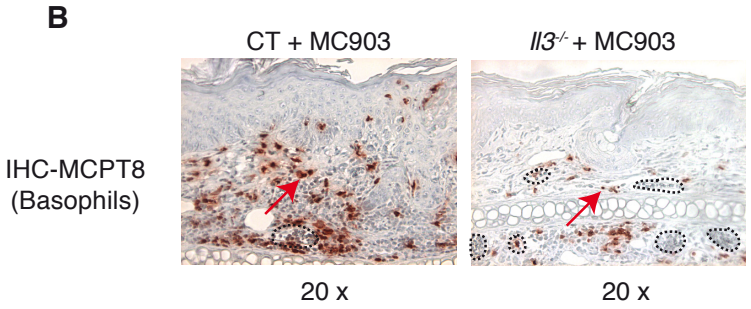

C
Itgam
Itgb2
Itga2b
Itgb3
p=0.15
Itga4
Itgb7
Itga5
Itgb1
Itgae
PSGL1
Itgal
Relative mRNA level
Basophils
Neutrophils
Eosinophils
TCRβ cells
Ct + FITC
Il3-/- + FITC

Figure 7

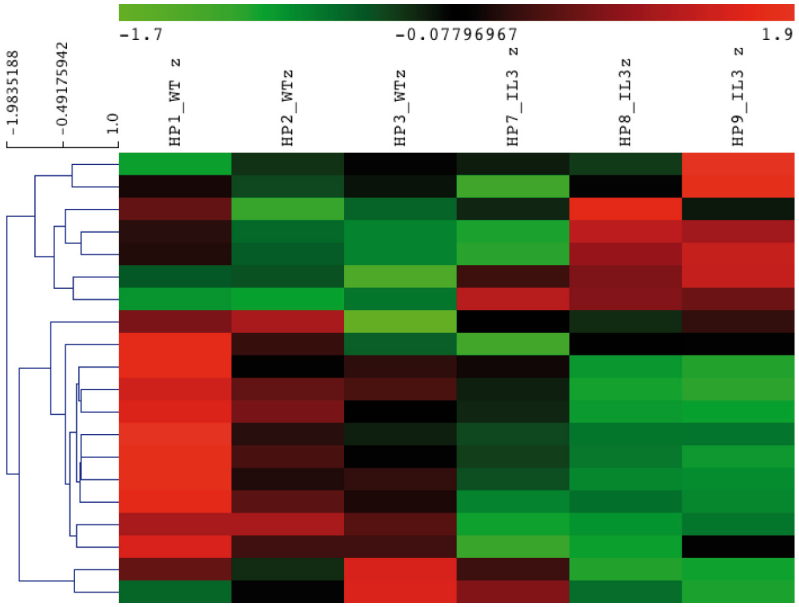
Figure 8. Analyses of integrin expression in basophils sorted from MC903-induced atopic dermatitis (AD) skin.

(**A**) Experimental protocol. Mouse ears were topically treated with MC903 in ethanol every other day from D0 to D10 and analysed at D11. (**B**) IHC staining of basophils in RE of MC903-treated *Il3*$^{-/-}$ and Balb/c wildtype control (CT) mice. Arrow points to one of the positive cells of IHC staining. Objective used: 20x. Dashed circles outline blood vessels. (**C**) Heatmap generated with MeV software, showing the comparison of the expression of PSGL-1 (encoded by gene Selplg) and integrins in basophils sorted from MC903-treated CT and *Il3*$^{-/-}$ mouse dermis (n=3 per group). The expression of genes was determined by Z score=[Value-Mean]/[Standard deviation]. Integrin genes shown in the heatmap are those with the value of "normalized and divided by gene length in kb" >50. Integrins with values <50 were considered as low expression and were not included in the heatmap (Itg3bp; Itgad; Itga11; Itgb1bp2; Itgax; Itgb8; Itgb2l; Itga10; Itga7; Itga3; Itgb4 and Itgb6). P-values from the comparison of two groups of RNA-seq data and adjusted p-values calculated using the Benjamini and Hochberg method (Benjamini, 1995) are shown. (**D**) RT-qPCR analyses of the sorted basophils.





C



	p-value	Adjusted p-value
Itga6	0.38	0.9
Itgav	0.7	0.9
Itga9	0.4	0.9
Itga5	0.5	0,9
Itgbl1	0.7	0.11
Itga8	0.001	0.06
Itga4	0.0002	0.02
Itgb5	0.9	0.9
Itgb1bp1	0.1	0.7
Itgal	0.006	0.1
Itgam	0.0005	0.03
Itgb7	0.0004	0.03
Itgb1	0.04	0.4
Itgb2	0.004	0.1
Itgb3	0.009	0.1
Selplg (PSGL-1)	7E-7	0.0004
Itgae	1.8E-14	1.2E-10
Itga1	0.006	0.1
Itga2	0.1	0.6
Itga2b	0.5	0.9

Figure 8

D

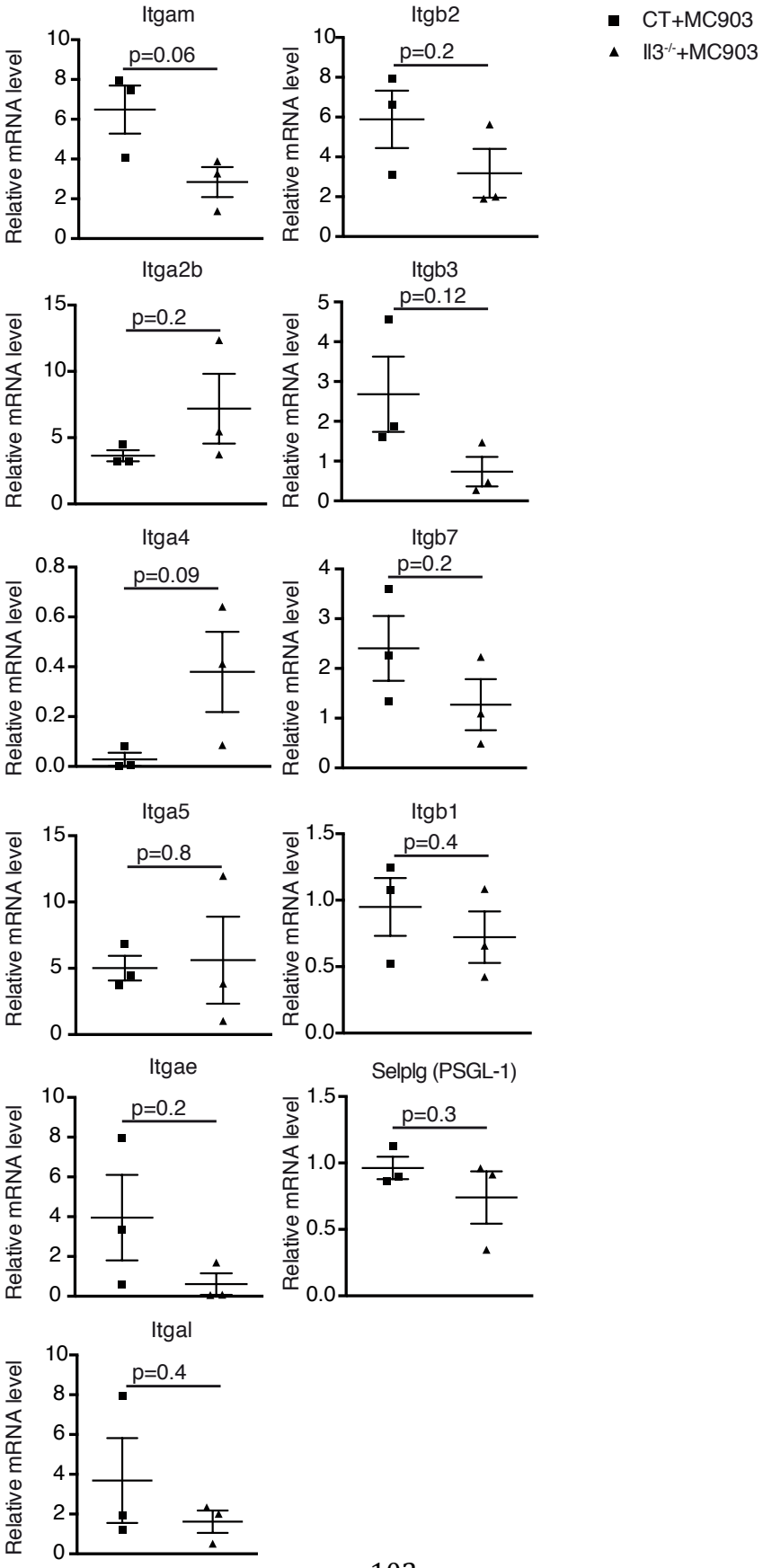

CT+MC903
Il3-/-+MC903
Itgam
p=0.06
Itgb2
p=0.2
Itga2b
p=0.2
Itgb3
p=0.12
Itga4
p=0.09
Itgb7
p=0.2
Itga5
p=0.8
Itgb1
p=0.4
Itgae
p=0.2
Selplg (PSGL-1)
p=0.3
Itgal
p=0.4
Relative mRNA level

Figure 8

Figure 9. All-trans RA treatment prior to FITC challenge promotes basophil recruitment to ACD skin.

(A) RT-qPCR analyses of ALDH1A3 in basophils, neutrophils, eosinophils and T cells sorted from FITC-treated Il3$^{-/-}$ and CT ears. (**B**) RNA-seq data showing the value of "normalised and divided by gene length in kb" for ALDH1A3 in the sorted basophils from MC903-treated *Il3*$^{-/-}$ and CT mice. **(C)** *Il3*$^{-/-}$ and CT mice were sensitized with 1% FITC (in DPB:acetone) on LE at Day D0, D1 and D2. Right ears (RE) were then challenged at D6 with 1% FITC (in DPB:acetone) as described in Fig. 1A. Mice were topically treated with all-trans retinoic acid (at-RA) on RE 2h before FITC-challenge or intraperitoneally (i.p.) injected 24h before FITC-challenge. RE sections from *Il3*$^{-/-}$ and CT mice were used for MCPT8 IHC. Blue arrows point to one of the positive cells outside of blood vessels; green arrows point to one of the positive cells inside blood vessels. Objective used: 20x. Dashed circles outline blood vessels. A comparison of total number of positive signals inside and outside of blood vessels from one ear section is shown.

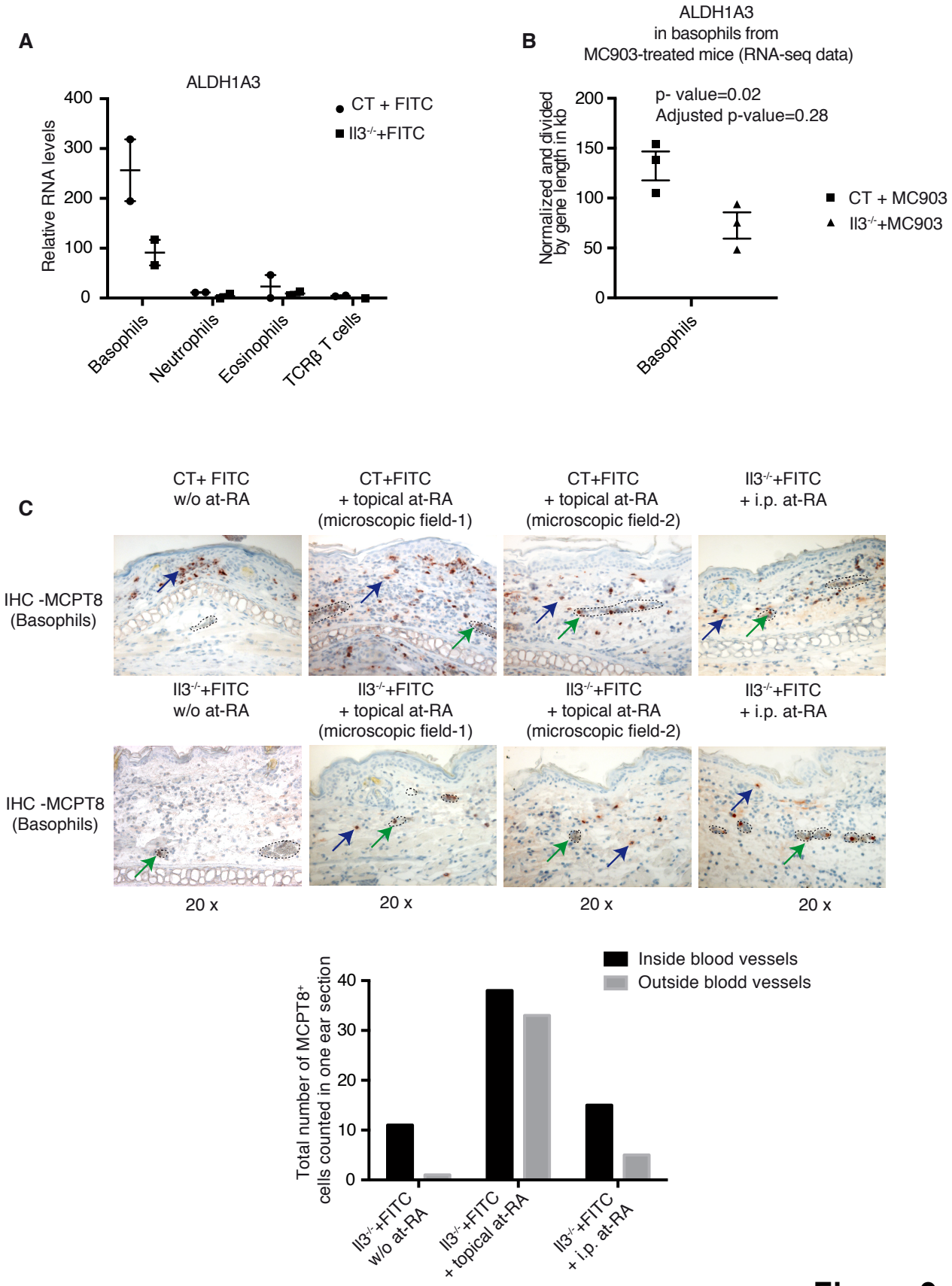

A
ALDH1A3
Relative RNA levels
400
300
200
100
0
CT + FITC
Il3-/-+FITC
Basophils
Neutrophils
Eosinophils
TCRβ T cells
B
ALDH1A3
in basophils from
MC903-treated mice (RNA-seq data)
p- value=0.02
Adjusted p-value=0.28
Normalized and divided by gene length in kb
200
150
100
50
0
CT + MC903
Il3-/-+MC903
Basophils
C
CT+ FITC
w/o at-RA
CT+FITC
+ topical at-RA
(microscopic field-1)
CT+FITC
+ topical at-RA
(microscopic field-2)
Il3-/-+FITC
+ i.p. at-RA
IHC -MCPT8
(Basophils)
Il3-/-+FITC
w/o at-RA
Il3-/-+FITC
+ topical at-RA
(microscopic field-1)
Il3-/-+FITC
+ topical at-RA
(microscopic field-2)
Il3-/-+FITC
+ i.p. at-RA
IHC -MCPT8
(Basophils)
20 x
20 x
20 x
20 x
Inside blood vessels
Outside blodd vessels
Total number of MCPT8+
cells counted in one ear section
40
30
20
10
0
Il3-/-+FITC
w/o at-RA
Il3-/-+FITC
+ topical at-RA
Il3-/-+FITC
+ i.p. at-RA

Figure 9

Figure 10. A schematic representation of our hypothesis on FITC-induced basophil extravasation to ACD skin.

Upon FITC-treatment, $CD4^+$ T cells secrete IL-3 (**1**), which binds to IL-3 receptor complex on basophils (**2**), and induces expression of ALDH1A2 or ALDH1A3 (**3**), allowing the production of RA by basophils (**4**). In turn, RA secreted by basophils activates RAR/RXR receptor heterodimer in an autocrine manner, which stimulates the expression of PSGL-1, ITGAM, ITGB2, ITGA2B, ITGB7 and others by basophils (**5**). This results in the interaction between basophils and endothelial cells, leading to the extravasation of basophils to ACD skin through rolling, adhesion and trans-endothelial migration (TEM) (**6**).

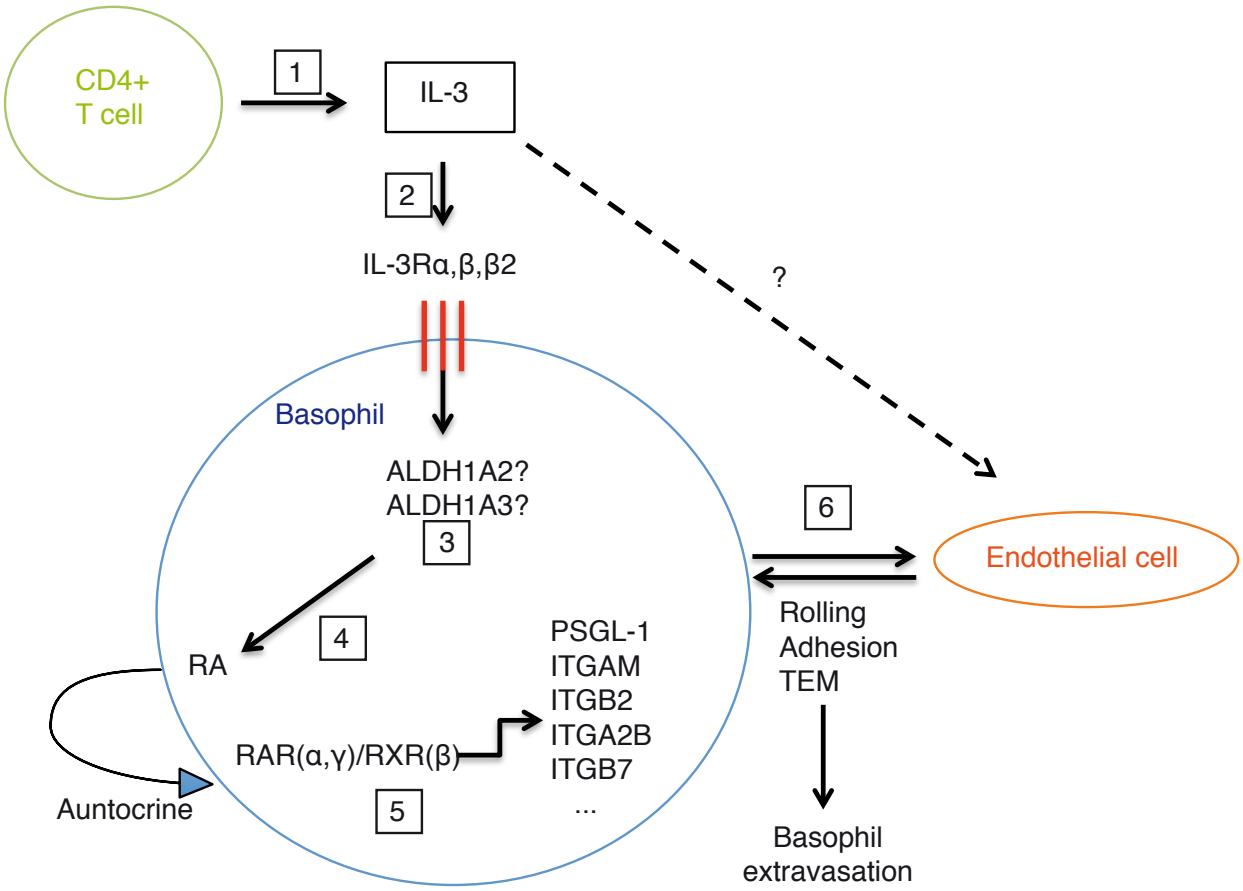


Figure 10

Part 2

Depletion of basophils in Mcpt8DTR mice leads to a systemic reduction of eosinophils and neutrophils

Carole EL Hachem et al. (manuscript in preparation).

Abstract

Mice expressing the diphtheria toxin receptor (DTR) in specific cell types are key tools for functional immunology studies. MCPT8^{DTR} mice express the DTR under the control of MCPT8 gene promoter, allowing an inducible depletion of basophils, a characteristic cellular component in allergy. Using this *in vivo* tool, we found that basophil depletion led to a decrease of eosinophils and neutrophils in allergic contact dermatitis (ACD) induced by hapten sensitization and challenge. Although such finding appeared to fit with a recent report that basophils play a critical role in eosinophil recruitment to skin, other lines of evidence we obtained contradicted this conclusion. Our further investigation found that DT injection actually caused a systemic reduction of eosinophils and neutrophils in blood, spleen and bone marrow of MCPT8^{DTR} mice. By performing a kinetic characterization, we revealed that the systemic reduction of eosinophils and neutrophils is time-dependent. Furthermore, we showed that hematopoietic stem cell-derived granulocyte-macrophage progenitors (GMPs), which give rise to neutrophils, eosinophils and basophils, were also depleted upon DT injection, possibly due to the activity of MCPT8 gene promoter in these cells. Finally, we identified a lower dose of DT that could deplete efficiently basophils but not GMPs, which further evidenced that basophils were not essential for eosinophil or neutrophil recruitment in ACD skin. Together, our study adds new insights towards a faithful recognition of the role of basophils in tissue inflammation. It also alerts that the interpretation based on MCPT8 promoter-driven DTR/DTA tools should be carefully (re)-evaluated, and points out several cautions, which should be taken for using DTR-based mouse tools in functional immunology studies.

Introduction

Basophils are one characteristic cell type in Type 2 inflammation including parasite infection and allergies. They account for less that 1% of blood leukocytes and were initially neglected and considered minor compared to tissue-resident mast cells, with which they share certain features such as the expression of the high-affinity IgE receptor (FcεRI) and the release of allergic mediators such as histamine and leukotrienes (Borriello et al., 2014; Karasuyama et al., 2011). The study of basophils had long been hampered due to their rarity and the lack of tools for their detection and functional analysis. During the last decade, tools have been developed for the *in vivo* study of basophil function. These include 1) depleting antibodies MAR-1 (specific to FcεRI) (Denzel et al., 2008) and Ba103 (specific to CD200R3) (Obata et al., 2007); and 2) genetically engineered mice such as mouse lines expressing the diphtheria toxin receptor (DTR) under the control of the mast cell protease 8 (MCPT8) promoter which was shown to be basophil specific (Poorafshar et al., 2000; Ugajin et al., 2009), thus allowing a selective depletion of basophils upon DT injection (Wada et al., 2010), as well as Basoph8xDTA (MCPT8-Cre x ROSA-DTA) mice for a constitutive depletion of basophils (Sullivan et al., 2011). These mouse lines have been widely used as powerful tools for elucidating the role of basophils in fundamental immune pathologies such as tick infestation (Wada et al., 2010), IgE-mediated allergic inflammation (Cheng et al., 2015; Egawa et al., 2013), atopic dermatitis (Leyva-Castillo et al., 2013), eosinophilic esophagitis (Venturelli et al., 2016), helminth infection (S. Kim et al., 2013) and anaphylaxis (Reber et al., 2013), highlighting important roles of basophils in the development of Th2 immune responses and in the crosstalk with other cells at the site of inflammation.

Our initial aim of this study was to investigate the role of basophils in allergic skin inflammation. To this aim, we employed an experimental protocol to induce allergic contact dermatitis (ACD), in which skin is sensitized and challenged with a hapten fluorescein isothiocyanate (FITC), leading to abundant recruitment of basophils, accompanied by Th2 cells, eosinophils and neutrophils. For basophil depletion, $MCPT8^{DTR}$ mice were injected with DT (named $MCPT8^{DEP}$) and subjected to FITC

treatment. Of interest, we observed that the depletion of basophils led to a decrease for eosinophils and neutrophils in ACD skin, which suggested that basophils were responsible of eosinophil and neutrophil recruitment to the skin. This observation appeared to be a good fit with a recent publication, concluding that basophils play a critical role for eosinophil recruitment in IgE-dependent allergic inflammation using MCPT8-Cre x ROSA-DTA (Cheng et al., 2015). However, other evidence that we obtained contradicted this conclusion, which led us to perform a careful characterization of DT-injected MCPT8^{DTR} mice. In this paper, we report our surprising finding on a time-dependent systemic reduction of eosinophils and neutrophils in these mice, and then how we dig out that in addition to basophils, granulocyte-macrophage progenitors (GMPs) could be depleted upon DT injection, thus providing a plausible explanation of our observations. We also report how we sought to achieve a specific depletion of basophils but not GMPs, by which we conclude that basophils do not play an essential role in recruiting eosinophils and neutrophils in allergic skin.

METHODS

Mice

Balb/c mice were purchased from Charles River Laboratories. MCPT8^{DTR} mice were as described (Wada et al., 2010). 4C13R dual reporter mouse (Roediger et al., 2013) were kindly provided by Dr. William E Paul (NIH, USA). Both mouse lines have been backcrossed to Balb/c background (>99.5%). Eight to twelve-week-old female mice were used for experiments. Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the animal care and ethic committee of animal experimentation of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) and Institut Clinique de la Souris (ICS).

Basophil depletion in mice

For DT-mediated depletion of basophils, MCPT8^{DTR} mice received an i.p. injection of DT (100ng, 300ng, 750ng or 1µg per 25g body weight), as indicated in experimental schemes in figures.

For antibody-mediated depletion of basophils, wild-type Balb/c mice were injected i.p. twice daily for 3 days from D1 to D3 with 5 µg of Armenian hamster-anti-mouse FcεRIα antibody (clone MAR-1, eBioscience) or isotype antibody control (Armenian hamster IgG, clone eBio299Arm, eBioscience) (Denzel et al., 2008).

FITC treatment

FITC (≥97.5% HPLC) (Sigma) was first dissolved in acetone (2%) then mixed with equal volume of DBP (Sigma) to get a final concentration of 1% solution (FITC in 1:1 DBP/acetone). Mice were sensitized with 25µl of FITC solution on the left ear (LE) followed by challenge on the right ear (RE) with 25µl of FITC solution, as indicated in experimental schemes in figures.

Cell preparation for FACS analyses

For preparation of dermal cells, REs were split into two halves, floated on a solution of Dispase (4mg/ml in PBS, Gibco), with epidermis side up, and incubated at 37°C for 1h. Dermis was then separated from epidermis and was further incubated at 37°C for 1h in

a solution of 1 mg/ml collagenase D (Roche), DNaseI 0.25 mg/mL (Sigma) and 2.5% of foetal calf serum (ThermoFisher) in PBS. Cells were then passed through a cell strainer (EASYstrainer 70µm, Greiner bio-one), resuspended in FACS buffer (1% of FCS + 2mM EDTA in PBS), counted and used for FACS staining.

For preparation of blood cells, 400 µl of blood was collected from mice by retro-orbital bleeding in EDTA-coated tubes, then merged with the same volume of Dextran (2% in PBS, Sigma-Aldrich) and incubated at 37°C for 30 min. The upper phase was transferred into new tubes, and 600 µl of FACS buffer was added. After the centrifugation step, the pellet was incubated for 2 minutes at room temperature (RT) in ACK lysis buffer (Ammonium-Chloride-Potassium: NH4Cl 0.15M; KHC03 1mM; Na_2EDTA 0.1mM), then washed with FACS buffer and used for FACS staining.

For preparation of splenocytes, mouse spleens were cut into pieces and passed through a cell strainer (EASYstrainer 70µm, Greiner bio-one). Cells were then incubated for 5 min at RT in ACK lysis buffer, then washed with FACS buffer, counted and used for FACS staining.

For preparation of bone marrow cells, one tibia and one femur were isolated from each mouse and flushed with a solution of 0.5% BSA and 2mM EDTA in PBS, using a 25G needle and a cell strainer (EASYstrainer 70µm, Greiner bio-one). Cells were then incubated for 5 minutes at RT in ACK lysis buffer, then washed with FACS buffer, counted and used for FACS staining.

Antibody staining and FACS analyses

All blood cells and $2x10^6$ cells from dermis, spleen or bone marrow were used for antibody (Ab) staining. Cells were first incubated with CD16/CD23 antibody (mouse Fc block) (ebiosciences), followed by antibody stainings with surface markers (see table of antibodies used for Flow cytometry) in 25µl of FACS buffer. Cells were then washed with FACS buffer, incubated for 3 min with DAPI (final concentration: 1 µg/ml) for exclusion of dead cells before passing on LSRII (BD).

Bone marrow progenitor analyses and sorting

After mouse euthanization, both femurs and both tibias together with hips and sternum were collected, mashed into a mortar and cells were passed through a 70µm cell strainer. Red blood cells were then lysed with ACK buffer. Bone marrow cells were washed with FACS buffer and incubated with surface Abs for 15 min on ice. Cells were analysed with LSRII (BD), or sorted with FACS ARIA II (BD) after incubation with DAPI for exclusion of dead cells.

Spleen basophil cell sorting

For basophil isolation, splenocytes were prepared as described above. Basophils were enriched using CD49b Microbeads (Miltenyi Biotec). $CD49b^{+}CD45^{low}FC\varepsilon RI\alpha^{+}$ cells were then sorted with FACS ARIA II (BD).

Antibodies used for Flow cytometry

Name	Fluorophore	Clone	Company	Dilution
CD49b-biotin		DX5	eBioscience	0.5:25
Streptavidin	BV605		BD Biosciences	0.5:25
CD45	APC-eFluor780	30-F11	eBioscience	0.05:25
TCR-beta	PerCP-Cy5.5	H57-597	eBioscience	1:25
Siglec-F	Alexa Fluor647	E50-2440	BD Biosciences	1:25
Gr1	PE	RB6-8C5	eBioscience	0.02:25
CD34	AlexaFluor 700	RAM34	eBioscience	4:25
ESAM-1	APC	1G8/ESAM	Biolegend	1.25:25
CD106 (VCAM-1)	PE	429	eBioscience	1.25:25
IgE-biotin		R35-72	BD Biosciences	0.5:25
CD19	PerCP-Cy5.5	eBio1D3	eBioscience	1:25
CD3	FITC	145-2C11	eBioscience	1:25
CD45R/B220	PE-Cy7	RA3-6B2	Biolegend	1:25
CD16/CD32 (Fc block):		93	eBioscience	0.5:25
FcεRIα	Alexa fluor 647	MAR-1	eBioscience	1:25
Antibodies for progenitor stainings				
CD3	-	17A2	Biolegend	1:100
CD11b	-	M1/70.15	BD	1:100

			Biosciences	
CD16/32	-	93	eBioscience	1:100
CD19	-	1D3	BD Biosciences	1:100
Gr1	-	RB6-8C5	BD Biosciences	1:100
Ter119	-	Ter119	BD Biosciences	1:100
CD4	-	GK1.5	BD Biosciences	1:100
CD8	-	53.6.7	eBioscience	1:100
B220	-	RA3-6B2	BD Biosciences	1:100
CD11c	-	N418	eBioscience	1:100
Ter119	-	Ter119	BD Biosciences	1:100
Anti-Rat	DyLight405		Jackson Im	1:40
Flt3	Biotin	A2F10	eBioscience	1:100
c-Kit	APC	2B8	BD Biosciences	1:100
c-Kit	APC-Cy7	2B8	Biolegend	1:100
Sca-1	PE-Cy7	D7	BD Biosciences	1:100
CD127	PE-CF594	SB/99	BD Biosciences	1:100
CD115	APC	AFS98	eBioscience	1:100
CD34	FITC	RAM34	BioLgend	1:100
c-Kit	APC	2B8	BD Biosciences	1:100
CD16/32	PE-Cy7	93	eBioscience	1:100
IgG2a,k (isotype control)	FITC	R35-95	BD Biosciences	1:100
IgG2a,k (isotype control)	PE-Cy7	eBR2	eBioscience	1:100

Histology

Mouse ears were fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) were stained with haematoxylin and eosin.

Immunohistochemistry and immunofluorescence staining

For immunohistochemistry (IHC) staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), paraffin sections were treated with 0.6% H_2O_2 (in PBS) to block endogenous peroxidase activity before antigen retrieval with either Pepsin (Invitrogen) incubated for 10 min at 37°C (for IHC of MBP), or with citric buffer (0.1M citric acid + 0.1M of sodium citrate, pH 6) boiled in the microwave (700W, 70% for 5 min) (for IHC of MCPT8). Slides were then blocked with normal rabbit serum (Vector Laboratories) and incubated with primary antibody overnight at 4°C, followed by incubation with biotinylated rabbit anti-rat IgG (Vector Laboratories) for 1h at RT and treatment of AB complex (Vector Laboratories) for 30 min at RT. Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

For immunofluorescence staining of CD4 and NIMP14, 10 µm cryosections were fixed in 4% paraformaldehyde, permeabilized with acetone, and blocked with normal goat serum. Slides were then incubated with primary antibody, followed by CY3-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch) and mounted with Vectashield medium (Vector Laboratories) containing DAPI (for nuclear staining; Invitrogen).

Antibody	**Vendor**	**Clone**
CD4	BD Biosciences	Rat-anti mouse GK1.5
NIMP-R14	Abcam	Rat anti-mouse NIMP-R14
MBP	Provided by Dr James J. Lee	Rat anti-mouse mAb
MCPT8	BioLegend	Rat anti-mouse TUG8

RNA extraction and RT-qPCR analyses of sorted cells

Cells sorted from bone marrow or spleen were centrifuged at 2000 rpm, 4°C for 6 min, resuspended and vortexed in lysis buffer from NucleoSpin RNA XS kit (Macherey-Nagel) then stocked at -80°C. RNA was extracted from these cells following the manufacturer's instruction, and was reverse transcribed by using random oligonucleotide

hexamers and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. Primers used are: for HPRT, 5'-TGGATACAGGCCAGACTTTG-3' and 5'-GATTCAACTTGCGCTCATCTTA-3'; for MCPT8, 5'-GTGGGAAATCCCAGTGAGAA-3' and 5'-TCCGAATCCAAGGCATAAAG-3'.

Statistical analysis

Data were analysed using SigmaStat (Systat Software, Point Richmond, CA, USA) by Student's t-test or the Mann–Whitney rank sum non- parametric test depending on results from the Kolmogorov–Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance.

Results and discussion

Depletion of basophils leads to a decreased infiltrate of eosinophils and neutrophils in mouse ACD skin

To induce ACD in mice, we employed an experimental protocol in which mice were sensitized with hapten FITC (in DBP-acetone) on the left ear (LE) at day (D)3, 4 and 5, and challenged with the same solution on the right ear (RE) at D9 (Fig. 1A) (Shigeno et al., 2009). To deplete basophils, MCPT8^{DTR} mice (Wada et al., 2010) were injected with diphtheria toxin (DT) (called hereafter MCPT8^{DEP} mice) at D0, followed by repeated DT injection every 4 days to maintain the depletion of basophils (Fig. 1A). DT-injected wildtype littermates were used as control (CT) mice. Histological analyses with haematoxylin and eosin (H&E) staining showed that, while FITC-challenged RE from CT mice exhibited a thickened skin with immune cell infiltrate in the dermis (compare untreated CT with CT+FITC, Fig.1B), the inflammation was much less in that from MCPT8^{DEP}+FITC mice (compare MCPT8^{DEP}+FITC with CT+FITC, Fig. 1B). Immunohistochemistry (IHC) and immunofluorescence (IF) stainings identified the infiltrate of basophils, eosinophils, neutrophils and CD4^{+} T cells in FITC-treated CT skin (compare untreated CT with CT+FITC, Fig. 1B). An efficient depletion of basophils was evident in MCPT8^{DEP} mice, as basophils were barely detected in untreated-MCPT8^{DEP} or MCPT8^{DEP}+FITC skin (Fig. 1B). Interestingly, we observed that the number of eosinophils and neutrophils, but not CD4^{+} T cells, was substantially decreased in the RE from MCPT8^{DEP}+FITC mice compared to that from CT+FITC (Fig. 1B). We next performed flow cytometry analyses to quantify immune cells in dermis of RE (Fig. 1C, see gating strategies in Fig. S1A). Results confirmed that with the depletion of basophils, both eosinophils and neutrophils were largely diminished in the dermis of RE from FITC-treated MCPT8^{DEP} mice, while the number of TCRβ^{+} T cells (we used TCRβ instead of CD4 antibody for labelling, as CD4 surface expression was decreased during dermal cell preparation) and mast cells remained unchanged (Fig. 1D).

As basophils have been reported to play a role for Th2 cell differentiation (Leyva-Castillo et al., 2013; Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009), we examined whether Th2 cells (TCRβ^{+} T cells expressing Th2 cytokines IL-4 or IL-13)

could be reduced in FITC-treated MCPT8^{DEP} skin. To this aim, MCPT8^{DTR} mice were crossed with 4C13R dual reporter mice, which express the cyan fluorescent protein AmCyan under the control of *Il4* regulatory elements and the red fluorescent protein dsRed under the control of *Il13* regulatory elements (Roediger et al., 2013), to generate MCPT8^{DTR}/4C13R^{Tg} mice. In FITC-treated CT/4C13R^{Tg} mice, we found that IL-4 and IL-13 were produced by both basophils and TCRβ^{+} T cells, although basophils were the major producers of these two cytokines (Supp Fig. S2A). Depletion of basophils did not result in any decrease of frequency or number of IL-4 or IL-13-expressing TCRβ^{+} T cells (compare MCPT8^{DEP}/4C13R^{Tg} + FITC with CT/4C13R^{Tg} + FITC, Supp Fig. S2A). These data indicated that the depletion of basophils in MCPT8^{DEP} mice did not affect IL-4 and IL-13 expression by Th2 cells in the skin; in other words, the decrease of eosinophils and neutrophils was not caused by a reduced Th2 response in MCPT8^{DEP} mice.

Recently, it has been shown that basophils play an important role in regulating eosinophil tissue entry, as depletion of basophils in Basoph8 x DTA (MCPT8-Cre x Rosa-DTA) mice led to a reduction of skin eosinophils in a mouse model of IgE-mediated allergic skin inflammation (Cheng et al., 2015). As it was reported in this paper that IL-4 from basophils promoted eosinophil accumulation by modulating endothelial expression of VCAM-1 (Cheng et al., 2015), we examined VCAM-1 expression in endothelial cells (ECs) of FITC-treated RE by FACS. Results showed that frequency for VCAM-1^{+} ECs (CD45^{-}ESAM-1^{+}CD34^{+}) was higher in CT+ FITC compared to untreated CT, but no difference was observed between CT+FITC and MCPT8^{DEP}+FITC (Supp Fig. S2B). Therefore, despite a similar observation that basophil depletion reduces eosinophil recruitment in the inflamed ACD skin, the mechanism reported by Cheng et al. could not explain our observation.

Actually, results from our study further contradicted the conclusion that basophils mediate eosinophil recruitment in skin. The depletion of basophils prior to the FITC challenge by administrating DT at D4, to our surprise, did not lead to any change on the number of eosinophils and neutrophils (Supp Fig. S3A), despite the fact that basophils were efficiently depleted. In addition, when basophils were depleted posterior to the FITC challenge (by administrating DT at D7), eosinophils or neutrophils did not either show a reduction (Supp Fig. S3B). Finally, depletion of basophils in WT Balb/c mice by

i.p. injection of an antibody specific to FcεRIα (MAR-1) (Denzel et al., 2008) also failed to cause any decrease of eosinophil or neutrophil numbers in FITC-treated skin (supp Fig. S3C).

Taken together, we observed on one hand that DT-mediated basophil depletion in MCPT8^{DTR} mice led to a decreased number of eosinophils and neutrophils in skin with allergic inflammation; on the other hand, we obtained several lines of evidence indicating that basophils do not mediate eosinophil or neutrophil recruitment, in contrast to recent reports (Cheng et al., 2015; Nakashima et al., 2014). Therefore, there should be other explanation for the decrease of eosinophils and neutrophils observed in the ACD skin of MCPT8^{DEP} mice.

Depletion of basophils leads to a systemic reduction of eosinophils and neutrophils in a time dependent manner

Searching for an explanation for our observation, we found with surprise that in addition to the skin site, the blood, the spleen and the bone marrow (BM) all exhibited a significant reduction of both eosinophils and neutrophils in FITC-treated MCPT8^{DEP} mice (Fig. 2A-D). This led us to ask whether depletion of basophils in MCPT8^{DEP} mice, without FITC treatment, may already result in a systemic reduction of eosinophils and neutrophils; and if yes, whether this would be associated with the time point of analyses upon DT administration. To examine these, we designed a kinetic experiment in which DT was given as in Fig 1A (at D0, D4 and D8), and CT and MCPT8^{DEP} mice were analysed at different time points (D3, D5, D8 or D10) (Fig. 3A). Results showed that, at D3, basophils were efficiently depleted in blood, spleen and bone marrow from MCPT8^{DEP} mice, while eosinophils and neutrophils at these sites were all comparable between MCPT8^{DEP} and CT (Fig. 3B). This result was in good agreement with the initial characterization of MCPT8^{DEP} by Wada *et al*., showing the specific depletion of basophils among mature blood cells (Wada et al., 2010). However, when examined at D5, neutrophils but not eosinophils were found reduced in blood, spleen and bone marrow; while at D8 and D10, both eosinophils and neutrophils were reduced at all these sites (Fig. 3B). Therefore, DT administration not only depleted basophils in

MCPT8^{DTR} mice, but also led to a systemic reduction of eosinophils and neutrophils in a time-dependent manner.

Granulocyte-macrophage progenitors (GMPs) are depleted in the bone marrow of MCPT8^{DEP} mice

Based on the above results, we wondered whether GMPs, the hematopoietic stem cell (HSC)-derived common progenitors of eosinophils, neutrophils and basophils, could be reduced in the bone marrow of MCPT8^{DEP} mice. To examine this, MCPT8^{DEP} and CT mice were analysed at D3 and D5 for HSC-derived progenitors in bone marrow (Fig. 4A, see gating strategies in supp Fig. S1B). We observed a significant decrease of GMPs at both D3 and D5 in the bone marrow from MCPT8^{DEP} mice (Fig. 4B-C). In addition, monocyte-dendritic cell progenitors (MDPs) and common dendritic cell progenitors (CDPs) showed also a reduction (Fig. 4B-C). In contrast, common myeloid progenitors (CMPs) (which are upstream of GMPs), CMP-derived megakaryocyte-erythrocyte progenitors (MEPs), as well as common lymphoid progenitors (CLPs) were not reduced but rather increased in the bone marrow from MCPT8^{DEP} mice (Fig. 4B-C). This result provided a plausible explanation for the systemic reduction of eosinophils and neutrophils in MCPT8^{DEP} mice, and moreover suggests that other myeloid lineaged cells (e.g. monocytes and dendritic cells), which are derived from GMPs, MDPs or CDPs (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012), may also be affected in MCPT8^{DEP} mice.

We suspected that the reduction of GMPs may reflect a DT-mediated depletion of these cells, for example, due to the activity of MCPT8 promoter in these cells. We then examined the microarray database from Immgen (www.immgen.org) (Heng, Painter, & Immunological Genome Project, 2008). Among immune cell populations, a highest expression of MCPT8 was seen in basophils (the post-normalization expression value is ≈4000; https://www.immgen.org/Protocols/ImmGen%20QC%20Documentation_ALL-DataGeneration_0612.pdf), as previously reported (Poorafshar et al., 2000; Wada et al., 2010), whereas MCPT8 RNA levels were much lower in mast cells (≈250), as well as in most of mature immune cells including T cells, dendritic cells, eosinophils and neutrophils (<40) (not shown). However, we noticed a considerable expression of

MCPT8 in bone marrow GMPs (≈690) and MDPs (≈280) (Fig. 4D). As Immgen data were obtained from C57BL/6J mice, while Balb/c mice were used in our study, we further examined MCPT8 gene expression by RT-qPCR in progenitors sorted from the bone marrow of Balb/c mice. Results confirmed that among the bone marrow progenitors examined, GMPs expressed the highest MCPT8 level, which was followed by MDPs (Fig. 4E).

Altogether, these results suggest that bone marrow GMPs, which give rise to neutrophils, eosinophils and basophils, are depleted in MCPT8^{DEP} mice possibly due to the activity of MCPT8 gene promoter in these cells. The decreased number of MDPs and CDPs could be explained by either a weak expression of MCPT8 in MDPs (and MDPs can give rise to CDPs) (Gabrilovich et al., 2012), or the fact that MDPs could be derived from GMPs (H. Iwasaki & Akashi, 2007).

Basophils are not required for eosinophil or neutrophil recruitment in ACD skin

Despite the expression of MCPT8 in GMPs, its level was still lower compared to basophils (Fig. 4D&E). We thus wondered whether by reducing the dose of DT, GMPs could be exempted from depletion, while basophils could still be efficiently depleted. To test this, MCPT8^{DTR} and CT mice were injected with different doses (100ng, 300ng and 750ng) of DT, and subjected to FITC treatment (Fig. 5A). At D10, RE, blood, spleen and bone marrow were sampled for analyses. Results showed that basophils were not efficiently depleted with 100ng of DT, but both 300ng and 750ng DT resulted in an efficient depletion of basophils in dermis, blood, spleen and bone marrow (Fig. 5B). Results obtained from the 750ng of DT-injected MCPT8^{DTR} mice were similar to those from 1μg of DT injected-mice (Fig. 1 and Fig. 2), showing that eosinophils and neutrophils were both reduced at all sites; results from 300ng of DT-injected MCPT8^{DTR} mice showed that eosinophils were not reduced in FITC-challenged RE, nor at other sites (a minor decrease of neutrophils was still observed in bone marrow) (Fig. 5B). In agreement with that, bone marrow analyses of 300ng of DT-injected MCPT8^{DTR} mice indicated that GMPs were not significantly reduced (Fig. 5C), different with 1 μg DT-injection (Fig. 4B). These data thus indicate that a specific depletion of basophils, but not GMPs, could be achieved when the dose of DT is optimised; and by this way, we

provide one more piece of evidence that basophils do not play an essential role for the recruitment of eosinophils and neutrophils in allergic skin.

Conclusion

In our study with an initial aim to investigate the role of basophils in allergic skin, we find unexpectedly that DT administration to MCPT8^{DTR} mice, not only depletes basophils, but also reduces bone marrow GMPs, possibly due to the activity of MCPT8 gene in these cells. Consequently, this leads to a systemic reduction of neutrophils and eosinophils in a time-dependent manner. This not only explains our initial observation that infiltrate of eosinophils and neutrophils was highly reduced when DT-injected MCPT8^{DTR} mice were subjected to FITC treatment (Fig. 1), but also explains our observation that reduction of eosinophils or neutrophils in ACD skin was not seen when DT was administrated prior or posterior to the FTIC challenge, because the systemic reduction of eosinophils and neutrophils (as a consequence of GMP depletion) is time-dependent (Fig. S3A & B). These pieces of evidence, together with that from 300ng DT injection (Fig. 5) and MAR-1-mediated basophil depletion (Fig. S3C), allow us to finally conclude that basophils do not play an essential role in eosinophil and neutrophil recruitment to allergic skin. This conflicts with a recent report (Cheng et al., 2015) showing that basophils are crucial for eosinophil accumulation in allergic skin. As MCPT8-Cre x ROSA-DTA mice were used in that previous study for basophil depletion, which was under the control of MCPT8 promoter, it remains to be examined whether their observation could reflect a consequence of the depletion of GMPs. Nevertheless, our finding has highlighted the caution with which basophil-depletion studies using MCPT8^{DTR} mice, as well as DTA systems under the control of MCPT8 gene promoter, should be interpreted, particularly because DT dose and administration / analysing time all varied in different studies (Egawa et al., 2013; Leyva-Castillo et al., 2013; Reber et al., 2013; Venturelli et al., 2016; Wada et al., 2010).

Over the last decades, the DTR-based cell depleting mouse models have been proven to be powerful tools to explore the *in vivo* function of a cell type, which are widely used in the research of immunology. Our study has exemplified several cautions which should be taken with DTR-based mouse tools: first, analyses of the specificity of DTR-

driven gene promoter (for designing), and of the specificity of cell depletion (for characterizing), should include not only mature immune cells, but also progenitor cells; second, the dose of DT needs to be carefully determined in order to achieve a selective depletion; and finally, kinetic analyses upon DT administration are also necessary for characterizing DTR-based tools and using properly these powerful tools in functional immunology studies.

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Figure 1. Depletion of basophils leads to a decreased infiltrate of eosinophils and neutrophils in mouse ACD skin.

(A) Experimental protocol. MCPT8^{DTR} and their littermate wildtype control mice were intra-peritoneally (i.p.) injected with diphtheria toxin (DT) at day (D) 0, D4 and D8. Mouse left ears (LE) were sensitized with FITC at D3, D4 and D5. Right ears (RE) were then challenged at D9 with FITC and sampled for analyses at D10. MCPT8^{DEP}, DT-injected MCPT8^{DTR} mice; CT, DT-injected wildtype control mice. **(B)** Hematoxylin and eosin (H&E), immunofluorescence (IF) and immunohistochemistry (IHC) staining of RE sections. Arrow points to one of the positive cells. Bar=50µm. **(C)** Representative FACS plots of CD45^{+}TCRβ^{+} T cells, CD45^{low}CD49b^{+} basophils, CD45^{hi}CD49b^{+} mast cells, CD45^{+}TCRβ^{-} Siglec-F^{+}Gr1$^{low\text{-}neg}$ eosinophils, and CD45^{+}TCRβ^{-}Gr1^{hi} neutrophils in RE dermis from FITC-treated CT and MCPT8^{DEP} mice. **(D)** A comparison of frequency and number for basophils, eosinophils, neutrophils, TCRβ^{+} cells and mast cells in REs from untreated and FITC-treated CT and MCPT8^{DEP} mice. **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

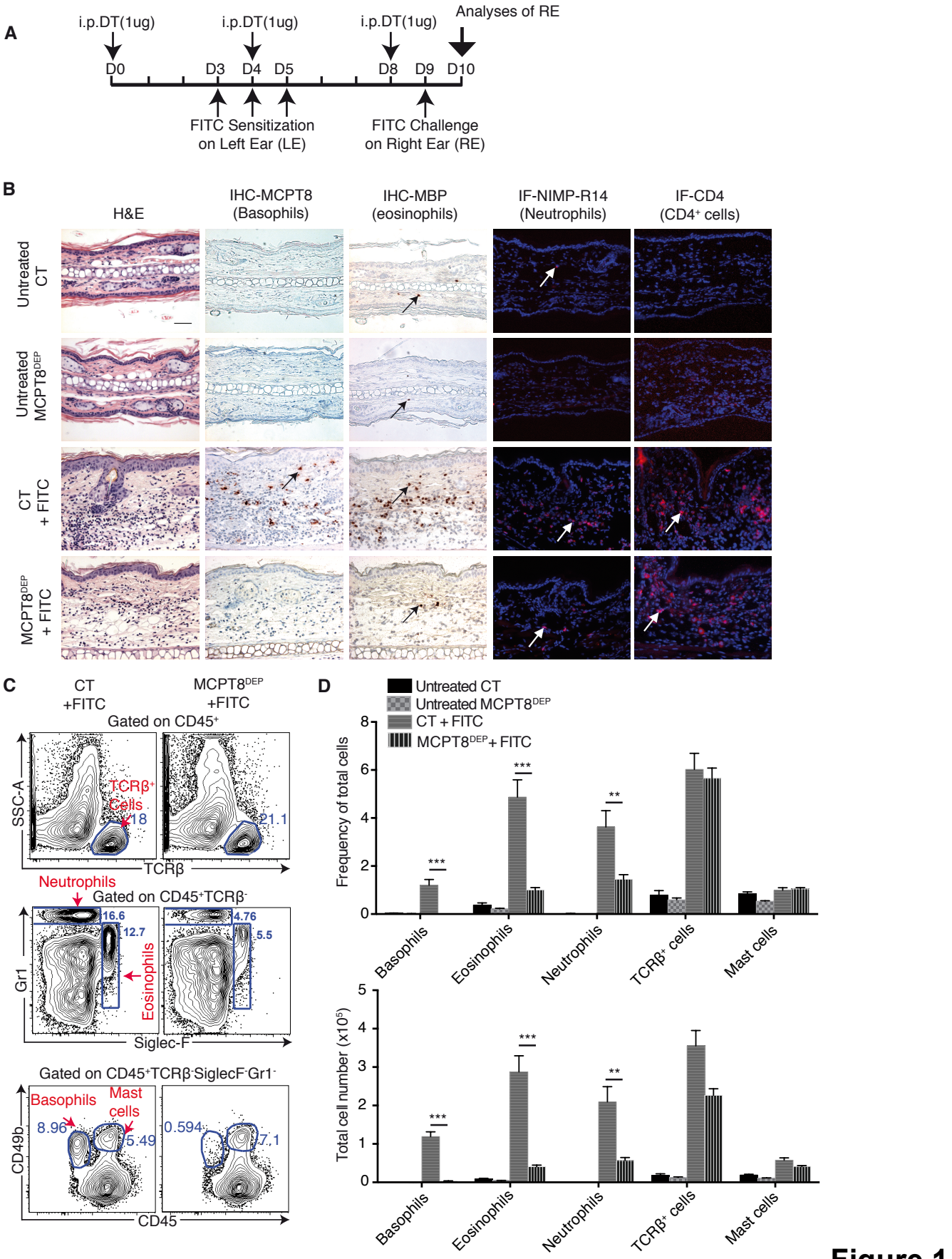
A
i.p.DT(1ug)
i.p.DT(1ug)
i.p.DT(1ug)
Analyses of RE
D0
D3
D4
D5
D8
D9
D10
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
B
H&E
IHC-MCPT8 (Basophils)
IHC-MBP (eosinophils)
IF-NIMP-R14 (Neutrophils)
IF-CD4 (CD4+ cells)
Untreated CT
Untreated MCPT8DEP
CT + FITC
MCPT8DEP + FITC
C
CT +FITC
MCPT8DEP +FITC
Gated on CD45+
SSC-A
TCRβ+ Cells
1.18
21.1
TCRβ
Neutrophils
Gated on CD45+TCRβ-
16.6
12.7
4.76
5.5
Gr1
Eosinophils
Siglec-F
Gated on CD45+TCRβ-SiglecF-Gr1-
Basophils
Mast cells
8.96
5.49
0.594
7.1
CD49b
CD45
D
Untreated CT
Untreated MCPT8DEP
CT + FITC
MCPT8DEP + FITC
Frequency of total cells
Total cell number (x10^5)
Basophils
Eosinophils
Neutrophils
TCRβ+ cells
Mast cells

Figure 1

Figure 2. Eosinophils and neutrophils are reduced in the blood, spleen and bone marrow in FITC-treated MCPT8^{DEP} mice.

(A) Mice were subjected to the experimental protocol as described in Fig.1A, except that blood, spleen and bone marrow (BM) were sampled for analyses at D10. **(B-D)** Representative FACS plots and frequency of total cells for basophils, eosinophils and neutrophils in blood **(B)**, spleen **(C**) and BM **(D)**. MCPT8^{DEP}, DT-injected MCPT8^{DTR} mice; CT, DT-injected wildtype control mice. *P≤0.05, **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

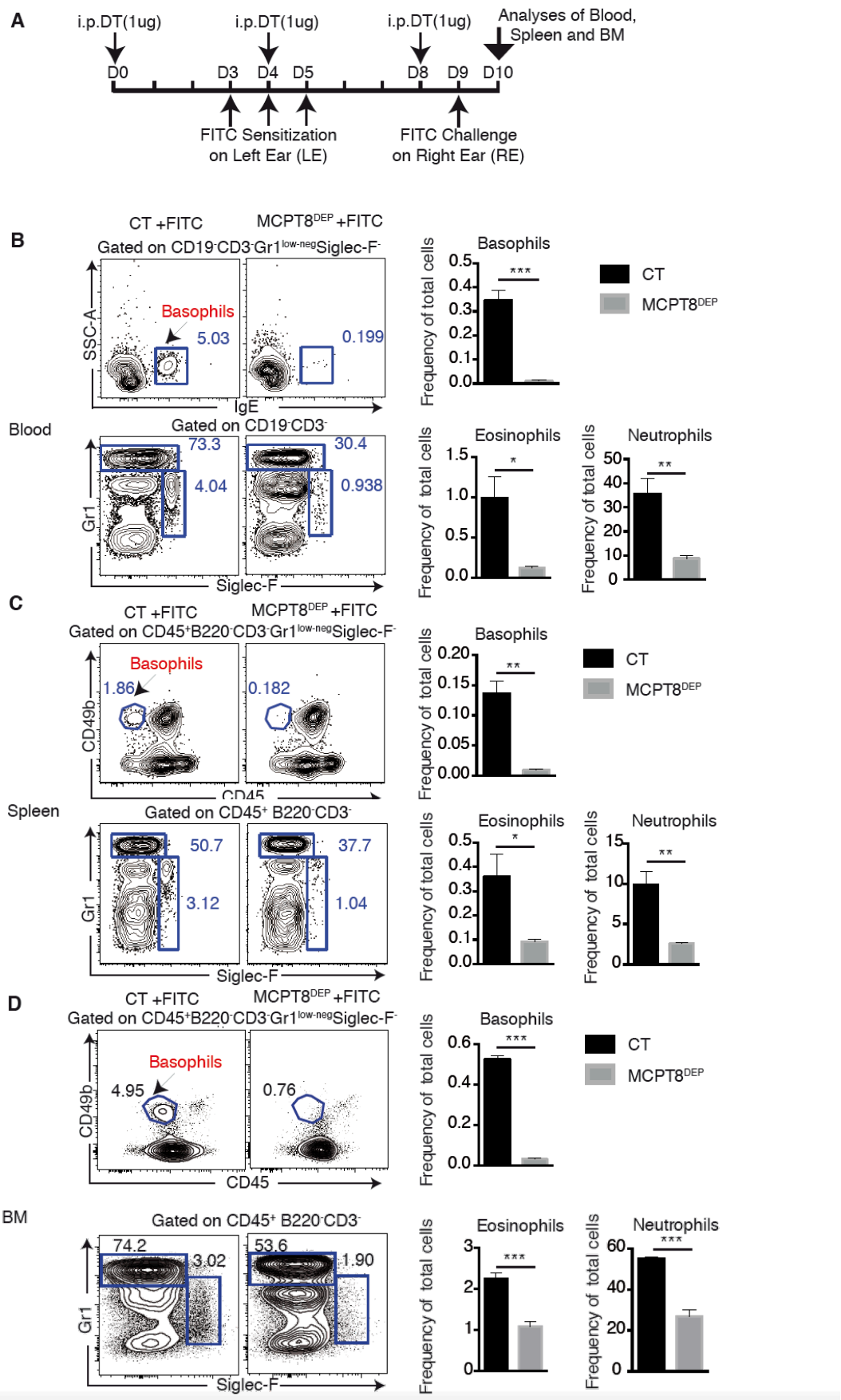
A
i.p.DT(1ug)
i.p.DT(1ug)
i.p.DT(1ug)
Analyses of Blood, Spleen and BM
D0
D3
D4
D5
D8
D9
D10
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
B
CT +FITC
MCPT8DEP +FITC
Gated on CD19-CD3-Gr1low-negSiglec-F-
Basophils
5.03
0.199
SSC-A
IgE
Blood
Gated on CD19-CD3-
73.3
4.04
30.4
0.938
Gr1
Siglec-F
Basophils
Eosinophils
Neutrophils
Frequency of total cells
CT
MCPT8DEP
C
CT +FITC
MCPT8DEP +FITC
Gated on CD45+B220-CD3-Gr1low-negSiglec-F-
Basophils
1.86
0.182
CD49b
CD45
Spleen
Gated on CD45+ B220-CD3-
50.7
3.12
37.7
1.04
D
CT +FITC
MCPT8DEP +FITC
Gated on CD45+B220-CD3-Gr1low-negSiglec-F-
Basophils
4.95
0.76
CD49b
CD45
BM
Gated on CD45+ B220-CD3-
74.2
3.02
53.6
1.90

Figure 2

Figure 3. Depletion of basophils leads to a systemic reduction of eosinophils and neutrophils in a time-dependent manner.

(A) Experimental protocol. MCPT8^{DTR} and their littermate wildtype control mice were i.p. injected with DT at D0, D4 and D8. Blood, spleen and bone marrow (BM) were sampled and analysed at D3, D5, D8 and D10. MCPT8^{DEP}, DT-injected MCPT8^{DTR} mice; CT, DT-injected wildtype control mice. **(B)** Frequency of total cells for basophils, eosinophils and neutrophils at the indicated time points in the blood, spleen and BM. *P≤0.05, **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

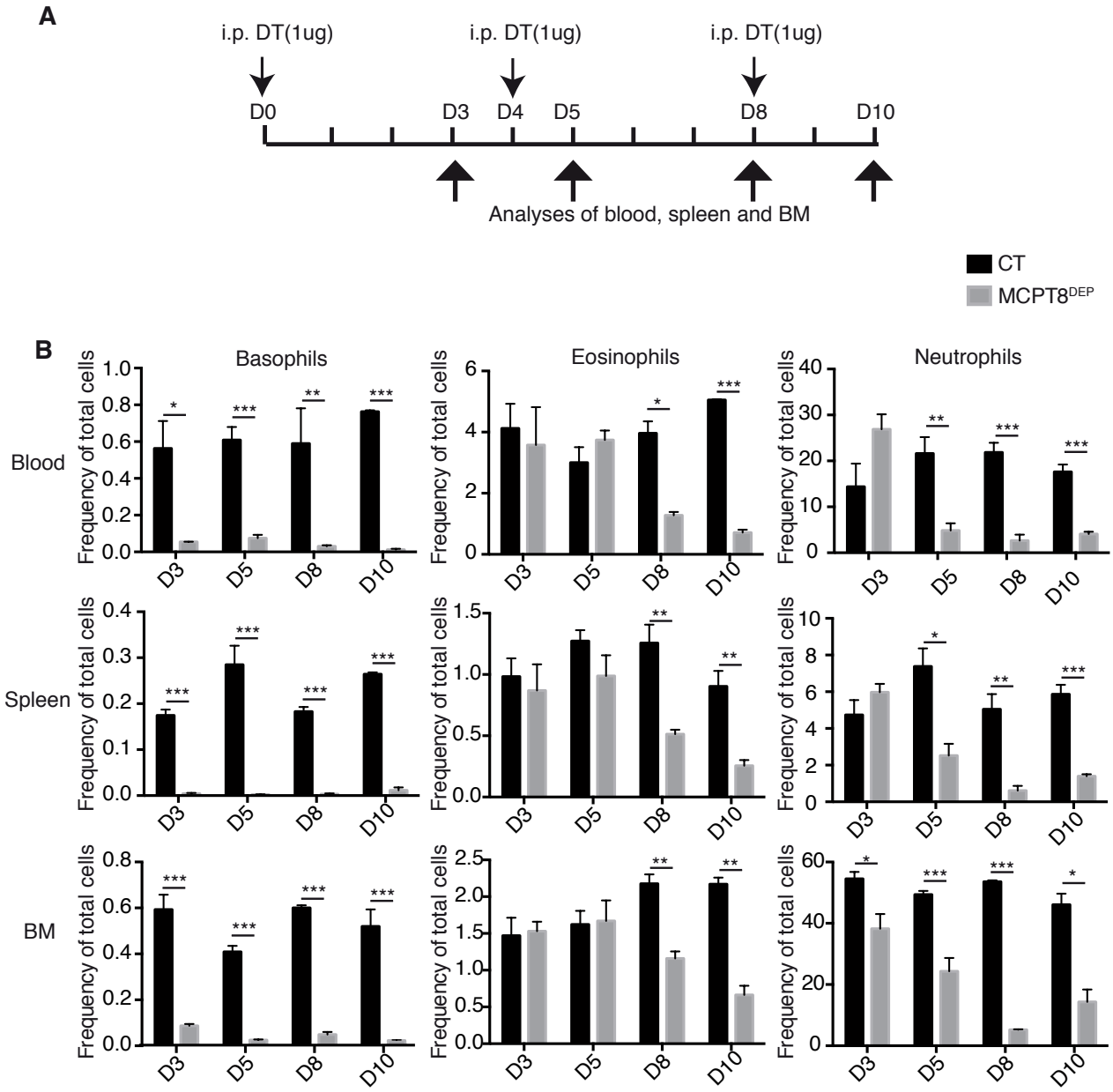
A
i.p. DT(1ug)
i.p. DT(1ug)
i.p. DT(1ug)
D0
D3
D4
D5
D8
D10
Analyses of blood, spleen and BM
CT
MCPT8DEP
B
Basophils
Eosinophils
Neutrophils
Blood
Spleen
BM
Frequency of total cells
D3
D5
D8
D10

Figure 3

Figure 4. Hematopoietic stem cell-derived GMPs are reduced in the bone marrow of MCPT8^{DEP} mice.

(A) Experimental protocol. MCPT8^{DTR} and their littermate wildtype control mice were i.p. injected with DT at day D0 and D4. Bone marrow were sampled for progenitor analyses at D3 or D5. MCPT8^{DEP}, DT-injected MCPT8^{DTR} mice; CT, DT-injected wildtype control mice. **(B-C)** Representative FACS plots and summary of frequency of total cells for Lin$^-$ c-kit$^+$CD34$^+$CD16/32$^-$ common myeloid progenitors (CMPs), Lin$^-$c-kit$^+$CD34$^+$CD16/32$^+$ granulocyte-macrophage progenitors (GMPs), Lin$^-$c-kit$^+$CD34$^-$CD16/32$^-$ megakaryocyte-erythrocyte progenitors (MEPs), Lin$^-$Flt3$^+$CD115$^+$c-kit^{hi} monocyte-dendritic cell progenitors (MDPs), Lin$^-$ Flt3$^+$CD115$^+$c-kit^{hi} common dendritic progenitors (CDPs) and Lin$^-$ CD127$^+$Sca-l^{lo}c-kit$^+$ common lymphoid progenitors (CLPs), at D3 **(B)** and D5 **(C). (D)** Immgen Data showing MCPT8 expression levels in BM progenitors, basophils from blood and spleen (www.immgen.org). **(E**) Progenitors were sorted from the BM and basophils were sorted from the spleen of Balb/c wildtype female mice, and were analysed for MCPT8 mRNA level by RT-qPCR**.** *P≤0.05, **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

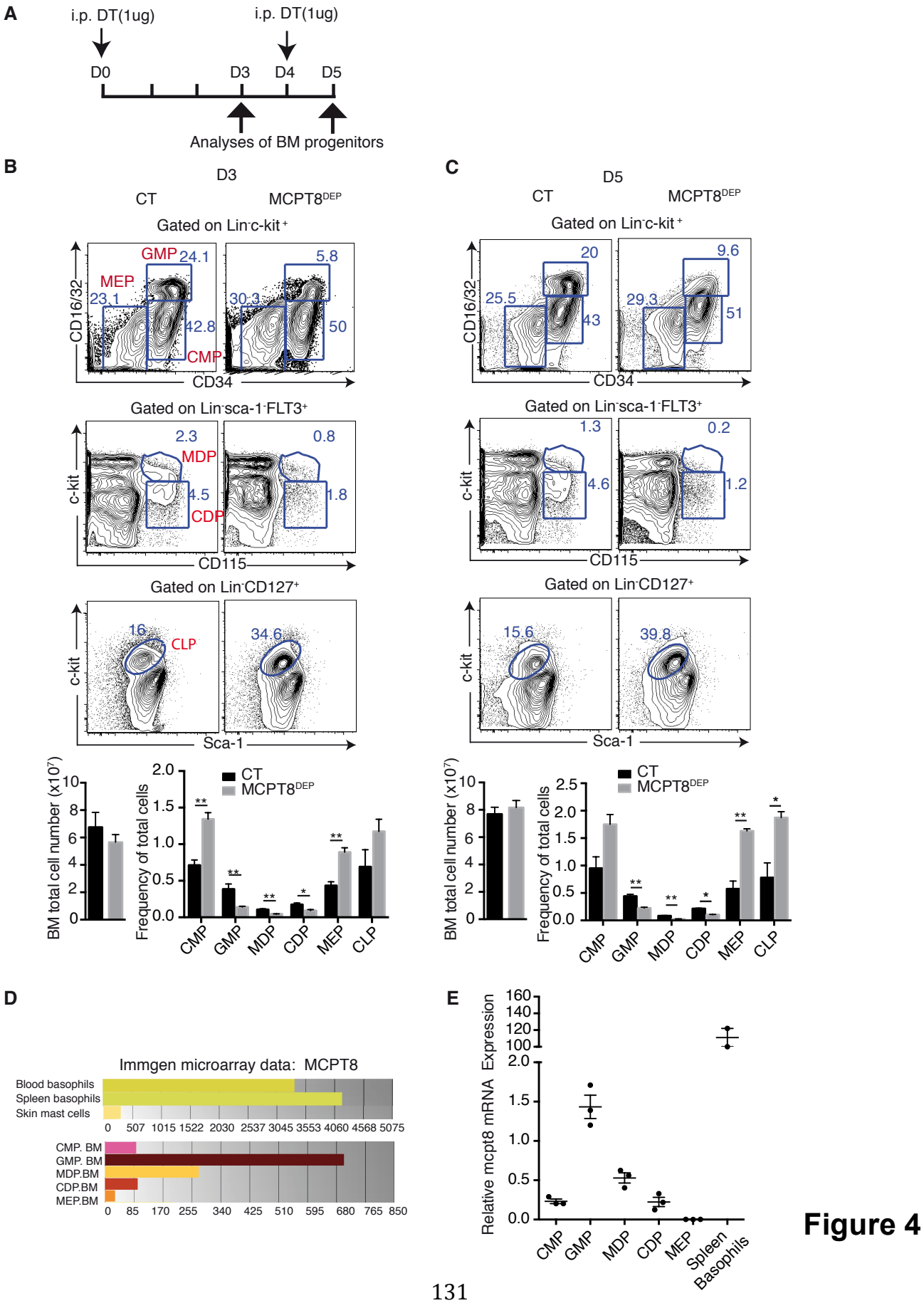
A
i.p. DT(1ug)
i.p. DT(1ug)
D0
D3
D4
D5
Analyses of BM progenitors
B
D3
CT
MCPT8DEP
Gated on Lin-c-kit+
GMP
MEP
CMP
24.1
23.1
42.8
30.3
5.8
50
CD16/32
CD34
Gated on Lin-sca-1-FLT3+
MDP
CDP
2.3
4.5
0.8
1.8
c-kit
CD115
Gated on Lin-CD127+
CLP
16
34.6
Sca-1
BM total cell number (x10^7)
Frequency of total cells
CT
MCPT8DEP
CMP
GMP
MDP
CDP
MEP
CLP
C
D5
CT
MCPT8DEP
Gated on Lin-c-kit+
20
25.5
43
9.6
29.3
51
Gated on Lin-sca-1-FLT3+
1.3
4.6
0.2
1.2
Gated on Lin-CD127+
15.6
39.8
D
Immgen microarray data: MCPT8
Blood basophils
Spleen basophils
Skin mast cells
CMP. BM
GMP. BM
MDP.BM
CDP.BM
MEP.BM
E
Relative mcpt8 mRNA Expression
Spleen Basophils

Figure 4

Figure 5. Basophils do not play an essential role in eosinophil or neutrophil recruitment to allergic skin.

(A) Experimental protocol. MCPT8^{DTR} and their littermate wildtype control mice were i.p. injected with different doses of DT at D0, D4 and D8. Mouse left ears (LE) were sensitized with FITC at D3, D4 and D5. Mouse right ears (RE) were then challenged at D9 with FITC. RE, blood, spleen and BM were sampled for analysis at D10. MCPT8^{DEP}, DT-injected MCPT8^{DTR} mice; CT, DT-injected wildtype control mice. **(B)** Comparison of frequency of total cells for basophils, eosinophils and neutrophils in the dermis of RE, blood, spleen and BM, between CT and MCPT8^{DEP} mice. **(C)** Mice were i.p. injected with 300ng of DT at D0, and BM progenitors were analysed by FACS at D3. BM total cell number and frequency of total cells for progenitors are shown. *P≤0.05, **P≤0.01, ***P≤0.001 (Student's *t*-test). Values are mean ± SEM (n≥3 mice per group).

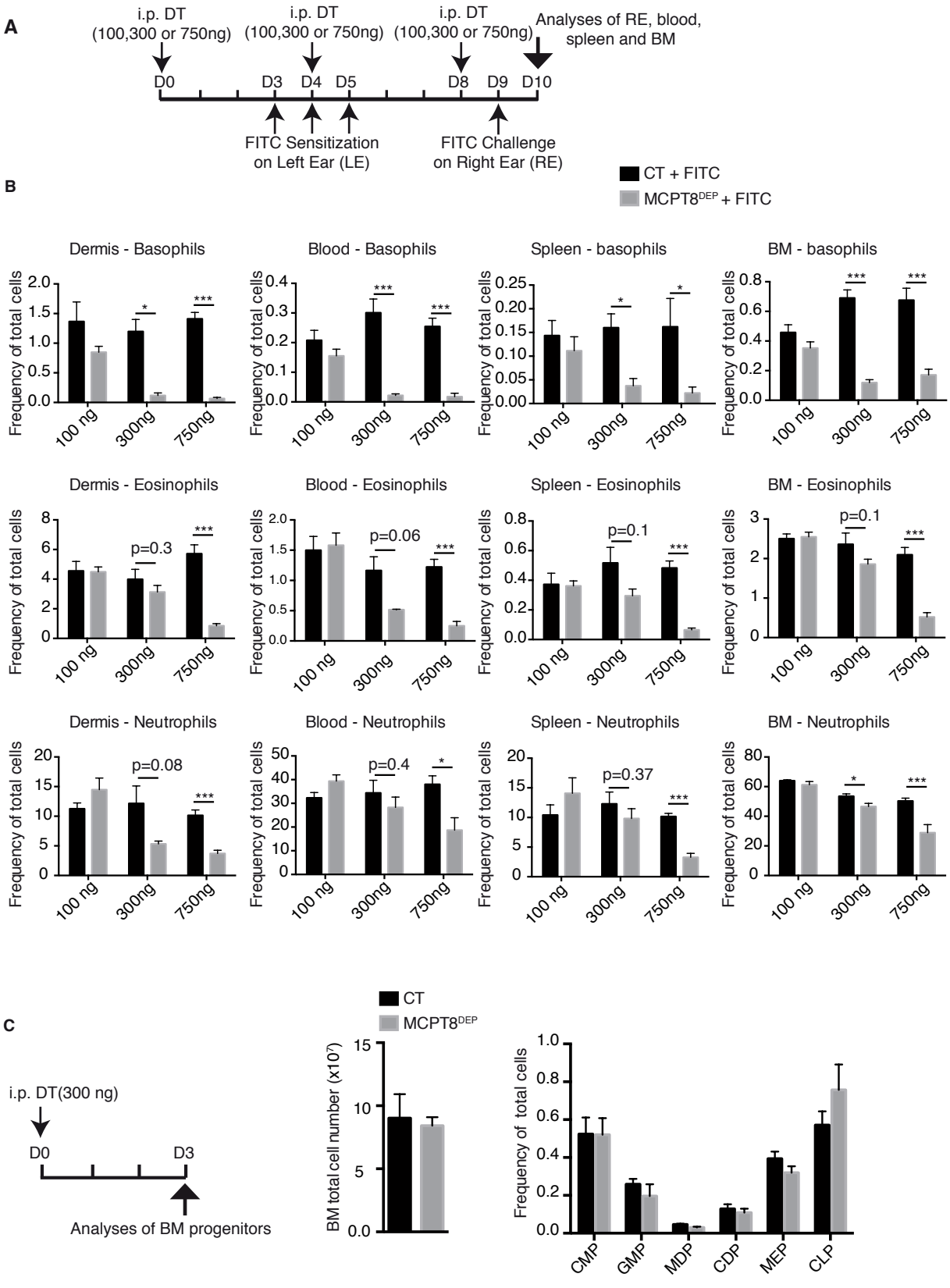
A
i.p. DT (100,300 or 750ng)
i.p. DT (100,300 or 750ng)
i.p. DT (100,300 or 750ng)
Analyses of RE, blood, spleen and BM
D0
D3
D4
D5
D8
D9
D10
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
B
CT + FITC
MCPT8DEP + FITC
Dermis - Basophils
Blood - Basophils
Spleen - basophils
BM - basophils
Dermis - Eosinophils
Blood - Eosinophils
Spleen - Eosinophils
BM - Eosinophils
Dermis - Neutrophils
Blood - Neutrophils
Spleen - Neutrophils
BM - Neutrophils
Frequency of total cells
100 ng
300ng
750ng
p=0.3
p=0.06
p=0.1
p=0.08
p=0.4
p=0.37
C
CT
MCPT8DEP
i.p. DT(300 ng)
D0
D3
Analyses of BM progenitors
BM total cell number (x10^7)
Frequency of total cells
CMP
GMP
MDP
CDP
MEP
CLP

Figure 5

Figure S1. Gating strategies of flow cytometry analyses.

(A) Gating strategies for immune cells in skin. REs were sampled and dermal cells were prepared for FACS analyses. After excluding debris, doublets, and dead cells, hematopoietic cells were gated as $CD45^{+}$. $TCR\beta^{-}$ cells were then gated for eosinophils (Siglec-F^{+} $Gr1^{int}$), and neutrophils (Siglec-F^{-} $Gr1^{hi}$) which were validated to be Ly6-$G^{+}SSC^{low}$. The $Gr1^{-}$siglec-F^{-} cells were gated for basophils ($CD45^{low}CD49b^{+}$) and mast cells ($CD45^{hi}CD49b^{+}$). **(B)** Gating strategies for bone marrow progenitors. Common myeloid progenitors (CMPs) were gated as Lineage Lin-1(CD11c, CD3, CD11b, CD19, CD127, Gr1, Sca-1, Ter119, CD4, CD8, B220)^{neg}c-$kit^{hi}CD34^{hi}CD16/32^{int}$; megakaryocyte-erythroid progenitors (MEPs) were gated as Lin-1^{neg}c-$kit^{hi}CD34^{neg}CD16/32^{neg}$; granulocyte-macrophage progenitors (GMPs) were gated as Lin-1^{neg}c-$kit^{hi}CD34^{hi}CD16/32^{hi}$ (Akashi, Traver, Miyamoto, & Weissman, 2000); monocyte and dendritic cell progenitors (MDPs) were gated as Lineage Lin-2(CD11c, CD3, CD11b, CD16/32, CD19, Gr1, Sca-1, Ter119, CD4, CD8, B220)$^{neg}FLT3^{+}CD115^{+}$c-kit^{hi} and common dendritic cell progenitors (CDPs) were gated as Lin-$2^{neg}FLT3^{+}CD115^{+}$c-kit^{low} (Liu et al., 2009); Common lymphoid progenitors (CLPs) were gated as Lineage Lin-3 (CD11c, CD3, CD11b, CD16/32, CD19, Ter119, CD4, CD8, Gr1, B220)$^{neg}CD127^{+}$c-kit^{+}Sca-1^{low} (Kondo, Weissman, & Akashi, 1997).

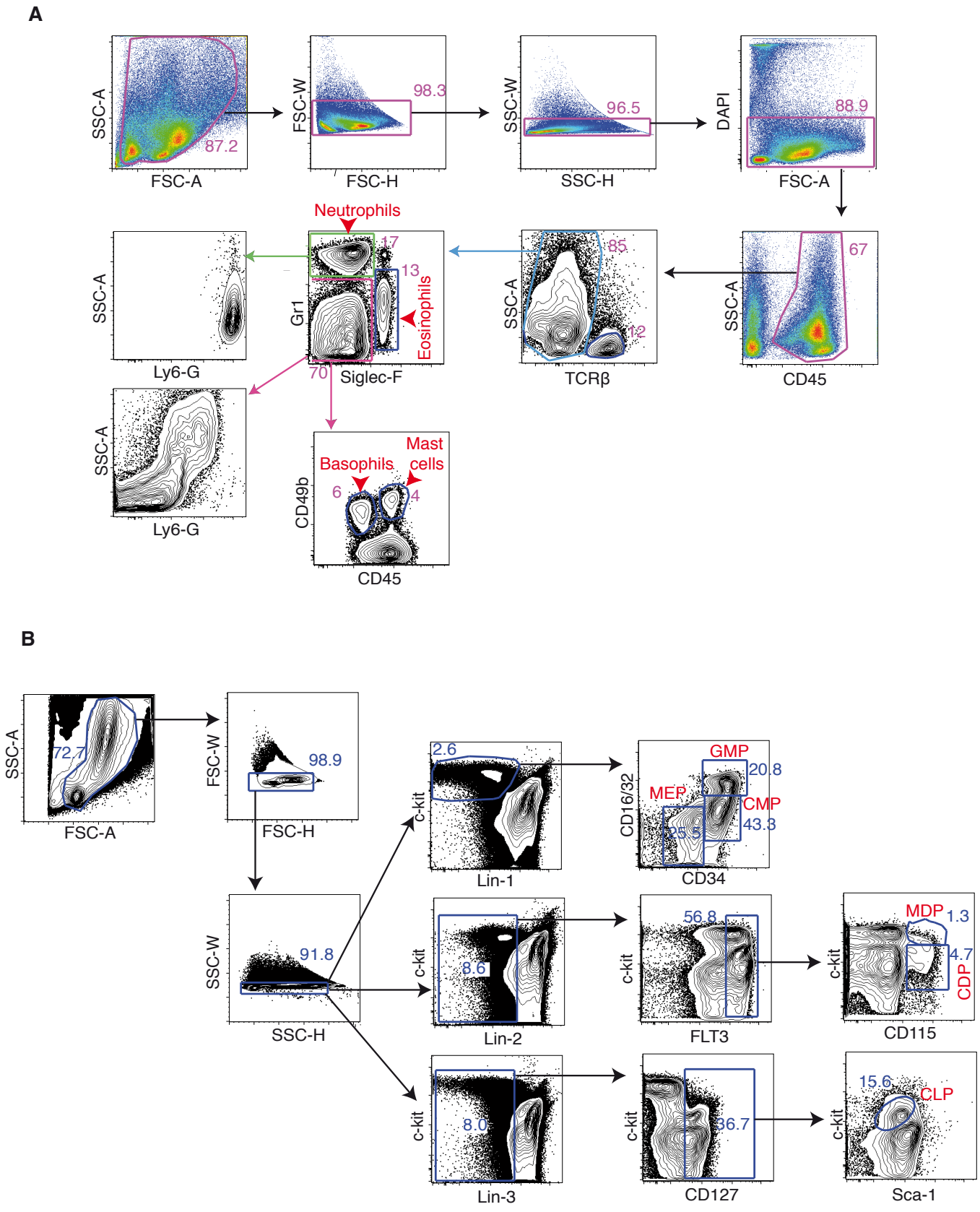
A
SSC-A
FSC-A
87.2
FSC-W
FSC-H
98.3
SSC-W
SSC-H
96.5
DAPI
FSC-A
88.9
SSC-A
CD45
67
SSC-A
TCRβ
85
12
Neutrophils
Gr1
17
13
Eosinophils
Siglec-F
70
SSC-A
Ly6-G
SSC-A
Ly6-G
CD49b
CD45
Basophils
6
Mast cells
4
B
SSC-A
FSC-A
72.7
FSC-W
FSC-H
98.9
SSC-W
SSC-H
91.8
c-kit
Lin-1
2.6
CD16/32
CD34
GMP
20.8
MEP
CMP
43.8
25.5
c-kit
Lin-2
8.6
c-kit
FLT3
56.8
c-kit
CD115
MDP
1.3
4.7
CDP
c-kit
Lin-3
8.0
c-kit
CD127
36.7
c-kit
Sca-1
15.6
CLP

Figure S1

Figure S2. The decrease of eosinophils and neutrophils was not due to a reduced Th2 differentiation or a decrease in VCAM-1 expression by endothelial cells in FITC-treated MCPT8^{DEP}mice.

MCPT8^{DEP}/4C13R^{Tg} and CT/4C13R^{Tg} mice were subjected to the FITC-treatment as described in Fig. 1A and RE were sampled at D7. **(A)** Left panel: Representative FACS plots and comparison of total cell number of IL-4 (AmCyan) and IL-13 (dsRed)-expressing basophils (CD45^{low} TCRβ^{-}Siglec-F^{-}CD49b^{+}) and TCRβ^{+} T cells in the dermis of FITC-treated CT/4C13R^{Tg} mice, showing that basophils are the major producers of IL-4 and IL-13. Right panel: representative FACS plots and a comparison of frequency of parent (TCRβ^{-} cells), as well as number of IL-4 (AmCyan) and IL-13 (dsRed)- expressing TCRβ^{+} T cells in the dermis of CT/4C13R^{tg} and MCPT8^{DEP}/4C13R^{Tg} mice, showing that basophil depletion doesn't change IL-4 and IL-13 expression by TCRβ^{+} T cells. (**B)** Representative FACS plots and comparison of frequency of total for VCAM-1^{+} endothelial cells (ECs, CD45^{-}ESAM-1^{+}CD34^{+}) in RE of CT and MCPT8^{DEP}, showing that upon FITC treatment, VCAM-1^{+} cells are increased similarly in both CT and MCPT8^{DEP} mice, compared with untreated CT mice. Values are mean ± SEM (n≥3 mice per group).

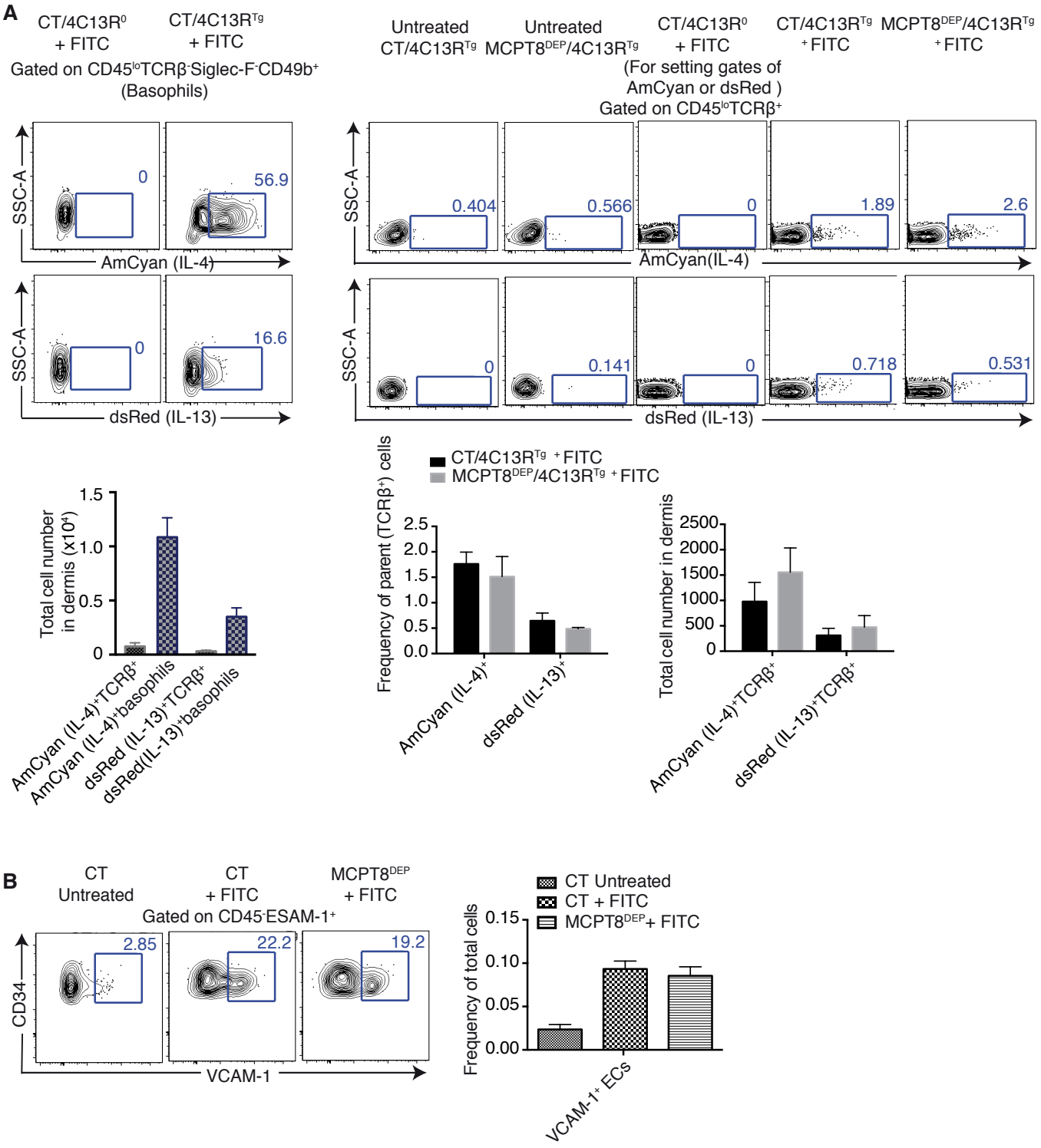
A
CT/4C13R⁰ + FITC
CT/4C13R^Tg + FITC
Gated on CD45^loTCRβ⁻Siglec-F⁻CD49b⁺ (Basophils)
SSC-A
AmCyan (IL-4)
dsRed (IL-13)
0
56.9
16.6
Untreated CT/4C13R^Tg
Untreated MCPT8^DEP/4C13R^Tg
CT/4C13R⁰ + FITC
CT/4C13R^Tg + FITC
MCPT8^DEP/4C13R^Tg + FITC
(For setting gates of AmCyan or dsRed)
Gated on CD45^loTCRβ⁺
AmCyan(IL-4)
0.404
0.566
1.89
2.6
0.141
0.718
0.531
Total cell number in dermis (x10⁴)
AmCyan (IL-4)⁺TCRβ⁺
AmCyan (IL-4)⁺basophils
dsRed (IL-13)⁺TCRβ⁺
dsRed(IL-13)⁺basophils
Frequency of parent (TCRβ⁺) cells
Total cell number in dermis
B
CT Untreated
CT + FITC
MCPT8^DEP + FITC
Gated on CD45⁻ESAM-1⁺
CD34
VCAM-1
2.85
22.2
19.2
Frequency of total cells
VCAM-1⁺ ECs

Figure S2

Figure S3. Basophils do not mediate eosinophil and neutrophil recruitment in skin. MCPT8^{DTR} and their littermate wildtype control mice were sensitized with FITC on left ear (LE) at D0, D1 and D2, and challenged with FITC on right ear (RE) at D6. To deplete basophils, mice were either i.p. injected with DT at D4 (depletion prior to the FITC challenge) **(A),** or at D7 (depletion posterior to the FITC challenge) **(B).** Representative FACS plots and comparisons of total cell number and frequency of total cells are shown for basophils, eosinophils, neutrophils and mast cells in RE at D7 **(A)** or at D10 **(B).** MCPT8^{DEP}: DT-injected MCPT8-DTR mice; CT: DT-injected wildtype control mice. **(C)** Balb/c mice were i.p. injected with MAR-1 or isotype antibodies twice per day on D1, D2 and D3 to deplete basophils, and subjected to FITC sensitization on LE at D0, D1 and D2, and challenge on RE at D6. REs were analysed at D7. Representative FACS plots, total cell number and frequency of total cells are shown for basophils, eosinophils, neutrophils and mast cells.

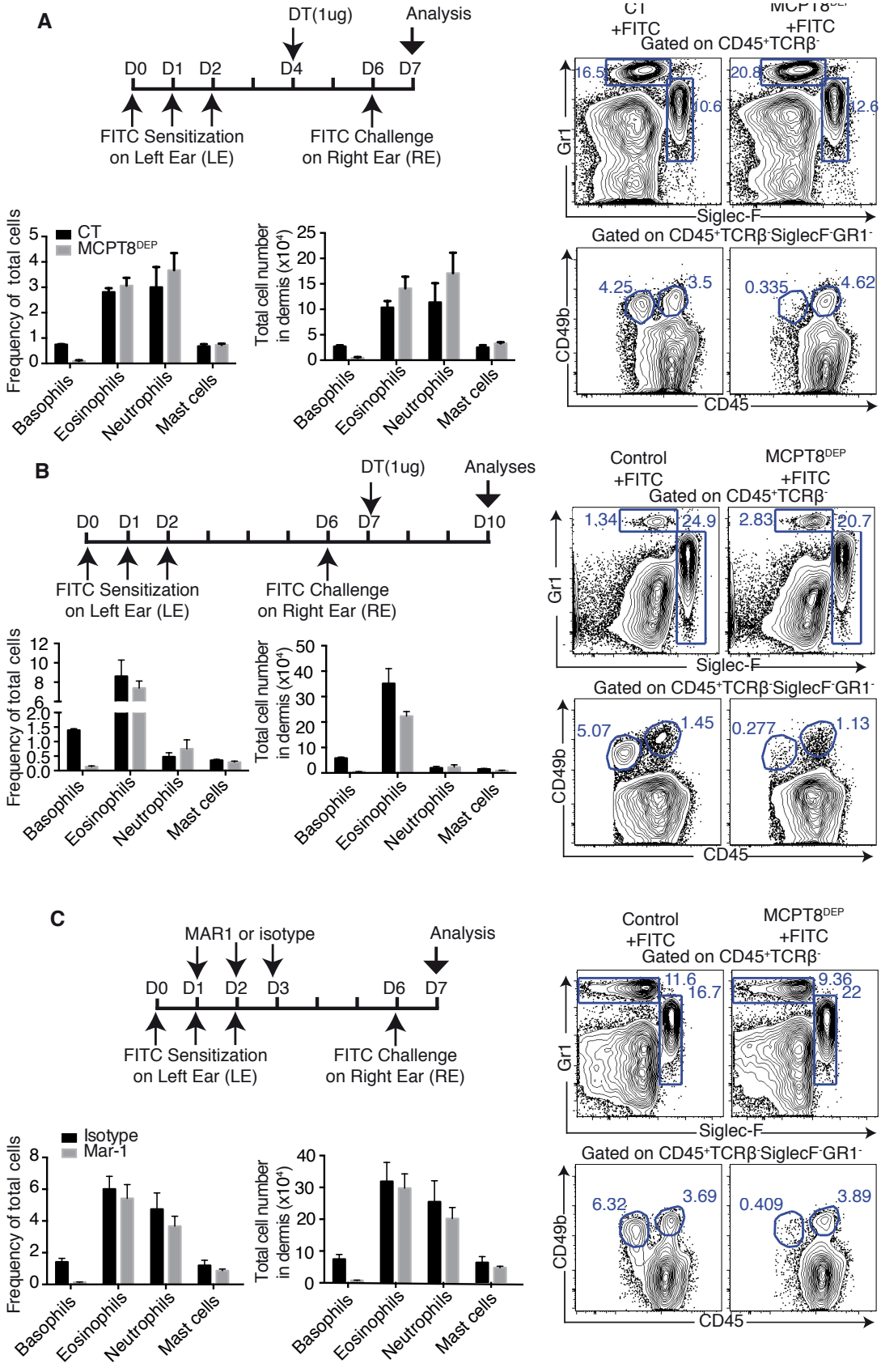
A
DT(1ug)
Analysis
D0 D1 D2 D4 D6 D7
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
CT
MCPT8DEP
Frequency of total cells
Total cell number in dermis (x10⁴)
Basophils
Eosinophils
Neutrophils
Mast cells
CT +FITC
MCPT8DEP +FITC
Gated on CD45+TCRβ-
16.5
10.6
20.8
12.6
Gr1
Siglec-F
Gated on CD45+TCRβ-SiglecF-GR1-
4.25
3.5
0.335
4.62
CD49b
CD45
B
DT(1ug)
Analyses
D0 D1 D2 D6 D7 D10
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
Control +FITC
MCPT8DEP +FITC
Gated on CD45+TCRβ-
1.34
24.9
2.83
20.7
Gated on CD45+TCRβ-SiglecF-GR1-
5.07
1.45
0.277
1.13
C
MAR1 or isotype
Analysis
D0 D1 D2 D3 D6 D7
FITC Sensitization on Left Ear (LE)
FITC Challenge on Right Ear (RE)
Isotype
Mar-1
Control +FITC
MCPT8DEP +FITC
Gated on CD45+TCRβ-
11.6
16.7
9.36
22
Gated on CD45+TCRβ-SiglecF-GR1-
6.32
3.69
0.409
3.89

Figure S3

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Carole EL HACHEM

Étude des basophiles dans l'inflammation allergique de la peau

Résumé

L'inflammation allergique de la peau est un état dans lequel l'hôte réagit de manière excessive à des allergènes en induisant une inflammation de type Th2. Un certain nombre d'acteurs cellulaires et moléculaires ont été impliqués dans cette pathologie, mais la façon dont ils agissent dans le réseau inflammatoire demeure largement inconnue. Les basophiles ont été reconnus pour leurs fonctions effectrices en allergie, cependant, comment ils sont recrutés et activés, ainsi que comment ils interagissent avec d'autres cellules dans l'inflammation allergique cutanée demeurent mal caractérisés. L'objectif de ce travail de thèse est d'étudier le recrutement, l'activation et la fonction des basophiles dans le réseau inflammatoire de la peau allergique. En employant un modèle expérimental murin de dermatite de contact allergique, combiné à des outils génétique de souris, d'immunologie et d'approches biologiques cellulaires/moléculaires, l'étude de ma thèse a démontré que l'IL-3 joue un rôle crucial dans l'extravasation des basophiles dans la peau allergique des souris (Partie I), et que l'invalidation des basophiles chez les souris Mcpt8DTR entraîne une réduction systémique des éosinophiles et des neutrophiles (Partie II). Ces études fournissent ainsi de nouvelles connaissances sur le recrutement, l'activation et la fonction des basophiles dans l'inflammation allergique de la peau.

Mots clés: allergie, peau, inflammation, basophiles, IL-3, souris

Abstract

Allergic skin inflammation is a state in which the host overreacts to otherwise innocuous allergens by inducing T helper type 2 inflammation. A number of cellular and molecular players have been implicated in the generation of allergic skin inflammation, but how they act and crosstalk in the inflammatory network remains largely unknown. Basophils have been recognized for their effector functions in allergy, however, how they are recruited to the inflamed tissue, get activated and crosstalk with other cells in inflammatory skin remain still incompletely understood. The objective of this PhD study is to investigate the recruitment, activation and function of basophils in the inflammatory network of allergic skin. Using experimental mouse model for allergic contact dermatitis, combined with mouse genetic tools, immunology and cellular/molecular biology approaches, the study of my thesis discovered that IL-3 plays a crucial role in basophil extravasation to mouse allergic skin (Part I), and that the depletion of basophils in Mcpt8DTR mice leads to a systemic reduction of eosinophils and neutrophils (Part II). These studies provide novel insights into the recruitment, activation and function of basophils in allergic skin inflammation.

Keywords: allergy, skin, inflammation, basophils, IL-3, mouse