The role of diacylglycerol acyltransferase 2 (DGAT2) in group 2 innate lymphoid cell function

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July 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

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ABSTRACT

Group 2 innate lymphoid cells (ILC2) are critical for initiation of the type 2 immune response at mucosal barrier sites, such as the lung. This response is essential for tissue repair and defense against extracellular pathogens; however, an overactive response can lead to immunopathologies, including allergies and asthma. Thus, the study of ILC2 regulation could lead to the identification of novel targets to modulate type 2 immune responses and dampen lung inflammation. Using an RNA-sequencing approach to identify novel genes important in ILC2 function, we show that IL-33 induced upregulation of diacylglycerol acyltransferase 2 (DGAT2), an enzyme that catalyzes the final step of triacylglycerol (TAG) synthesis. We hypothesized that DGAT2-mediated TAG synthesis is critical for ILC2 function, and aimed to **(a)** confirm the kinetics of DGAT2 expression in ILC2; **(b)** determine the effect of pharmacological DGAT2 inhibition in ILC2 *in vitro*; **(c)** investigate whether DGAT2 inhibition could dampen allergic lung inflammation in mice; and **(d)** characterize metabolic alterations in ILC2 upon IL-33-induced activation. We demonstrate here a critical role of DGAT2 in ILC2 biology and ILC2-mediated allergic airway inflammation. DGAT2 is rapidly upregulated upon IL-33 stimulation of ILC2, and pharmacological inhibition of DGAT2 *in vitro* significantly decreases ILC2 viability, proliferation, and type 2 cytokine production. In a preclinical mouse model, DGAT2 inhibition reduced allergic airway inflammation and ILC2 proliferation. Furthermore, there is an increase in lipid uptake, lipid storage, and oxidative metabolism upon ILC2 activation by IL-33. DGAT2 inhibition increases lipid storage and decreases oxidative metabolism, suggesting an inability to channel fatty acids into downstream metabolic pathways, such as β-oxidation of fatty acids, production of bioactive lipids, and phospholipid synthesis to sustain ILC2 proliferation. Further characterization of the unique metabolic requirements of ILC2s will contribute to our understanding of this recently

discovered cell population that is central to polarization of the type 2 immune response and could lead to the development of novel targets for the treatment of type 2 immunopathologies.

RÉSUMÉ

Les cellules lymphoïdes innées de type 2 (ILC2; « *group 2 innate lymphoid cells* ») sont essentielles à l'initiation de la réponse immunitaire de type 2 au niveau des sites de barrière muqueuse, tels que les poumons. Cette réponse est essentielle pour la réparation des tissus et la défense contre les pathogènes extracellulaires; cependant, une réponse hyperactive ou dérégulée peut entraîner des immunopathologies, notamment des allergies et de l'asthme. Ainsi, l'étude des ILC2 pourrait identifier de nouvelles cibles pour moduler les réponses immunitaires de type 2 et diminuer l'inflammation pulmonaire. Nous avons utilisé une approche de séquençage de l'ARN dans le but d'identifier de nouveaux gènes jouant un rôle important dans le fonctionnement des ILC2 et avons démontré que l'enzyme diacylglycérol acyltransférase 2 (DGAT2), qui catalyse la dernière étape de la synthèse du triacylglycérol (TAG), est régulée à la hausse lors de l'activation de ces cellules par l'IL-33. Nous avons émis comme hypothèse que la synthèse de TAG médiée par DGAT2 est critique pour la fonction des ILC2 et nous avons comme objectif de **(a)** confirmer le mécanisme d'expression de DGAT2 dans les ILC2; **(b)** déterminer l'effet de l'inhibition pharmacologique de DGAT2 dans les ILC2 in vitro; **(c)** déterminer si l'inhibition de DGAT2 pourrait atténuer l'inflammation pulmonaire; et **(d)** caractériser les altérations métaboliques dans les ILC2 après l'activation par l'IL-33. Nous démontrons, ici, un rôle essentiel de DGAT2 dans la biologie des ILC2 et l'inflammation allergique. L'expression de DGAT2 est rapidement augmentée après la stimulation par l'IL-33. De plus, l'inhibition pharmacologique de DGAT2 dans les ILC2 diminue leur viabilité, prolifération et production de cytokines de type 2. Dans un modèle murin préclinique, l'inhibition de DGAT2 réduit l'inflammation allergique au niveau des poumons et de la prolifération des ILC2. De plus, les ILC2 absorbent, entreposent plus de lipides et augmentent leur métabolisme oxydatif après une activation par l'IL-33. L'inhibition de DGAT2 augmente

l’entreposage des lipides et diminue le métabolisme oxydatif, suggérant une incapacité à utiliser des acides gras dans les voies métaboliques subséquentes, telles que la β-oxydation des acides gras, la production de lipides bioactifs et la synthèse des phospholipides pour soutenir la prolifération. Une caractérisation plus complète des besoins métaboliques uniques des ILC2 contribuera à notre compréhension de cette population cellulaire récemment découverte qui est essentielle pour la polarisation de la réponse immunitaire de type 2 et pourrait conduire au développement de nouvelles cibles pour le traitement des immunopathologies de type 2.

ACKNOWLEDGEMENTS

First and foremost, thank you to Dr. Jörg H. Fritz for having faith in me from day one as a second-year undergraduate student who wanted to volunteer to prepare buffers and rack pipette tips. Since then, he has supported me as an undergraduate summer student, an honours student, and finally, a Master's student. It has been a long journey, and I thank him and all the other members of the Fritz lab. In particular, thank you to (soon-to-be Dr.) Barbara C. Mindt, who taught me nearly everything I know about lab work, and Dr. Claudia U. Duerr, who is one of the kindest, most brilliant people I have met in academia. Thank you as well to my advisory committee members, Dr. Martin Richer and Dr. Selena Sagan, for their guidance over the years; and to Julien Leconte and Camille Stegen, the flow cytometry technicians behind countless sorts.

I also want to thank the students I have trained over the years for their hard work; for reminding me to never stop questioning my methods, rationale, and knowledge; and for helping me discover my love of teaching. Of all these students, I particularly want to thank Maike T. Köllner, who quickly became a valuable lab member and a good friend. Thank you to all my friends—especially those on the lab floor—for their constant support, whether I ran out of an antibody, needed an opinion on my analysis, or simply wanted company in the student room. Thank you to my parents, who do not understand my work but wholeheartedly support me nonetheless; and to my brother, who never tires of my discussions of immunology, only to suggest how I could do my work more efficiently.

My Master's thesis was generously funded by fellowships from the Canadian Institute of Health Research (CIHR), les Fonds de recherche du Québec – Santé (FRQS), and the McGill Department of Microbiology and Immunology Graduate Excellence Award, for which I am incredibly thankful.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACAT	cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
ACP	acyl-carrier protein
AGPAT	acylglycerol-3-phosphate acyltransferase (AGPAT)
AHR	airway hyperreactivity
AnA	Antimycin A
APC	antigen-presenting cell
ARAT	retinol acyltransferase
AREG	amphiregulin
ARG1	arginase 1
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BCR	B cell receptor
BM	bone marrow
CACT	carnitine acylcarnitine translocase
cAMP	cyclic AMP
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
CDP	cytidine-5’-diphosphate
CGRP	calcitonin gene-related peptide
CHILP	common helper innate lymphoid progenitor
CLP	common lymphoid progenitor
CLR	calcitonin receptor-like receptor
CMT	Charcot-Marie-Tooth disease
CoA	coenzyme A
COX	cyclooxygenase
CPT	carnitine palmitoyl transferase

CRTH2	chemokine receptor homologous molecule expressed on T_H2 lymphocytes
CSR	class switch recombination
CXCR	C-X-C motif chemokine receptor
CysLT1R	cysteinyl leukotriene receptor 1
DAG	diacylglycerol
DAMP	danger-associate molecular pattern
DC	dendritic cell
DGAT	diacylglycerol acyltransferase
ECAR	extracellular acidification rate
Eomes	eomesodermin
ER	endoplasmic reticulum
ETC	electron transport chain
FA	fatty acid
$FABP_c$	cytoplasmic fatty acid-binding protein
$FABP_m$	membrane-associated fatty acid-binding protein
FA-CoA	fatty acyl-CoA thioester
FACS	fluorescence-activated cell sorting
FALC	fat-associate lymphoid clusters
FAT	fatty acid translocase
FATP	fatty acid transport protein
FBS	fetal bovine serum
FCCP	carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazine
FcεRI	Fc ε receptor I
GALT	gut-associated lymphoid tissue
GI tract	gastrointestinal tract
GILP	global innate lymphoid precursor
GPAT	glycerol-3-phosphate acyltransferase
GPCR	G protein-coupled receptor
G3P	glycerol-3-phosphate
IAV	Influenza A virus
ICOS	inducible T cell co-stimulator

ICOSL	ICOS ligand
Id2	inhibitor of DNA binding protein 2
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILCP	common precursor to ILCs
IL-1RAcP	IL-1 receptor accessory protein
ImmGen	Immunological Genome Project
i.n.	intranasal
i.p.	intraperitoneal
JNJ	JNJ-DGAT2-A
KLRG1	killer cell lectin-like receptor G1
LD	lipid droplet
LDL	low-density lipoprotein
Lin	lineage
LO	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
LTi cell	lymphoid tissue inducer cell
MAG	monoacylglycerol
MAM	mitochondrial-associated membrane
MAPK	mitogen-activated protein kinase
MBOAT	membrane-bound *O*-acyltransferase
MHC	major histocompatibility complex
MOGAT	monoacylglycerol acyltransferase
MPP	multipotent progenitor cell
MyD88	myeloid differentiation primary response 88
M1	classically activated macrophage
M2	alternatively activated macrophage
NBNT	non-B non-T

NFκB	nuclear factor κ B
NK cell	natural killer cell
NK-T cell	natural killer T cell
NLR	Nod-like receptor
NMU	neuromedin U
OCR	oxygen consumption rate
Oligo	oligomycin
PAMP	pathogen-associated molecular pattern
PAP	phosphatidate phosphatase
PBMC	peripheral mononuclear cell
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PF	PF-06424439
PG	prostaglandin
PGHS	prostaglandin H synthase
PI	phosphatidylinositol
PLA2	phospholipase A2
PLC	phospholipase C
PLZF	promyelocytic leukaemia zing finger protein
pNK	NK cell precursor
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PS	phosphatidylserine
RAG	recombination-activating gene
RLH	RIG-I-like helicase
RORα	retinoic acid receptor-related orphan receptor α
Rot	rotenone
SR-B2	scavenger receptor B2
siRNA	small interfering RNA
SM	sphingomyelin
STAT	signal transducer and activator of transcription

S1P	sphingosine-1-phosphate
TAG	triglyceride
TCA	tricarboxylic acid
TCR	T cell receptor
TGF-β	transforming growth factor β
T_H cell	T helper cell
T_{reg}	regulatory T cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor-associated factor 6
TSLP	thymic stromal lymphopoietin
TSLPR	TSLP receptor
UPR	unfolded protein response
WT	wild-type
2-DG	2-deoxyglucose
7-AAD	7-aminoactinomycin D

CONTRIBUTION OF AUTHORS

Protocols for isolation and expansion of murine bone marrow and lung ILC2s were established by Dr. Claudia U. Duerr, Connor D. A. McCarthey, and Barbara C. Mindt in Dr. Jörg H. Fritz's laboratory at McGill. Isolation and expansion of cells used in most murine experiments presented here (with exceptions listed below) were performed by myself.

The RNA-sequencing dataset **(Fig 7A)** was generously provided for my thesis by Barbara C. Mindt and Dr. David Langlais. The RNA kinetics data **(Fig 7B, C)** were provided by Barbara C. Mindt. Experimental setups for viability assays and Annexin V staining were established by Dr. Claudia U. Duerr, Barbara C. Mindt and Connor D. A. McCarthey, and adapted by myself.

The human peripheral ILC2 isolation and expansion protocol was established by Maike T. Köllner in Dr. Jörg H. Fritz's laboratory at McGill. ILC2 expansion for the experiments presented here was performed by either Maike T. Köllner or myself, and experiments were set up together.

The *in vivo* intranasal IL-33 administration and immunophenotyping protocol was established by Dr. Claudia U. Duerr and Barbara C. Mindt in Dr. Jörg H Fritz's laboratory at McGill. Barbara C. Mindt wrote the necessary animal use protocols and assisted with the intranasal administration. Data acquisition and analysis was performed by myself.

Lipid uptake assays, lipid staining assays, proliferation assays, and Seahorse analysis of ILC2s were established by myself. Preliminary lipidomics analysis (data not shown) was completed by Dr. Shama Naz in Dr. Dajana Vuckovic's laboratory at Concordia University.

All figures were edited by myself from graphs imported from GraphPad Prism and FlowJo software. All illustrations are my own original work. Dr. Jörg H. Fritz assisted in editing this thesis and provided valuable guidance throughout this project. Jorge Flores and Laurance Boubonnière assisted with French translation of the abstract.

CHAPTER 1 – INTRODUCTION & LITERATURE REVIEW

1.1 Introduction to Immunology

The immune system comprises a vast array of leukocytes derived from the primary lymphoid organs, the bone marrow and the thymus. Tissue-resident and circulating cells that make up the innate immune system provide immediate recognition of pathogens and instruct downstream adaptive responses, which provide a more robust, antigen-specific response (*1*).

The innate immune response is the body's initial defense against pathogens; this response distinguishes self from non-self through recognition of common molecular patterns expressed by bacteria, viruses, and fungi (*2*). These patterns were first called pathogen-associated molecular patterns (PAMPs), although the larger category of danger-associated molecular patterns (DAMPs) was proposed as a category to include mediators derived from sterile injury and tissue damage (*3, 4*). PAMPs are recognized by a variety of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), nuclear oligomerization domation (NOD)-like receptors (NLRs), and RIG-I-like helicases (RLHs). These PRRs can recognize a range of non-self structures including lipopolysaccharide (LPS) expressed on the surface of bacteria, peptidoglycan in the cell wall of bacteria, CpG islands in DNA, and foreign genetic material such as single- and double-stranded RNA (*5-8*). Engagement and dimerization of TLRs on the cell surface or within endosomes initiates downstream signaling mediated by nuclear factor κ B (NFκB) or mitogen-activated protein kinase (MAPK), which can lead to the production of inflammatory cytokines, interferons (IFNs), chemokines, and antimicrobial peptides (*5, 9-14*).

Upon recognition by the innate immune system, many pathogens will be engulfed by phagocytic cells, including macrophages, granulocytes, and dendritic cells (DCs) (*15*). Macrophages are the mature form of circulating monocytes, and are found in a variety of tissues

where they can rapidly clear foreign bacteria or apoptotic cells (*16*). Granulocytes include eosinophils, neutrophils and basophils, which are abundant in the blood, but only localize to tissues during an immune response. DCs are also phagocytic cells; however, their primary function is antigen presentation for activation of T and B lymphocytes rather than pathogen clearance (*17*, *18*). Phagocytic cells can take up microbes either through phagocytosis, receptor-mediated endocytosis, or in the case of DCs, macropinocytosis (*19*) .

DCs serve as the primary link between the early, innate immune response, and the adaptive response, which is a more robust, antigen-specific response with the capability of generating antigen-specific T cell and B cell memory, preventing reinfection by the same pathogen. After taking up pathogens, they travel to secondary lymphoid organs, primarily the draining lymph nodes, for antigen presentation to circulating T and B lymphocytes (*20*, *21*). Thus, they are considered professional antigen-presenting cells (APCs). Macrophages and B cells can also serve as APCs (*22*); however, DCs are considered the principal cell involved in activation of naïve T cells (*17*). Macrophages are primarily scavenger cells that clear pathogens and dead cells, and B cells can internalize antigen after binding to an antigen-specific surface immunoglobulin. Basophils and eosinophils have also been described to act as APCs *in vitro* (*23-30*), although their contribution to T cell activation *in vivo* is debated, and DCs are still considered the primary cell involved in antigen presentation to T cells and activation of adaptive immunity (*31*).

Circulating T cells have a vast array of antigen specificities, originating from gene rearrangement of their T cell receptors (TCRs) (*32*, *33*). Upon activation by an APC, they rapidly proliferate to generate a large quantity of cells with identical antigen specificities, a process known as clonal expansion. For this process to occurs, three signals of activation are necessary:

presentation of the T cell's cognate antigen by an APC; co-stimulation provided by an APC; and polarizing cytokines (*13, 15*).

T cells recognize antigens as peptides presented by major histocompatibility complex (MHC) molecules (*34, 35*). MHC class I molecules are present on most nucleated cells and present peptides from proteins synthesized in the cytosol (*36-38*) and are recognized by T cells expressing the T cell co-receptor CD8 ($CD8^+$ T cells) with cytotoxic ability. Upon activation, they target cells infected with viruses or intracellular bacteria (*34, 39*). In some cases, DCs can also present exogenous antigens on MHC class I molecules after phagocytosis of infected cells, a phenomenon known as cross-presentation (*40-42*). This allows initial activation of $CD8^+$ T cells within the secondary lymphoid organs, prior to clonal expansion and targeting of infected cells (*43*). MHC class II molecules, however, are generally only present on DCs, macrophages and B cells, allowing them to present engulfed and processed antigen from sites of infection (*34, 44, 45*). Antigens presented by MHC class II molecules are recognized by $CD4^+$ T cells, called helper T cells (T_H cells) because they lack cytotoxic capabilities, and instead secrete cytokines to accelerate and polarize the immune response.

Furthermore, MHC molecules are highly polymorphic, allowing T cells to differentiate between self- and non-self (*46, 47*). In most cases, for antigen recognition to occur, there must be presentation by a self MHC molecule, a concept known as MHC restriction. However, some T cells, called alloreactive T cells, can recognize antigen presented by non-self MHC, which initiates the rejection of foreign tissue such as grafts and organ transplant (*47*).

The second signal in T cell activation, co-stimulation, is provided by an APC in close proximity to the MHC-TCR interaction. In the case of a T cell interacting with peptide-MHC on an APC, co-stimulation is presented by the same APC that is presenting antigen. Lastly, APCs

such as DCs also deliver cytokines to the T cell to further direct or "polarize" the immune response; this is considered the third signal in T cell activation. The cytokines produced by APCs are often determined by the engagement of PRRs during antigen encounter; however, they are also influenced by cytokines produced by activated neighbouring cells (*13*).

Downstream of T cell activation, B cells mediate the humoral immune response upon activation and differentiation into antibody-secreting plasma cells (*48*). Before activation and differentiation to plasma cells, B cells express membrane-bound immunoglobulin, also called the B cell receptor (BCR), which has the same antigen specificity as the antibody it will secrete upon activation. Upon BCR binding to its cognate antigen, the BCR and antigen are internalized, processed, and presented by MHC class II molecules (*49*). Upon encountering T cells with the same T cell specificity, T cells provide activation signals to B cells, inducing B cell proliferation and differentiation (*50*). In turn, B cells can also provide cytokine signals to enhance T cell polarization (*51*). B cells can either immediately form plasmablasts that provide early, less specific antibody production, or they can enter germinal centres within the lymph nodes and differentiate to plasma cells (*48, 49, 52*). Plasma cells undergo somatic hypermutation and affinity maturation, during which the region coding for the variable region of the antibody undergoes rapid mutation, and B cells with higher antigen specificity are positively selected (*53, 54*).

The antibodies produced by plasmablasts and plasma cells have three main functions: neutralization to prevent adherence of e.g. a bacterium or a toxin; opsonization to enhance phagocytosis; and activation of the complement system, which further enhances opsonization and can lead to lysis of certain bacteria (*15, 55*). Five classes, or isotypes, of antibodies exist: immunoglobulin M (IgM), IgG, IgA, IgD and IgE. IgM is the first isotype of antibody produced upon antigen exposure and B cell activation; however, B cells undergo class switch recombination

(CSR), during which the portions of the genome coding for short sequences of the antibody are excised, leading to production of other antibody classes (*56*). Although it is generally of lower affinity than antibodies produced after class switching, IgM forms pentamers, which increases its avidity for antigen. IgG is the primary antibody isotype that circulates in plasma, while IgA is found in mucosal secretions at epithelial surfaces, where it functions as a neutralizing antibody (*57, 58*). IgD is also important in mucosal immunology, since circulating IgD can bind the surface of myeloid cells such as basophils and mast cells, and its crosslinking induces the release of antimicrobial, proinflammatory and B cell-stimulating factors (*58-60*). IgE is found primarily bound to the surface of mast cells via the Fc ε receptor I (FcεRI), and antigen binding induces mast cell degranulation (*61*).

Following the primary adaptive immune response, there is a contraction phase during which activated $CD4^+$ T cells, $CD8^+$ T cells, and some plasma cells either undergo apoptosis, or become long-lived memory cells. Long-lived plasma cells and the circulating antibodies they produce provide immediate antigen-specific protection against re-infection (*62*), and memory B cells circulate at a higher frequency than naïve B cells prior to infection (*63*). In the germinal centers in secondary lymphoid organs, somatic hypermutation and affinity maturation occur, followed by selection, leading to higher-affinity antibodies upon re-infection (*53, 64*). B cells can also undergo class switch recombination to generate higher affinity antibodies with more specialized purposes (*56*). Similarly, the affinity of TCRs is also refined during time. Thus, the secondary adaptive response is not only more rapid, but also more robust.

1.2 T Cell Polarization and Type 2 Immunity

Although immune responses to different pathogens share similar characteristics and patterns of immune cell activation, different categories of pathogens lead to different downstream immune responses, which can be divided into general categories with common patterns of cytokine secretion. The earliest patterns to be characterized were the type 1 and type 2 immune responses, which were described based on $CD4^+$ T cell activity upon activation (*65, 66*). Type 1 immunity is an inflammatory response directed towards virally infected cells and intracellular bacteria (*67*), which begins with macrophage polarization by PRR engagement towards an inflammatory phenotype, historically referred to as an M1 or "inflammatory" macrophage (*16*). M1 secrete interleukin (IL)-12 and IFN-γ, which act on T cells to polarize them towards a T helper type 1 (T_H1) phenotype, driven by the expression of the transcription factor T-bet (*68*). Upon polarization, T_H1 cells secrete IL-2 and IFN-γ to enhance downstream anti-viral and inflammatory responses (*69*).

Type 2 immunity, however, evolved as a response to parasitic helminths, and is also important in the response to other extracellular pathogens and in tissue repair following viral infection (*70*). This response is often activated by signals released by epithelial cells during tissue damage, which activate downstream innate and adaptive immune responses (*71*). Many of the cytokines secreted by innate immune cells and activated T cells during this response actively suppress type 1 inflammation, preventing excessive tissue damage and restoring immune homeostasis (*72, 73*). Macrophages in this response are referred to as "alternatively activated" or "M2" macrophages (*74-76*) and are activated primarily by IL-4, an important cytokine driving T_H2 differentiation through expression of the transcription factor GATA3 (*77-81*). Other cell types that are recruited to barrier sites, such as basophils, can also secrete IL-4 and contribute to T cell

polarization (*82*). Although DCs were originally considered to promote T_H1 polarization, it is now clear that DCs presenting antigens during a type 2 response, such as the response to helminths or tissue damage, also contribute to T_H2 polarization (*13*). T_H2 cells secrete IL-4, IL-5, and IL-13, which contribute to inflammatory cell infiltration; airway hyperreactivity; tissue repair through fibroblast recruitment; and eosinophil maturation and recruitment to barrier sites (*83-87*).

The type 2 immune response is also heavily mediated by IgE bound to FcεRI on the surface of mast cells and basophils (*88*). IgE is produced CSR in B cells during initial allergen challenge. While it is found in very low levels in the circulation and has little neutralizing ability, it binds FcεRI with high affinity (*56*). Mast cells are long-lived, tissue-resident cells that bind IgE produced during initial immune challenge; whereas basophils are short-lived cells recruited to inflammatory sites (*61*). Upon antigen binding, mast cells and basophils degranulate and release large amounts of inflammatory mediators into the tissue environment (*61, 89, 90*). One such inflammatory mediator is histamine, which exacerbates many hallmarks of allergic disease, including inflammation, smooth muscle contraction, bronchoconstriction, mucus production, and airway hyperreactivity (*91*). During evolution, this response was important in the clearance of parasitic helminths, other mucosal pathogens, and substances such as venoms (*92, 93*). However, an overactive type 2 response can lead to severe immunopathologies, such as in allergic disease or asthma (*72, 73*).

Since the first descriptions of the type 1-type 2 paradigm, many additional subsets of T_H cells have been described. One such subset are T_H17 cells, which polarize in response to IL-1, IL-6, IL-23, and transforming growth factor (TGF)-β and secrete IL-17 upon activation (*94-96*). This response is most prominent in the gastrointestinal (GI) tract, where it might have an immunopathogenic role and lead to the development of immunopathology (*97, 98*). Interestingly,

regulatory T cells (T_{reg}) differentiate in response to TGF-β, but in the absence of IL-6 and IL-23 (*96, 99, 100*). T_{reg} cells generally express the transcription factor FoxP3 (*101*), and secrete IL-10, which has a broadly dampening effect on many immune cell types, and serves to downregulate activated immune cells after pathogen clearance, limit immunopathology from an overactive or extended immune response, and prevent autoreactivity to self-antigens (*102-104*). The importance of T_{reg} cells was apparent when mice deficient in T cells displayed marked multi-organ autoimmunity (*102*). T cell differentiation also appears to be highly plastic in response to local signals, although epigenetic modification may contribute to sustained T cell polarization (*105*). Nevertheless, immune responses are not as black-or-white as the initial type 1-type 2 paradigm suggested; instead, they are highly plastic and respond to a vast array of tissue signals in order to efficiently respond to tissue damage and a wide variety of pathogens.

1.3 Innate Lymphoid Cells

1.3.1 Discovery of ILC2

Between 2001 and 2012, several reports described a previously unknown lymphoid cell population that respond to the alarmins IL-25 and IL-33 and produce type 2 signature cytokines, including IL-5 and IL-13. The first reports by Fort *et al.* (*106*) and Hurst *et al.* (*107*) closely followed the discovery of IL-25 (IL-17E) as a novel cytokine involved in type 2 immunity (*106*). When administered to mice intraperitoneally (i.p.) or intranasally (i.n.), IL-25 induced production of the type 2 cytokines IL-4, IL-5 and IL-13, and histological characteristics of a type 2 response, including eosinophilia in the lung; increased mucus production in the lung, stomach and gastrointestinal (GI) tract; increased serum IgE and IgG_1; and increased airway hyperreactivity (AHR) (*106, 107*). IL-25 was also found to be upregulated in the lung and the gut following

infection with *Aspergillus fumigatus* and *Nippostrongylus brasiliensis*, respectively (*107*). However, when administered *ex vivo* to T cells and B cells, there was no detectable change in their production of type 2 cytokines (*106*). When administered *ex vivo* to unfractionated splenocytes, there were detectable levels of IL-5 and IL-13 produced, regardless off the presence of T and B cells (*106*). *In vivo*, the response to IL-25 was unaffected by the absence of T and B cells, natural killer (NK) cells, or mast cells (*107*). Upon IL-25 administration i.n. to wild type (WT) and recombination-activating gene (RAG)-deficient mice that lack T and B cells, a small population positive for intracellular IL-5 was identified, although this population did not express any definitive lineage markers (*107*). Although these cells were not isolated in either report, Fort *et al.* classified them as MHC-II^{high}CD11c^{low}F4/80^{low}CD4^{-}CD8α^{-} cells, dissimilar to any previously characterized leukocyte population (*106*).

These cells were further characterized in 2006 upon generation of a mouse deficient in IL-25 (*Il25*$^{-/-}$ mice) that showed a significantly impaired type 2 response upon infection with *N. brasiliensis* (*108*). *Il25*$^{-/-}$ mice were unable to expel the parasite from the gut, unlike WT mice that had cleared the infection within 10 days. This delay correlated with significantly lower levels of IL-5 and IL-13 early in infection in *IL25*$^{-/-}$ mice compared to WT. By day 10 of infection, however, IL-5 and IL-13 levels were elevated compared to WT, suggesting a compensatory mechanism. The authors reported the presence of a c-Kit^{+} and FcεRI^{-} non-B non-T cell (NBNT) population that was dependent on IL-25 and absent in *Il25*$^{-/-}$ mice but restored upon recombinant IL-25 (rIL-25) administration. These cells were confirmed as the source of IL-4, IL-5 and IL-13 early in *N. brasiliensis* infection, and upon purification by fluorescence-activated cell sorting (FACS) following either rIL-25 treatment or *N. brasiliensis* infection, they expressed mRNA of type 2 signature cytokines IL-4, IL-5 and IL-13 (*108*).

The next description of these cells came in 2010, when $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells were found in clusters in the mouse and human mesentery that the authors referred to as fat-associated lymphoid clusters (FALC) (*109*). Unlike lymph nodes, these lymphoid clusters surrounded by adipocytes rather than a capsule. Within these clusters, a Lin^{-} lymphocyte population was identified that resembled the IL-25-responsive populations previously described (*106*)(*107*)(*108*). These lymphocytes were further characterized to express CD45, IL-7Rα, Thy-1.2 (Thy1, CD90.2), and ST2 (IL-1RL1, a subunit of the IL-33 receptor) (*109*). Although present in *Rag2*$^{-/-}$ mice, they were absent in *Il2rg*$^{-/-}$ and *Il7*$^{-/-}$ mice, indicating that their development was independent of RAG-induced rearrangement of a TCR or BCR, but dependent on IL-2 and IL-7. Furthermore, these cells were shown to have 'helper' function in the proliferation of B1 cells and in IgA production by splenic B cells. Further mouse models of *N. brasiliensis* infection in *Rag2*$^{-/-}$ mice supported the hypothesis that these cells were critical in the very early response to infection, before the production of type 2 signature cytokines by T_H2 cells. Since they proposed that this lymphocyte population served as a 'helper' cell to T_H2 cells, Moro *et al.* proposed the term 'natural helper cells' (*109*).

Shortly thereafter, two reports published similar findings. Neill *et al.* (*110*) and Barlow *et al.* (*111*) identified similar cell populations, which they termed 'nuocytes', using *Il13-eGFP* reporter mice. Nuocytes were described as an early source of IL-13 in response to IL-25 or IL-33 administration *in vivo*, or infection with *N. brasiliensis*. These cells were determined to be positive for both ST2 and IL-17RB (IL-25R). In mice deficient for both of these receptors, there was no early IL-13 production in response to IL-25 or IL-33. Consistent finding by other groups (*106, 107, 112*), these cells were still present in *Rag2*$^{-/-}$ mice (*110, 111*). Upon purification by fluorescence-activated cell sorting (FACS), nuocytes could be expanded *ex vivo* with IL-33 and

IL-7 while maintaining high expression of all surface markers and without differentiation to other known leukocyte lineages. Adoptive transfer of WT nuocytes could restore the anti-helminth response in *Il17rb*$^{-/-}$ mice that otherwise had severe impairment of worm expulsion. In *Il13*$^{-/-}$ mice, adoptive transfer of WT nuocytes was sufficient for worm expulsion, indicating that these cells are a potent enough source of IL-13 to initiate worm expulsion. However, in *Rag2*$^{-/-}$ mice, nuocyte levels decrease later in infection, demonstrating that T cells are necessary for complete expulsion of the parasite (*110*).

In the same issue of Nature, Saenz *et al.* (*113*) published similar findings, but suggested that this lymphocyte population, which they identified in the gut-associated lymphoid tissue (GALT) using *Il4-eGFP* mice, more closely resembled a multipotent progenitor cell (MPP)-like population The fourth paper in 2010 to describe an innate IL-13-producing lymphocyte population was published by Price *et al.* (*114*). IL-4 and IL-13 reporter mice were used to confirm previous findings that this innate, Lin^{-} cell population responded rapidly to IL-25, IL-33, and intestinal infection with *N. brasiliensis*, and the authors suggested the term innate helper type 2 cells (I_H2).

Although intranasal challenge with *A. fumigatus* was shown to induce IL-25 in the lung in one of the first descriptions of these cells (*107*), their potential importance in immunopathologies of the lung, such as allergy and asthma, was first described by three independent groups in 2011 and 2012 (*115-117*). Chang *et al.* first demonstrated that infection of WT and *Rag2*$^{-/-}$ mice with Influenza A Virus (IAV) resulted in AHR within five days of infection, characterized by neutrophil and macrophage infiltration, but not eosinophilia (*115*). The early development of AHR independent of RAG indicated that this mechanism was independent of adaptive T and B cells. However, IAV infection increased IL-33 levels in the lung, and mice deficient in the IL-33 receptor (ST2, *Il1rl1*$^{-/-}$ mice) failed to develop the same response. The authors identified a “natural helper”

cell population that was $Lin^{-}ST2^{+}$c-$Kit^{+}Sca\text{-}1^{+}Thy\text{-}1.2^{+}CD25^{+}$ and found this population to be a source of IL-13 during IAV infection. Depletion of this population with an anti-Thy1.2 neutralizing antibody in *Rag2*$^{-/-}$ mice resulted in a depleted AHR response during IAV infection, and viral challenge of *Il13*$^{-/-}$ mice did not result in AHR without adoptive transfer of WT natural helper cells (*115*). Shortly thereafter, Monticelli *et al*. confirmed the expansion of this cell population in the lung upon IAV infection, and suggested a role in the maintenance and restoration of integrity of the epithelial barrier during and following infection (*116*). Similarly, Bartemes *et al.* (*117*) found that IL-33 administration i.n. to WT or *Rag1*$^{-/-}$ mice resulted in early eosinophil recruitment and mucus hyperplasia, which parallels findings in the gut upon *N. brasiliensis* infection (*117*). These observations are also consistent with a previously published report that demonstrated that IL-33 can induce a type 2 response in the lung independently of adaptive immunity, but did not identify a mechanism for this effect (*118*). When non-purified lung cells were cultured with IL-33, Bartemes *et al*. saw that the cells producing IL-5 and IL-13 did not express lineage markers but were $Thy1.2^{+}CD25^{+}CD44^{+}$ (*117*), like the previously described Lin^{-} lymphocytes described in the gut and mesentery (*112*). Upon purification by flow cytometric sorting, these cells were also determined to express ST2 (IL-1RL1), CD127 (IL-7Rα), Sca-1, CD69, Thy1.2, CD9, CD38 and ICOS. *In vivo*, the fungal allergen *Alternaria alternata* also induced eosinophilia, epithelial hyperplasia, and activation of the $Lin^{-}CD25^{+}CD44^{+}$ population, as determined by CD25 expression and early production of IL-5 and IL-13 (*117*). Taken together, these reports suggest similar phenotypes and functional roles of these innate lymphocytes in the lung as in the gut upon cytokine challenge, allergen exposure, or infection.

Further classification of these cells revealed that they depend on the transcription factor GATA3, revealing similarities between their differentiation and the transcriptional programs

determining T_H cell differentiation, since T_H2 cells also depend on GATA3 for their differentiation and function (*119-121*). Furthermore, retinoic acid receptor-related orphan receptor α (RORα) was shown to be necessary for the development of this cell subset (*122*), and mice lacking RORα had functional T_H2 cells but not innate IL-5- and IL-13-producing cells (*123*). However, deletion of RORα in adult mice did not result in defects in type 2 cytokine production in ILC2, indicating that this transcription was essential for ILC2 differentiation, but not for effector function upon activation (*120*). In 2013, the uniform nomenclature of "group 2 innate lymphoid cells" or ILC2 was proposed for all innate, lineage-negative lymphocytes producing type 2 cytokines that require the transcription factors GATA3 and RORα, including cells previously called natural helper cells, nuocytes, multipotent progenitor type 2 cells, and I_H2 cells (*124*). Although the expression of surface markers can vary depending on tissue localization and activation status, murine ILC2 are classically defined as lineage-negative cells expressing c-Kit, Sca-1, CD45, CD127 (IL-7R), CD25 (IL-2R), CD90 (Thy-1), T1/ST2 (IL-33R), IL-25R (IL-17RB), ICOS, and KLRG1 (*124-126*).

ILC2s in humans were first characterized in 2011 by Mjösberg *et al.* (*127*), although there were earlier descriptions of Lin^- immature NK cells from human cord blood and peripheral blood capable of IL-5 and IL-13 production (*128, 129*). From early evidence of this immature NK cell population, it was concluded that the ability to produce IL-5 and IL-13 was lost upon differentiation to NK cells *in vitro* (*128*). However, these small subsets described as immature NK cells may have been the first descriptions of ILC2s in humans. Mjösberg *et al*. further characterized ILC2s in humans as Lin^-CD127^+ lymphocytes expressing CD161, an NK cell marker, and the chemokine receptor homologous molecule expressed on T_H2 lymphocytes (CRTH2) (*130*). These cells, like murine ILC2s, were present in the human lung and gut, responded to IL-25 and IL-33, and produced IL-5 and IL-13 upon stimulation (*130*). Shortly after this first characterization,

additional descriptions of ILC2s in human tissues emerged (*116*). Although the surface markers that define them differ, ILC2s in mice and humans show the same tissue localization and effector function during type 2 inflammation.

1.3.2 ILC Differentiation and Subsets

Although ILC2s have been only relatively recently described, NK cells and lymphoid tissue inducer (LTi) cells are previously described lymphocytes that have the main features of ILCs: they are of lymphoid morphology, but both cell types lack rearranged antigen receptors and lineage markers present on myeloid cells (*131, 132*). Thus, NK cells and LTi cells were recently re-classified as belonging to the larger family of ILCs (*124, 133, 134*).

All ILC lineages differentiate primarily in the bone marrow from the same common lymphoid progenitor (CLP) as do T and B lymphocytes. The CLP first differentiates into a global innate lymphoid precursor (GILP), which gives rise to NK cell precursors (pNK) and the common helper innate lymphoid progenitor (CHILP) (*135, 136*). pNK cells differentiate to NK cells through expression of the transcription factors inhibitor of DNA-binding protein 2 (Id2), eomesodermin (Eomes), and T-bet (*137, 138*). LTi cells differentiate from the CHILP, which express high levels of Id2 and IL-7Rα, to LTi cells through the expression of RORγt (*139*). The CHILP can further differentiate into the common precursor to ILCs (ILCP), which is defined by its expression of the transcription factor promyelocytic leukaemia zinc finger protein (PLZF) (*140*). Before the characterization of ILCs, PLZF was previously associated with natural killer T (NK-T) cells and is not expressed at any point in the development of NK cells or LTi cells (*140*). From the ILCP, ILCs can differentiate to ILC1s, ILC2s or ILC3s depending on the expression of the transcription factors RORα, GATA3, T-bet, and RORγt (*133, 141*).

NK Cells

NK cells, also called cytotoxic ILCs, were first described in 1975 as cytolytic cells with anti-tumour activity against mouse Moloney leukemia cells *in vitro* (*142*). These cells were further characterized to depend on the transcription factor T-bet and produce IFN-γ in the presence of IL-12 and IL-18 produced during viral infections (*138*). Their production of IFN-γ is critical in the type 1 immune response against viral infections (*133*); hence, some years after the discovery of other innate lymphocyte populations, NK cells were included in the classification of group 1 ILCs (ILC1s) (*124*). In addition to their production of IFN-γ, NK cells are distinct from other ILC1s in their anti-tumor and anti-viral properties, which are similar to those of $CD8^+$ cytotoxic T lymphocytes (*138*). NK cells constitute a high percentage (5-15%) of circulating lymphocytes and play a critical role in immunosurveillance towards virally infected or cancerous cells, towards which they can direct cytotoxic proteins such as perforin and granzyme (*143, 144*). These cells are a heterogeneous group in terms of surface receptor expression and tissue localization. While the majority of NK cells are found in the circulation at steady state (*145*), distinct subsets of NK cells can be found in vasculature associated with particular tissues, including the liver, kidneys, uterus, bone marrow and mucosal sites such as the lung and the gut (*144*).

ILC1s

The group of ILC1s also comprises non-cytotoxic ILC1s, which, like NK cells, produce IFN-γ upon stimulation with IL-12 and IL-18 (*124, 133, 134, 146*). However, there is some degree of functional plasticity between ILC1s and group 3 ILCs, influenced by cytokine signals in the tissue influencing the differential expression of the T-bet and RORγt (*139, 147, 148*). Furthermore, as development and differentiation of ILCs from the CLP was better characterized, ILC1s were found

to be more closely related to ILC2s and ILC3s than to NK cells, and are now considered to be their own subset of ILCs (*134*). While NK cells are most abundant in the circulation at steady state, ILC1s are considered tissue resident cells that expand during inflammation at sites of viral infection and dissemination, such as the liver, spleen, kidney, and mucosal barrier sites including the lung, gut and skin (*145*). They respond to myeloid-derived proinflammatory cytokines more rapidly than circulating NK cells during the antiviral response (*146*). Together, ILC1s and NK cells orchestrate early antiviral responses alongside myeloid cells, and contribute to polarization of the downstream adaptive immune response towards an antiviral, or T_H1 phenotype (*134, 145*).

ILC3s

Although group 3 ILCs are the most heterogeneous group of ILCs, they all depend on RORγt for differentiation, although some subsets later downregulate its expression (ex-RORγT^+ ILC3s) (*133, 149*). ILC3s are most abundant in the gut, where they play an important role in the T_H17 response, including the response to small extracellular bacteria and fungi, and autoimmunity (*150, 151*). They were first described as a subset of NK cells in the gut that produces IL-22 critical for mucosal immunity, since some subsets express the NK cell markers NKp46 (in mice) or NKp44 (in humans) (*152-154*). However, they do not produce IFN-γ or have any cytotoxic capacity; instead, they produce IL-22, IL-26 and leukaemia inhibitory factor (LIF) (*155*). Hence, they were originally termed NK-22 cells and postulated to have a role in immune homeostasis at barrier sites (*152*). However, ILC3s display functional heterogeneity, and have also been suggested to drive pathogenic responses and chronic inflammation in the gut, such as Crohn's disease and ulcerative colitis (*156, 157*).

LTi cells

LTi cells, which are critical during embryogenesis for the formation of secondary lymphoid organs (*132*), were first re-classified as a subset of group 3 ILCs due to their dependence on the transcription factor RORγt and their ability to produce IL-17A and IL-22 after stimulation (*158*). However, like NK cells, they are now considered to be a subset of ILCs distinct from group 3 ILCs due to developmental differences from ILC3s (*159*).

1.4 ILC2 Regulation

ILC2s, like other ILC subsets, lack RAG-dependent rearranged antigen-specific receptors, unlike T and B lymphocytes. Instead, they are regulated by a vast network of signals, including activating and suppressive cytokines, eicosanoids and neuropeptides (*125, 160, 161*) **(Fig 1).**

1.4.1 Positive Regulation of ILC2

The primary cytokines modulating ILC2 activation are the alarmins IL-33 and IL-25. IL-33 belongs to the IL-1 family of cytokines and is constitutively expressed by epithelial cells, but lacks the signal peptide sequence necessary for secretion (*162, 163*). In response to tissue injury by parasitic helminths, viruses, or allergens, IL-33 is rapidly released by the damaged cells (*164-166*). However, apoptosis of epithelial cells does not trigger IL-33 release, since caspases inactivate IL-33 prior to cell death (*167, 168*). Upon cleavage by proteases to its more active form, IL-33 binds to its receptor, a heterodimeric protein composed of ST2 and IL-1 receptor accessory protein (IL-1RAcP) (*169, 170*). ILC2s are characterized by high ST2 expression, although other cells in the type 2 immune response also express ST2, including mast cells, basophils, T_H2 cells, and some T_{reg} populations (*171-173*). Upon ST2 and IL-1RAcP binding by IL-33, the adaptor protein

myeloid differentiation primary response 88 (MyD88) is recruited to the intracellular Toll/interleukin-1 receptor (TIR) homology domains of ST2 (*174, 175*). This initiates a signaling cascade through either NF-κB or MAPK signaling, leading to GATA3 phosphorylation and transcription of type 2 signature cytokines (*120*). GATA3 further enhances ST2 expression, amplifying the response in a positive feedback loop (*173*). Although both mouse and human ILC2s respond to IL-33 to some degree, the magnitude of the response differs depending on the tissue source and the presence of other signals. For example, human ILC2s respond poorly to IL-33 alone *in vitro* in the absence of IL-2 (*176*); however, murine ILC2s respond to IL-33 alone, though the response is amplified by IL-2 or IL-7 (*117*).

IL-25, also called IL-17E, is a member of the IL-17 family of cytokines and is released primarily by tuft cells, a subset of epithelial cells found in the intestine and upper airways (*177-179*), but can also be released by mast cells upon engagement of FcεRI (*180*) and alveolar macrophages upon allergen challenge (*181*). Upon binding to its receptor, a heterodimeric receptor of IL-17RA and IL-17RB, the NF-κB and MAPK signaling pathways are activated through the adaptor proteins tumor necrosis factor-associated factor 6 (TRAF6) and Act-1 (*182*). *In vitro*, ILC2s show a very weak response to IL-25 alone, although intranasal administration *in vivo* leads to ILC2 proliferation and type 2 inflammation in the lung, albeit to a lesser degree than intranasal IL-33 administration (*183*).

In addition to IL-25 and IL-33, ILC2 activation can be enhanced or sustained by a variety of other cytokines, sometimes referred to as "co-stimulatory cytokines" (*160*) **(Fig 1)**. Many of these cytokines signal through the common gamma chain (γ_c), including IL-2, IL-4, IL-7 and IL-9, leading to activation of several signal transducer and activator of transcription (STAT) proteins, primarily STAT5 by IL-2, IL-7 and IL-9, and STAT6 by IL-4 (*184, 185*). This pathway is also

activated by thymic stromal lymphopoietin (TSLP), another alarmin released by epithelial cells and dendritic cells, which signals through a heterodimer of the IL-7 receptor α chain (IL-7Rα) and the TSLP receptor (TSLPR) (*186, 187*). STAT5 activation upregulates several genes contributing to sustained ILC2 activation, including GATA3 and IL-2 and IL-4 receptors (*188-192*), and STAT6 further contributes to GATA3 expression (*193*). IL-2 is produced primarily by activated $CD4^+$ T cells and acts on a variety of lymphoid cells as a survival signal (*194, 195*), while IL-7 and TSLP are produced primarily by epithelial cells, fibroblasts and stromal cells (*186*). Upon activation, ILC2s produce both IL-4 and IL-9, but IL-4 is also produced by basophils (*196, 197*), and IL-9 is produced by a variety of leukocytes, including $CD4^+$ T cells and mast cells (*198-203*). Thus, many signals from diverse leukocytes involved in the innate and adaptive immune response at mucosal barrier sites contribute to sustained ILC2 activation.

Like T cells, ILC2 activation can be further enhanced through engagement of co-stimulatory molecules (*125, 160*). The best characterized co-stimulatory molecule expressed by ILC2s is inducible T cell co-stimulator (ICOS, CD278), which was noted to be expressed by ILC2s early in their characterization (*110, 111, 117*). Previously, ICOS, a member of the CD28 superfamily, was known to be expressed on T cells, including activated T cells, memory T cells, and T_{reg} cells (*204*). ICOS deficiency in ILC2s led to increased apoptosis, impaired cytokine production, and decreased proliferation, and animals lacking ICOS or administered an anti-ICOS blocking antibody show significantly reduced ILC2 numbers at mucosal surfaces, as well as decreased pathology during lung inflammation (*205, 206*). Additionally, ILC2s express the ICOS ligand (ICOSL) (*205, 207*), which is canonically expressed on APCs (DCs, macrophages, B cells) to provide co-stimulation to T cells during activation (*204*). ICOS-ICOSL interaction was shown to be necessary for ILC2 survival in purified ILC2 cultures, indicating that ICOS engagement by ICOSL expressed by

neighbouring ILC2s was sufficient to sustain ILC2 activation through STAT5 activation (*205*). However, co-culture of ILC2s with a mast cell line expressing ICOSL also increased cytokine production by ILC2s in the presence of IL-33 and IL-2 compared to cytokine stimulation alone (*208*), suggesting that other cell types may also sustain ILC2 activation through ICOSL expression. ILC2 have further been suggested to directly interact with ICOS expressed on T cells, particularly T_{reg} cells, limiting acute inflammation and maintaining tissue homeostasis (*207, 209*).

1.4.2 Negative Regulation of ILC2

Certain cytokines, such as IFNs, IL-27, and IL-10, can also have suppressive effects on IL-33-induced ILC2 activation **(Fig 1)**. Both type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) mediate this suppressive effect through STAT1 activation (*207, 210, 211*). IL-27, a member of the IL-12 family of cytokines, also activates STAT1 to antagonize IL-33-induced ILC2 activation (*210, 212, 213*). During inflammation, IFNs are produced by T cells, NK cells and plasmacytoid DCs (*214-216*), while IL-27 is produced primarily by conventional DCs (*217, 218*). IL-10, produced primarily by regulatory T (T_{reg}) cells (*219*), is a broadly anti-inflammatory cytokine that suppresses ILC2 function, though this effect is much weaker than suppression by IFNs or IL-27, and varies depending on the stimulating cytokines provided (*209, 220*).

In contrast to the co-stimulatory molecule ICOS, which sustains ILC2 activation (*205-208*), the co-inhibitory receptor killer cell lectin-like receptor G1 (KLRG1) is expressed by ILC2s and has been suggested to restrain ILC2 activation (*221*). KLRG1 is also expressed on NK cells and activated T cells (*222*), which binds to E-Cadherin expressed primarily on epithelial cells and keratinocytes (*223*). Given that E-Cadherin expression on keratinocytes is downregulated in atopic dermatitis, a condition resulting from an excessive type 2 immune response in the skin, KLRG1-

E-Cadherin interactions in ILC2 may be critical in restraining ILC2 function, particularly in the skin (*221*).

1.4.3 Eicosanoids

In addition to cytokine signals, some bioactive lipids produced during inflammation, known as eicosanoids, can modulate ILC2 function (*160*). Eicosanoids comprise prostaglandins (PGs), prostacyclins, thromboxanes, leukotrienes (LTs) and lipoxins (LXs) (*224*). These molecules, synthesized from the long-chain FA arachidonic acid (AA), have many physiological effects, including mediating inflammation, maintaining blood pressure, and inducing blood clotting (*224, 225*). They are similar to hormones in that they bind to G protein-coupled receptors (GPCRs); however, they rapidly break down and thus only act in a paracrine matter, rather than being transported into the bloodstream (*224*). During allergic pulmonary inflammation, they are released primarily by mast cells, although eosinophils, DCs, and lymphocytes can also produce various eicosanoids (*226-228*).

Their effects are heterogeneous; some eicosanoids are pro-inflammatory, such as PDG_2 and LTA_4, while others such as PGE_2 and LXA_4 are considered pro-resolving (*229*). The receptor for PGD_2, CRTH2, is expressed on leukocytes involved in the type 2 immune response, including T_H2 cells (*230-232*), eosinophils (*233-235*), basophils (*236*), and ILC2s found in the peripheral blood (*130, 237*). However, both murine and human ILC2s downregulate CRTH2 expression upon tissue localization (*237*). The importance of PGD_2 and other eicosanoids in airway inflammation has been well documented (*226, 238-242*), and these pathways remain a promising treatment for asthmatic airway inflammation (*243-249*).

Several eicosanoids regulate ILC2 function, including PGs, LTs, and LXs. *In vitro,* PGD_2 has been demonstrated to mediate chemotaxis of human ILC2s and increase cytokine expression (*250-252*). Furthermore, ILC2s were recently shown to produce PGD_2 *de novo*, suggesting a feedforward mechanism in ILC2 activation (*253*). It has been suggested that PGD_2 may regulate ILC2 accumulation at mucosal sites through CRTH2 signaling (*237, 252, 254*), and a selective antagonist of CRTH2 currently in clinical trials for the treatment of asthma, Fevipiprant, has been shown to inhibit ILC2 migration towards PGD_2 *in vitro* (*255*). However, whether PGD_2 regulates ILC2 function in tissues where ILC2s downregulate CRTH2 expression is unclear. Additionally, LTD_4, LTC_4, and LTE_4 have been shown to stimulate ILC2s through their receptor, cysteinyl leukotriene receptor 1 (CysLT1R) (*256-259*). Conversely, PGI_2, PGE_2 and the pro-resolving eicosanoid LXA_4 have been shown to down-regulate cytokine production by activated ILC2 (*251, 260-262*). Thus, ILC2s appear to be heavily influenced by the network of eicosanoids released during inflammation at mucosal barrier sites.

1.4.4 Neuropeptides

Recent studies have highlighted interactions between ILC2s and the nervous system through the neuropeptides neuromedin U (NMU) and calcitonin gene-related peptide (CGRP). Cross-talk between the nervous system and the immune system has been well documented throughout the GI tract, which is highly enervated and often referred to as the "gut-brain axis" (*263*); however, until recently, the role of ILC2s in neuro-immune interactions was not well studied, and neuro-immune interactions in the lung remain poorly characterized.

Single-cell RNA sequencing of ILCs revealed *Nmur1* as a novel gene expressed in ILC2 and upregulated upon activation by IL-25 (*264, 265*). *Nmur1* codes for the receptor of NMU, which

has been previously described to be an important neuropeptide in the gut-brain axis (*266*). Administration of NMU and IL-25 in parallel *in vivo* elevated the inflammatory response and ILC2 proliferation in the lung, and *Nmur1* deficiency diminished ILC2 numbers in response to HDM challenge or *N. brasiliensis* infection (*264*, *265*). When stimulated with NMU *ex vivo*, ILC2s proliferated and rapidly expressed type 2 signature cytokines (*265*). Furthermore, ILC2s in the GI tract were found closely associated with NMU-secreting neurons by immunohistochemistry (IHC) (*265, 267*). It was determined that NMU can activate ILC2s and induce type 2 cytokine production independently of IL-33 and more rapidly than either IL-33 or IL-25 (*265, 267*).

More recently, CGRP was shown to downregulate NMU- and alarmin-induced ILC2 activation. CGRP was previously demonstrated to have either pro- or anti-inflammatory effects, depending on the tissue context (*268, 269*). Using single-cell RNA sequencing, a subset of ILC2s expressing a high level of *Il5* were also identified to express CGRP and its receptor, calcitonin receptor-like receptor (CLR) (*270, 271*). *In vitro*, CGRP was shown to antagonize NMU- or IL-33-induced ILC2 proliferation and IL-13 production, although it increased IL-5 production; *in vivo*, CGRP suppressed IL-33-mediated responses to allergens and *N. brasiliensis* (*270*). Mice deficient in CLR showed an elevated response to IL-33 and increased ILC2 proliferation *in vivo* (*272*). The effect of CGRP on ILC2s appears to be mediated by cyclic AMP (cAMP) production as a second messenger in CGRP signaling, since administration of a cell-permeable cAMP analogue to ILC2s *in vitro* mimicked the effect of CGRP (*270*), and CGRP stimulation of ILC2s induced cAMP response genes (*271*).

Although many questions remain regarding the interaction between ILC2s and the nervous system, these recent reports demonstrate a direct interaction between neurons in close proximity

to ILC2s at mucosal sites, and highlight the vast diversity of local signals that can influence ILC2 effector function and modulate downstream innate and adaptive immune responses.

1.4.5 Tissue Tropism and Migration

ILC2s are found primarily in mucosal tissues at barrier sites and are present in very low numbers in the circulation (*134, 273*). While ILC2s increase in number during immune challenge, this effect is thought to be mediated by rapid proliferation rather than recruitment from the bone marrow (*274, 275*). Parabiosis approaches have revealed that donor ILC2 do not accumulate in the lung after IL-33 challenge or *N. brasiliensis* infection (*213, 276*). Only two weeks post-*N. brasiliensis* infection a detectable change in donor-derived ILC2 was observed in the lung; however, the rapid increase in ILC2 numbers in the lung immediately after challenge cannot be attributed to ILC2 recruitment. Rather, prolonged inflammation, such as the type 2 response induced by *N. brasiliensis*, may lead to re-seeding of these tissue-resident cells from the bone marrow (*276*).

Nevertheless, ILC2 egress from the bone marrow has not yet been well characterized. ILC2 begin to accumulate in the lung during ontogeny (*277, 278*); however, knowledge of their localization to mucosal surfaces during development is limited. Stier *et al.* demonstrated that IL-33-deficient or ST2-deficient mice show retention of functional ILC2 precursors within the bone marrow, although this effect can be reversed by intravenous IL-33 administration in animals with functional ST2 (*279*). In the absence of IL-33, ILC2 precursors in the bone marrow retained high expression of C-X-C motif chemokine receptor 4 (CXCR4), which retains developing leukocytes in the bone marrow (*280*). This study further demonstrated that upon non-lethal irradiation, ILC2s

were repopulated from the donor animal in a parabiotic pair, emphasizing that some ILC2 migration is possible under certain conditions, and is most likely mediated by the IL-33-ST2 axis.

Furthermore, sphingosine-1-phosphate (S1P), an important factor in regulating T cell egress from the thymus and secondary lymphoid organs (*281*), was also shown to influence ILC2 trafficking (*282*). Parabiotic mice were also used in this study, and the authors found no exchange of ILC2s in the lung between parabiotic partners. Donor ILC2s were found in the small intestine, but this increase was only significant after 6-8 months of parabiosis. However, upon intraperitoneal IL-25 administration or *N. brasiliensis* infection, there was migration of ILC2s from the small intestine to the lung. Furthermore, ILC2s were found to express S1P receptors upon IL-25 challenge, and inhibition of the S1P signaling pathway significantly reduced ILC2 accumulation in the lung, but not ILC2 proliferation (*282*). Another group found expression of *S1pr1*, the gene coding for the S1P receptor, in naïve lung ILC2, although its expression decreased with IL-33 stimulation (*283*).

Three recent studies examined the roles of chemokines in ILC2 homing to the lung, notably the chemokine receptors C-C motif chemokine receptor 6 (CCR6) and CCR8. In one study, a selective CCR8 antagonist dampened ILC2 responses, although CCR8 did not mediate ILC2 migration *in vitro* (*284*). ILC2s were found to secrete one of the CCR8 ligands, CCL1, upon stimulation, and administration of exogenous CCL1 increased ILC2 survival. *In vivo*, the immune response to *N. brasiliensis* infection was considerably dampened in *Ccr8*$^{-/-}$ mice. Another recent study showed actin remodeling, ameboid-like movement of ILC2s, and ILC2 aggregation within the airways in regions of high expression of CCL8, another CCR8 ligand (*285*). ILC2 homing to the lung was also suggested to be mediated by CCR6 and its ligand, CCL20, during inflammatory conditions (*286*). Collectively, these data suggest that although ILC2s are still considered to be

tissue-resident cells, they have some migratory capacity, and the factors influencing their migration remain to be further characterized.

1.4.6 Effector Function

Just as the signals modulating ILC2 activation are diverse and come from a variety of cells, cytokines secreted by ILC2s upon stimulation act on various innate and adaptive immune cells. Upon activation, ILC2s secrete IL-4, IL-5, IL-6, IL-9, IL-13, and amphiregulin, to module downstream responses (*274, 287*) **(Fig 2).**

Although IL-4 production is observed from human ILC2 upon stimulation *in vitro* (*288*), and murine ILC2 express *Il4* mRNA, murine ILC2 produce very low amounts of IL-4 protein *in vitro* upon stimulation with IL-33 or IL-25 (*110, 114*). Whether there is sufficient IL-4 production *in vivo* to influence downstream immune responses is unclear. However, IL-4 produced by basophils and T_H2 cells during the type 2 response contributes to ILC2 activation and feeds back onto mast cells and basophils (*197*). IL-4 further contributes to the type 2 immune response by polarizing macrophages towards an M2 phenotype (*289, 290*). It also acts on mature B cells to induce CSR to produce IgE and IgG_1 (*291-293*), and protects B cells from apoptosis through STAT6 signaling (*294, 295*).

IL-5 is particularly critical for its role in inducing eosinophil maturation in the bone marrow (*84, 86*). Upon entry into the inflamed tissue, IL-5 contributes to eosinophil degranulation (*84, 85, 296*). Furthermore, IL-5 acts on B1 cells, which are a lineage of B cells that rapidly produce antibodies and are maintained through self-renewal in the peritoneal cavity (*297*). B1 cells are located in close proximity to ILC2s within FALC, and ILC2-derived IL-5 enhances early antibody production during lung inflammation or pleural infection (*298, 299*).

IL-9 was first characterized to act on mast cells to promote their expansion (*300*). Upon crosslinking of surface IgE on mast cells followed by degranulation, IL-9 can also enhance expression of type 2 signature cytokines by mast cells (*301*). Furthermore, IL-9 also acts on ILC2s to sustain STAT5 signaling and promote survival (*197, 199, 201, 202*).

IL-13 acts on many immune and non-immune cells present at mucosal barrier sites, including goblet cells, smooth muscle cells, epithelial cells, fibroblasts, macrophages, and dendritic cells. At steady state, mucous has a protective role in the lung and the GI tract; however, excessive production of mucus by goblet cells is characteristic of a type 2 immune response, and can become chronic in the case of allergy and asthma (*302*). IL-13 induces transcription of genes involved in the production, packaging, and secretion of mucin in goblet cells, leading to excess mucus production (*73*). Fibroblasts are critical in the type 2 response for wound healing; however, excessive fibroblast recruitment, collagen deposition, and fibrosis are characteristic of the dysregulated type 2 response in allergy and asthma (*302*). The mechanisms underlying this excessive response are not as well characterized as those underlying mucus hypersecretion by goblet cells, although it has been suggested that to be mediated in part by arginase-1 (ARG1) expression by macrophages in response to IL-13 (*303-305*). ARG1 hydrolyzes the amino acid L-arginine to generate precursors for the synthesis of collagen by fibroblasts, although it is unclear if this pathway is the primary pathway leading to fibrosis *in vivo* (*73, 306*). Notably, ILC2 also express Arg1, although it may be more important in regulation of metabolism and proliferation rather than having a direct effect on collagen deposition at barrier sites (*307*). Furthermore, IL-13 also acts on macrophages to induce production of TGF-β, another important cytokine in regulating tissue fibrosis (*308, 309*). In addition to inducing ARG1 and TGF-β expression, ILC2-derived IL-13 acts on macrophages to polarize them towards the “alternatively activated” or M2 macrophage

subtype (*310, 311*). IL-13 also induces production of eotaxins by epithelial cells, which enhance IL-5-induced eosinophilia (*312*). Furthermore, early production of IL-13 is critical for inducing an adaptive T_H2 response, since ILC2-derived IL-13 promotes migration of lung DCs to the draining lymph node for antigen presentation to naïve T cells (*313*).

Amphiregulin (AREG), which is also produced by epithelial and mesenchymal cells, has an important role in tissue repair following injury or chronic inflammation (*314*). Amphiregulin produced by ILC2 has a tissue restorative effect, such as in the response to lung damage upon influenza virus infection (*116*) or intestinal injury (*315*). Thus, although ILC2s secrete many cytokines that exacerbate type 2 responses at mucosal sites, they may also have an important role in the maintenance of tissue homeostasis.

In addition to producing cytokines to modulate downstream innate and adaptive responses, ILC2s can directly interact with T cells through expression of MHC-II and the costimulatory ligand OX40L as well as ICOS-ICOSL interactions previously discussed (*316-318*). Interaction with T_H2-polarized $CD4^+$ T cells via MHC-II provides a source of IL-2 to ILC2s that prolongs survival and acts synergistically with IL-33 and IL-25 to enhance ILC2 activation (*316*). Furthermore, IL-33 stimulation of ILC2s induces expression of OX40L (*317, 318*), an important costimulatory molecule that binds OX40 on activated T cells to promote T cell survival, polarization, and cytokine production (*319*). Deletion of OX40L on ILC2s resulted in diminished T_H2 and T_{reg} responses in the lung upon *N. brasiliensis* infection or allergen challenge (*317*).

1.4.7 Role in Lung Immunopathology

Although the type 2 immune response has a protective role in immunity against helminths and other extracellular pathogens and in tissue repair, its dysregulation can lead to

immunopathologies at mucosal surfaces, including allergy, asthma, atopic dermatitis, and rhinitis (*72*). Many of these immunopathologies were formerly considered to be mediated by T_H2 cells in the adaptive response; however, ILC2s are now considered to be early mediators of the innate and adaptive type 2 responses that can lead to pathology when prolonged or dysregulated (*126, 275, 320, 321*). Thus, ILC2s are promising clinical targets in the treatment of allergies, asthma, and other type 2-mediated immunopathologies. Two common treatments for these conditions are corticosteroids and adrenergic agonists (*322, 323*), both of which may directly influence ILC2 function (*324-327*). However, it is unclear whether ILC2s are the primary target of these therapies *in vivo*, and both classes of drugs can have detrimental effects with long-term use (*323, 328*).

Corticosteroids are often provided as a first line of treatment for patients with allergies or asthma, and they are often effective in reducing inflammations and its symptoms (*323*). While there are some adverse effects reported from long-term corticosteroid use (*328, 329*), the greater concern with prolonged use is the development of resistance. Although corticosteroids have been shown to inhibit ILC2s *in vitro* and decrease the number of circulating ILC2 in asthmatic patients (*325*), ILC2s may also influence corticosteroid resistance through TSLP signaling. Stimulation with TSLP induces resistance to corticosteroids in ILC2 both *in vitro* and in mouse models (*326, 327*). Furthermore, higher TSLP levels in the bronchoalveolar lavage (BAL) fluids from asthmatic patients correlated with corticosteroid resistance (*330*). Nevertheless, whether ILC2s are a primary target of corticosteroids in the treatment of asthma and allergy is unclear.

In addition to corticosteroids, adrenergic agonists (or β_2 agonists) are now commonly used as asthma treatments due to their action on smooth muscle cells as a bronchodilator (*322, 323*). ILC2s also express the β_2-adrenergic receptor, and treatment with an adrenergic agonist reduced ILC2 function *in vitro* and the immune response to *N. brasiliensis* infection or *Alternaria* extract

administration (*324*). It remains unknown whether their effect on ILC2 has a significant impact on the resolution of lung inflammation in asthma patients; however, further characterization of the many factors modulating ILC2 function may lead to further drug targets for the treatment of type 2 immunopathologies.

1.5 Lipid Metabolism

1.5.1 Leukocyte Metabolism

Upon activation, T cells favour aerobic glycolysis over oxidative metabolism, like the Warburg effect seen in cancerous cells and other rapidly proliferating cells (*331, 332*). However, oxidative metabolism is a much more efficient method of adenosine triphosphate (ATP) generation, and it is unclear why this shift to glycolysis occurs upon T cell activation. One hypothesis suggests that while oxidative metabolism is more efficient, the mitochondrial biogenesis required takes far longer than switching to glycolytic metabolism (*333*). Glycolysis also supplies intermediates for the synthesis of lipids, which are necessary for rapidly proliferating cells to increase the area of their phospholipid membranes (*334, 335*). Conversely, after the retraction phase of adaptive immunity, memory T cells favour oxidative metabolism (*332, 333*). In particular, tissue-resident memory $CD8^+$ T cells have been shown to favour exogenous lipid uptake, lipolysis and β-oxidation of fatty acids (FA) to fuel oxidative metabolism (*336-339*). Furthermore, metabolic differences may have a role in driving differentiation of $CD4^+$ T cell subsets, such as T_{reg} and T_H17 cells (*332, 337, 340*).

Macrophages, like T cells, shift from oxidative metabolism to glycolysis when activated to a conventional or "M1" phenotype characterized by the production of nitric oxide (NO) and other inflammatory mediators (*333*). Conversely, alternatively activated macrophages, or "M2"

macrophages display marked metabolic differences, relying more on oxidative metabolism fueled by lipolysis and β-oxidation (*341*).

Nevertheless, recent findings in the field of immunometabolism have challenged this previously accepted model of T cell and macrophage metabolism. Previous studies of β-oxidation of FA in T cells and macrophages have used Etomoxir, an inhibitor of carnitine palmitoyl transferase (CPT)-1a, to inhibit β-oxidation and demonstrate impaired polarization. However, more recent studies employing genetic deletion of *Cpt1a* either in macrophages or T cells demonstrate no impairment of macrophage polarization or generation of a memory T cell phenotype (*342, 343*). Furthermore, one of these studies demonstrates significant off-target effects of Etomoxir at high concentrations through depletion of cytoplasmic coenzyme A (CoA) (*343*). Thus, there is debate in the field as to whether the currently accepted role of FA β-oxidation in leukocytes is valid.

1.5.2 Lipid Uptake

FAs can rapidly diffuse through the plasma membrane by inserting themselves into the outer leaflet of the membrane upon dissociation from their physiological carrier protein, albumin (*344*). During adsorption into the plasma membrane, FA are protonated and interact with membrane phospholipids so that their carboxyl head, the charged portion of the FA, is at the aqueous interface, and the hydrophobic tail is parallel to the hydrophobic phospholipid tails (*345-347*). FA will "flip-flop" to the other leaflet prior to desorption from the membrane and entry of the lipid into the aqueous phase of the cytoplasm (*348*). After desorption, they are activated by esterification to cytoplasmic CoA, essentially trapping the FA within the cell (*349*). They can then bind to cytoplasmic lipid-binding proteins such as fatty acid-binding protein ($FABP_c$) family members or

caveolin-1, which maintain solubility of the FA and serve as chaperones to channel FA into specific metabolic processes (*350, 351*).

In addition to characterization of the "flip-flop" mechanism of crossing cell membranes, FA were thought to be taken up by three different transport proteins: fatty acid translocase (FAT), membrane-associated fatty acid-binding protein ($FABP_m$), also called adipocyte fatty acid-binding protein (aP2) in adipocytes, and fatty acid transport protein (FATP) (*345, 346, 348, 352*). FAT was later characterized as the glycoprotein CD36, also called scavenger receptor B2 (SR-B2), important in the uptake of oxidized low-density lipoprotein (LDL) by macrophages (*352, 353*). CD36, which is also present on other leukocytes, enterocytes, adipocytes, and cardiac and skeletal muscle (*346*), likely serves as an acceptor for FA and an anchor point for $FABP_m$, accelerating FA uptake (*346, 354*). Recent evidence also suggests that the intracellular domain of CD36 may accelerate intracellular lipid metabolism through esterification of FA (*355*). $FABP_m$ is a family of soluble proteins with no transmembrane domains, found in many tissues including adipocytes, hepatocytes, myocytes, and jejunum enterocytes (*356-363*). Anti-$FABP_m$ antibodies reduced uptake of extracellular FA in hepatocytes (*360, 361*) and 3T2-L1 adipocytes (*350, 364*). However, more recent studies suggest that $FABP_m$ is not essential for FA uptake, but accelerates dissociation of extracellular FA from albumin (*351*). FATP is a highly conserved family of transmembrane proteins with FA-binding domains, FATP1-6 (*365-371*), that was later characterized as a family of acyl-CoA synthetases that esterify FA upon uptake (*372-376*). The expression of these proteins can be modulated by metabolic cues such as insulin signaling (*377-380*) and the transcription factors peroxisome proliferator-activated receptor α (PPARα) and PPARγ (*381, 382*), which modulate expression of a wide array of metabolic genes (*383*). Additionally, there is some evidence that some cytokines, including IL-6 and TNF-α, might stimulate FA uptake, although

there are no changes in the gene expression of any of these proteins that might accelerate FA uptake (*384*).

Despite the evidence that CD36, $FABP_m$ and FATP proteins enhance FA uptake, the precise mechanism of their roles remains unclear. Synthetic protein-free model membranes efficiently take up FA (*348, 385, 386*), and small interfering RNA (siRNA) knockdown of FATPs does not consistently decrease FA uptake (*387*), suggesting that these proteins are dispensable, or redundant and compensatory mechanisms for FA uptake have evolved. However, mice deficient in CD36 have disrupted lipid metabolism (*388, 389*). Since expression of different FATP family members is tissue-specific, deletion of different FATPs do not necessarily show the same phenotype (*349*). In general, mice lacking FATP family members do not have defects in lipid metabolism, but show altered insulin sensitivity (*390*). Although there is no conclusive working model of protein-mediated FA uptake, various models have been suggested (*349, 385, 386, 391*). One hypothesis is that protein-mediated FA uptake is necessary when the extracellular FA concentration is low, but at higher concentrations, these proteins become less important in FA uptake (*371, 386, 389, 391*). It has also been suggested that they may play a regulatory role at higher extracellular FA concentrations, limiting FA uptake based on the metabolic demands of the cell (*392, 393*). Nevertheless, given the speed at which uptake occurs, the tight coupling to esterification, and the possibility of compensatory mechanisms when disrupting metabolic processes, measurement of FA uptake alone is difficult, and the precise role of these proteins in FA uptake remains to be established (*394*).

Regardless of extracellular FA concentration, intracellular free FA concentration remains low, suggesting that FA are metabolized immediately upon uptake to prevent lipotoxicity from an excess of free FA (*391, 395-398*). FA feed into a variety of metabolic pathways such as storage as

triacylglycerol (TAG), phospholipid synthesis, and β-oxidation for energy generation (discussed in detail in Chapters 1.5.4 through 1.5.7).

1.5.3 *De Novo* Fatty Acid Synthesis

In most cell types, the majority of FA are taken up from the extracellular environment rather than synthesized *de novo* (*387*). However, liver and adipose tissue can convert carbohydrates to FA, as well as some specialized cells such as those in sebaceous glands (*399*). Unlike β-oxidation, which occurs in the mitochondria, FA synthesis occurs primarily in the cytoplasm, and the growing FA chain is linked to acyl-carrier protein (ACP) rather than CoA (*224*). The FA synthesis pathway begins with acetyl CoA, which is converted to malonyl CoA by acetyl-CoA carboxylases (ACCs) (*400*). In addition to being used as the substrate for FA synthesis, malonyl-CoA also negatively regulated β-oxidation, prohibiting FA synthesis and oxidation from occurring simultaneously (*401*). In total, seven malonyl-CoA molecules and one acetyl-CoA molecule are condensed to generate palmitate, a 16-carbon FA that can be further elongated or desaturated (*402*). In order for this process to occur, there must be an excess of acetyl-CoA derived from the tricarboxylic acid (TCA) cycle and NADPH as an electron donor (*224*). Newly synthesized FA can then be stored as TAG or can feed into phospholipid synthesis (discussed in detail in Chapters 1.5.4 and 1.5.5).

1.5.4 Triacylglycerol Synthesis and Lipid Droplet Formation

TAGs are a heterogenous group of neutral lipids made from a glycerol backbone and three FAs (*403*). They store lipids as an energy reservoir within cells, since excess intracellular free FA can lead to toxic effects (*397, 398, 404*) through endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) (*396, 405, 406*). In mammals, adipocytes are the primary

cell type for TAG storage, although hepatocytes, enterocytes, and myocytes also store TAG within lipid droplets (*403*). TAG synthesis can occur through two pathways: the glycerol phosphate pathway, also called the Kennedy pathway after it was first described by Eugene Kennedy and colleagues (*407*); or the monoacylglycerol (MAG) pathway (*408*).

The glycerol phosphate pathway of TAG synthesis **(Fig 3)** begins with glycerol-3-phosphate (G3P), which can be produced from the glycolytic intermediate dihydoxyaceteon-3-phosphate, or directly from glycerol (*399, 404*), and FA that have been activated by a FATP to make a fatty acyl-CoA thioester (abbreviated FA-CoA) (*409*). From G3P, three FA-CoA are added sequentially to form TAG. At the ER membrane, glycerol-3-phosphate acyltransferase (GPAT) adds one FA-CoA to make 1-acyl-*sn*-glycerol-3-phosphate, or lysophosphatidate. Acylglycerol-3-phosphate acyltransferase (AGPAT) then adds one more FA-CoA to generate phosphatidate, which can either be used for synthesis of acidic phospholipids, or for synthesis of a diacylglycerol (DAG). Dephosphorylation of phosphatidate by phosphatidate phosphatase (PAP) generates DAG, which can be channeled into the synthesis of other phospholipids, or TAG. (*399, 403, 404, 410, 411*).

The MAG pathway of TAG synthesis is important for enterocytes in the small intestine, where MAG is absorbed directly from the intestinal lumen (*408, 412*). It is then converted to DAG by monoacylglycerol acyltransferase (MOGAT) within enterocytes, a critical step in the absorption of dietary fat prior to export of TAG-containing chylomicrons (*404, 412*). Although the Kennedy pathway of TAG synthesis is ubiquitous, the MAG pathway is restricted to enterocytes, hepatocytes, and adipocytes (*408*). Unpublished RNA-sequencing data from our group suggests that the MAG pathway is not active in ILC2.

Regardless of the route of synthesis of DAG, diacylglycerol acyltransferases 1 and 2 (DGAT1, DGAT2) catalyze the final and rate-limiting step of TAG synthesis by addition of a third

FA (*330, 399, 403, 404, 413, 414*). However, these enzymes share no sequence homology (*330, 403*). The gene coding for DGAT1 was first identified by its sequence similarity to cholesterol acyltransferase (ACAT) enzymes (*415*), and later, the protein was shown to have DGAT activity (*416*). Like ACAT enzymes, this enzyme was identified as belonging to the larger family of membrane-bound *O*-acyltransferases (MBOAT) (*417*). However, DGAT1-deficient animals are not deficient in TAG, suggesting the presence of a second DGAT enzyme (*418*). This enzyme, DGAT2, was characterized shortly thereafter (*419, 420*), and other members of the same family were identified, including MOGAT enzymes and wax monoester synthases (*420, 421*).

Although DGAT1 and DGAT2 catalyze the same reaction, they are suggested to be non-redundant in their substrate specificity and the downstream TAG targeting. DGAT2 appears to be the enzyme responsible for the majority of TAG synthesis, since DGAT2 deficiency in mice results in hypolipidemia, a near complete loss of tissue triglycerides, and skin barrier abnormalities that lead to death shortly after birth (*422*). Two human patients have been identified with *DGAT2* mutations, both from the same family and exhibiting symptoms of Charcot-Marie-Tooth disease (CMT), a disorder of the peripheral nervous system leading to progressive muscle degeneration and sensory defects (*423*). The father showed significantly reduced serum TAG levels and diminished TAG within isolated fibroblasts compared to age- and sex-matched controls (*423*).

DGAT2 is closely associated with enzymes involved in *de novo* FA synthesis, and mice lacking these enzymes also have impaired TAG synthesis, suggesting that DGAT2 may be important for incorporating endogenous FA into TAG (*424, 425*). Conversely, DGAT1-deficient mice are viable, but are resistant to high-fat diet-induced obesity and show increased insulin and leptin sensitivity, suggesting that DGAT1 may be important in the storage of circulating dietary FA (*418, 426, 427*). *In vitro* studies of hepatocytes using gene silencing approaches and selective

DGAT1 and DGAT2 inhibitors support this hypothesis, since DGAT2 inhibition or silencing results in decreased radiolabeled glycerol or glucose incorporation into TAG, with no change in the incorporation of radiolabeled oleic acid. DGAT1 inhibition, however, resulted in decreased incorporation of both radiolabeled glycerol and radiolabeled oleic acid, demonstrating that DGAT1 incorporates exogenous FA into TAG (*428-430*). Further studies using selective inhibitors to distinguish the roles of DGAT1 and DGAT2 suggest that DGAT2 links glucose uptake to β-oxidation of FA, since FA synthesized from radiolabeled glucose were unable to be oxidized upon DGAT2 inhibition (*430*). Mouse models with intestinal overexpression of DGAT1 or DGAT2 further demonstrate that DGAT1 and DGAT2 are not functionally redundant and synthesize TAG for distinct subcellular pools during absorption of dietary fat within enterocytes (*431*). Studies in plant cells suggest that DGAT1 and DGAT2 are present in different ER subdomains (*432*), and DGAT2 tends to localize to the mitochondrial-associated membrane (MAM) subdomain of the ER (*433*). DGAT1 is also suggested to have a broader range of substrate specificity than DGAT2, since it has been shown to have MOGAT, wax synthase and retinol acyltransferase (ARAT) activities *in vitro* (*434, 435*). Thus, in addition to not sharing sequence homology, the DGAT enzymes appear to be non-redundant in terms of cellular functions and substrate specificity.

The conversion of DAG to TAG occurs at the ER membrane (*411, 421, 436*), and in the most widely accepted model of lipid droplet (LD) biogenesis, TAG accumulate in the hydrophobic space between the inner and outer leaflets of the ER membrane, forming a lens. Once a critical number of TAG accumulate in this lens, a single leaflet of the membrane buds off to form an LD surrounded by a phospholipid monolayer acting as a surfactant (*437-441*). Thus, LDs contain primarily TAG and some cholesterol derived from the ER membrane and proteins targeted to LDs from the cytoplasm, such as perilipins (*442-445*). LDs remain closely associated with the ER

membrane (*446*), and some ER membrane proteins can also localize to LDs, a process suspected to be mediated by some of the same machinery that mediates vesicle trafficking (*447-449*). At the LD phospholipid monolayer membrane, they contribute to local synthesis of TAG (*450, 451*). It has been demonstrated with fluorescence microscopy that DGAT2, but not DGAT1, is found associated with LDs (*433, 450, 452*). Overexpression of DGAT2 results in very large LDs, whereas its loss blocks LD expansion, suggesting that DGAT2 alone mediates local TAG synthesis and LD growth (*450, 451*). Although this is the most widely accepted model of LD formation, alternative models hypothesize that both the luminal and cytoplasmic leaflets of the ER membrane engulf the lipid lens when an LD is formed, creating a temporary pore in the ER membrane (*453*). This hypothesis would explain the presence of ER transmembrane proteins that do not have roles in lipid metabolism on the surface of LDs (*454*); however, there is little supporting evidence for this hypothesis, and pure LD preparations for proteomic analysis are difficult to obtain, leading to protein contamination, especially by ER proteins (*439*). Despite many recent advances in the study of LDs since the discovery and characterization of the DGAT enzymes, models of LD biogenesis remain predominantly untested and the role of proteins in lipid lens formation and LD budding remains unclear.

Nevertheless, LDs serve as an energy reservoir and protect cells from toxic effects of free FA accumulation (*397*). In response to the energy needs of the cell, TAG stored within LD can be broken down to DAG or FA for phospholipid synthesis, energy generation, lipid mediator synthesis, or transcriptional regulation of metabolism. This process is tightly regulated in cells that take up and store high levels of FA, such as adipocytes and hepatocytes (*455*). However, regulation of these pathways in leukocytes remains poorly characterized.

1.5.5 Phospholipid Synthesis

Phospholipid synthesis begins with DAG or phosphatidate, which can be either an intermediate of the Kennedy pathway of TAG synthesis, or the product of TAG lipolysis (*404, 407, 411*). Phospholipid synthesis must increase in rapidly proliferating cells to allow for membrane generation for daughter cells and organelles. Furthermore, phospholipids serve as reservoirs that are broken down to FA as precursors for the synthesis of eicosanoids (*226*).

Phospholipid synthesis is a complex process resulting in a diverse range of lipids that influence the function of membrane proteins. In mammalian cells, the most prominent phospholipids are phosphatidylcholine (PC) (45-55%), phosphatidylethanolamine (PE) (15-25%), phosphatidylinositol (PI) (10-15%), phosphatidylserine (PS) (5-10%), and sphingomyelin (SM) (5-10%), although this composition varies between tissues and subcellular organelles (*456*). PC and PE can be synthesized directly from DAG, or PE can be converted to PC (*456, 457*). PC and PE serve as precursors for PS and SM. PI and PS are directly synthesized from cytidine-5'-diphosphate (CDP)-DAG, which much be first generated from phosphatidate from the Kennedy pathway (*458*). Although some enzymes involved in phospholipid synthesis are cytoplasmic, they must localize to the ER membrane in order to be active (*459*). Additionally, these enzymes are found primarily in the MAM (*456*) where many enzymes involved in TAG synthesis are also found, suggesting that phospholipid synthesis, TAG synthesis, and β-oxidation may be tightly linked in response to the energy and proliferation needs of the cell (*460*).

1.5.6 Mitochondrial β-oxidation

Upon FA uptake or TAG breakdown, FA can serve as a rich energy source through β-oxidation in the mitochondria. Long chain FA-CoA pass across the mitochondrial membrane by

means of the carnitine shuttle (*461*). Once inside the mitochondrial lumen, saturated FA-CoA are sequentially broken down to acetyl CoA and reducing equivalents that enter the electron transport chain (ETC) (*462*). Thus, FA are a rich energy source for the cell, although the energy stored within them is not as readily available as the energy from other metabolites such as glucose from the rapid breakdown of glycogen (*224*).

The carnitine shuttle for long chain FA transport comprises CPT1 at the outer mitochondrial membrane, carnitine acylcarnitine translocase (CACT) that shuttles between the inner and outer mitochondrial membranes, and carnitine palmitoyl transferase-2 (CPT2) at the inner mitochondrial membrane. The shuttle is dependent on the non-essential amino acid carnitine, whose primary function is FA transport into the mitochondria, and can be synthesized endogenously or provided though diet (*246*). CPT1 has three isoforms in humans, CPT1a, CPT1b, and CPT1c, expressed primarily in the liver, muscle, and brain, respectively (*462*, *463*). However, CPT1a has been shown to be expressed in several other tissues, including leukocytes (*342*). CPT1 is considered the rate-limiting enzyme of β-oxidation (*464*, *465*), since it catalyzes the first step of FA entry into the mitochondria by conjugating a FA-CoA to carnitine (*461*). The resulting acylcarnitine is then shuttled across the inner mitochondrial membranes by CACT, and CPT2 removes carnitine to re-generate a FA-CoA molecule. Carnitine is shuttled back to the outer mitochondrial membrane by CACT, where it binds another FA-CoA for the cycle to repeat (*461*).

The mitochondrial β-oxidation cycle includes four steps: oxidation, hydration, a second oxidation, and thiolysis (*462*). At both oxidation steps, the electrons removed are transferred to ubiquinone to enter the ETC for ATP generation (*462*). In the final thiolysis step, one molecule of acetyl-CoA is removed, shortening the acyl-CoA ester by two carbons, and providing substrate for the TCA cycle or ketogenesis (*464*).

1.5.7 Eicosanoid Synthesis

There are two primary pathways of eicosanoid synthesis from AA: the cyclic pathway and the linear pathway (*224*). From these two pathways, eicosanoids can be further modified to make a broad range of lipid products (*225*). In the cyclic pathway, AA is first converted to PGH_2 through the action of cyclooxygenase (COX) and PGH synthase (PGHS) (*466*). PGH_2 can then be converted to other prostaglandins including PGE_2 and PGD_2, prostacyclins, and thromboxanes (*226, 467*). In the linear pathway, lipoxygenases (LOs) convert AA to LTA_4, which can then be converted to LTB_4, LTC_4, LTD_4, LTE_4, and lipoxins such as LXA_4 (*224, 225, 468*).

The canonical source for AA is through the breakdown of phospholipids by phospholipase A2 (PLA2) or phospholipase C (PLC), followed by the action of diacylglycerol lipase or diacylglycerol kinase (*224*). However, there is some evidence to suggest that mast cells, which have relatively large LD stores with no well-defined metabolic function, can also break down stored TAG through the action of adipose triglyceride lipase (ATGL) as an AA source for eicosanoid synthesis (*227, 469-471*). Furthermore, LDs increase in number and serve as the primary site of AA metabolism and eicosanoid synthesis in eosinophils during allergen challenge (*472-476*) and in activated macrophages upon infection by many different pathogens, including mycobacteria (*477-481*), *Trypanosoma cruzi* (*482-486*), *Toxoplasma gondii* (*487, 488*), and *Leishmania major* (*489*). LDs were also identified as the sites of eicosanoid synthesis in colon cancer cells removed from patients (*490*) and in a rat epithelial-derived cell line (*491*). Thus, the formation of LDs and the generation of eicosanoids may be tightly linked processes implicated in the innate immune response (*492*). However, whether these pathways are linked in ILC2s remains to be investigated.

CHAPTER 2 – OBJECTIVES & HYPOTHESIS

ILC2s are known to be critical regulators in initiation of the type 2 response at mucosal barrier sites, including the lung. While this response is essential in the defence against parasites, and a critical regulator of tissue repair, an overactive or dysregulated response can lead to immunopathologies of the lung, notably allergies and asthma. Given that ILC2s are some of the first cells involved in this response, they could be promising therapeutic targets in dampening lung inflammation in the treatment of type 2 immunopathologies.

Recently, there has been considerable research performed to understand the negative regulation of ILC2, such as by type I and type II IFNs (*207*, *210*, *211*), neuropeptides (*270*-*272*) and eicosanoids (*248*, *251*, *255*, *260*, *262*). However, more investigations are necessary into whether any of these factors contributing to ILC2 downregulation could be used therapeutically. Although corticosteroids administered in the treatment of asthma may act on ILC2s, drug resistance is a common occurrence (*326*, *327*). Nevertheless, the study of ILC2 regulation could lead to the identification of novel targets allowing modulation of type 2 immune responses in allergy and asthma.

To identify novel genes that could be potential targets to modulate ILC2 function, we performed an RNA-sequencing screen to identify genes that are differentially regulated upon ILC2 activation. One gene that was highly upregulated upon ILC2 activation with IL-33 was DGAT2, which catalyzes the final and rate-limiting step of TAG synthesis (discussed in detail in Chapter 1.5.4).

Based on this observation, we hypothesized that DGAT2-mediated TAG synthesis is critical for ILC2 function. Thus, we sought to investigate whether DGAT2 inhibition could modulate ILC2 function and allergic lung inflammation. We specifically aimed to:

(a) confirm the kinetics of DGAT2 expression in ILC2 at an mRNA and protein level;

(b) determine the effect of pharmacological DGAT2 inhibition on murine and human ILC2 function *in vitro*;

(c) investigate whether DGAT2 inhibition could dampen allergic lung inflammation in pre-clinical mouse models; and

(d) characterize metabolic alterations in ILC2 upon IL-33-induced activation.

Further elucidating the role of DGAT2 and the poorly characterized metabolic requirements of ILC2 could lead to the development of novel targets for the treatment of type 2 immunopathologies.

CHAPTER 3 – MATERIALS & METHODS

3.1 Reagents

All cytokines were purchased from R&D Systems. PF-06424439 (PF) DGAT2 inhibitor ([(3R)-1-[2-[1-(4-Chloro-1H-pyrazol-1-yl)cyclopropyl]-3H-imidazo[4,5-b]pyridin-5-yl]-3piperidinyl]-1-pyrrolidinylmethanonemethane-sulfonate**)** (*493*) was purchased from Sigma, and JNJ-DGAT2-A (JNJ) DGAT2 inhibitor (3-Bromo-4-[2-fluoro-4-[[4-oxo-2-[[2-(pyridin-2-yl)ethyl]amino]-1,3-thiazol-5(4H)ylidene]methyl]phenoxy]-benzonitrile) (*429*) was purchased from Tocris. 2-deoxyglucose (2-DG) was purchased from Sigma.

3.2 Animals

C57BL/6J mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME). Animals used in experiments were bred in-house under specific pathogen-free conditions with *ad libitum* access to food and water. *In vivo* experiments were conducted with female age-matched mice (8 – 16 weeks). *In vitro* experiments were conducted with primary cells obtained from older (>16 weeks) female mice. All experiments were performed in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University.

3.3 Human Subjects

Peripheral blood was drawn from healthy adult volunteers after obtaining written informed consent. All experiments were approved by the McGill Faculty of Medicine Institutional Review Board (study number A10-M46-19A) and carried out with the TCPS 2 policy.

3.4 Isolation and Culture of Murine ILC2

Bone marrow (BM) ILC2 progenitors were isolated as previously described Briefly, bone marrow was isolated from the femur and tibia of female mice. Red blood cells were lysed with Hybri-Max™ red blood cell lysis buffer (Sigma), and ILC2 progenitors were sorted as Lin(B220, CD3ε, CD5, CD11b, CD11c, CD19, FcεRIα, Ly-6C/G, TCRαβ, TCRγδ, TER-119)$^{-}$Sca-1^{+}c-Kit^{-}CD25^{+} cells on a BD FACSAria™ Fusion cell sorter **(Fig 4)**. Cells were cultured in complete medium (RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin and 55 μM 2-mercaptoethanol).

Lung ILC2 were isolated by mincing lungs and digesting in RPMI-1640 with 5% FBS, 0.2 mg/mL Liberase™ and 100 μg/mL DNase I (both from Roche). Red blood cells were lysed with Hybri-Max™ red blood cell lysis buffer (Sigma). ILC2 were sorted as live CD45^{+}Lin^{-} CD90^{+}ST2^{+}CD25^{+} on a BD FACSAria™ Fusion cell sorter **(Fig 5)**. All antibodies used for sorting are listed in Table 1.

ILC2 were either stimulated immediately or expanded for experiments requiring larger numbers of cells. For expansion, cells were cultured in complete media supplemented with IL-2, IL-7, IL-33, IL-25 (each at 50 ng/mL) and TSLP (20 ng/mL), as previously described (*210*). Cells were expanded for 14 days prior to resting for 3 days in complete media supplemented with IL-2 and IL-7 (each at 10 ng/mL). Prior to use in various experimental approaches, cells were washed and incubated in complete media without cytokines for 4 hours.

3.5 Isolation and Culture of Human Peripheral Blood ILC2

Whole blood was drawn from healthy donors, and peripheral mononuclear cells (PBMCs) were isolated using SepMate™ isolation tubes (STEMCELL Technologies) containing lymphocyte

separation medium (Wisent Bioproducts) according to the manufacturer's instructions. ILC2 were sorted as live $CD45^+$Lin(CD1a, CD3ε, CD11c, CD14, CD16, CD19, CD34, CD56, CD94, CD123, CD303, FcεRIα, TCRαβ, TCRγδ)$^-CD127^+CD294^+CD161^+$ cells on a BD FACSAria™ Fusion cell sorter **(Fig 6)**. Cells were cultured in complete media with the same composition as murine complete medium except for 10% human serum instead of FBS. ILC2 were expanded in complete media supplemented with IL-2 (40 ng/mL), prior to resting for 3 days in complete media supplemented with 10 ng/mL IL-2. All antibodies used for sorting are listed in Table 1.

3.6 RNA-Sequencing

BM-derived ILC2s were expanded for 5 days and rested for 72 h. Cells were then incubated for 4 hours in medium without cytokines prior to IL-33 stimulation (10 ng/mL) or medium alone for 4 hours. RNA was extracted with the Quick-RNA™ MicroPrep Kit (ZymoResearch) (manufacturer's instructions) and RNA-seq was performed as described previously (*494*). First, a Bioanalyzer RNA Pico kit (Agilent) was used to evaluate RNA integrity. Next, the KAPA Stranded RNA-Seq kit (Roche) was used to deplete rRNA and prepare RNA libraries for sequencing on an Illumina HiSeq 2500 sequencer in paired-end 50 bp configuration. The FastQC tool (Babraham Bioinformatics) was used to assess quality of reads, and Trimmomatic v.0.33 (*495*) was used to trim low-quality bases and adapter sequences (arguments: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 HEADCROP:4 LEADING:3 TRAILING:3 SLIDINGWINDOW:6:25 MINLEN:30). TopHat v2.0.9 with Bowtie v1.0.0 algorithms (*496, 497*) were used to map reads to mouse UCSC mm9 reference assembly. featureCounts (*498*) was used to quantify reads mapping to gene exons. IL-33-stimulated and non-stimulated data were compared with the edgeR Bioconductor package (*499*), excluding rRNA reads and genes with less than 10 counts per million

reads (CPM). Genes with changes in expression ≥ |2| fold and Benjamini-Hochberg adjusted *p* values ≤ 0.001 were considered significant. MeV software (*500*) was used to generate heatmaps of gene expression. Genes were arbitrarily grouped by pathway prior to clustering based on expression level by MeV software.

3.7 RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA from cultured bone marrow ILC2s was extracted using the Quick-RNA™ MicroPrep kit (Zymo Research) according to the manufacturer's instructions. RNA from lung tissue was extracted using mechanical disruption and TRIzol (Invitrogen) and further purified using the Quick-RNA™ MicroPrep kit (Zymo Research). cDNA was synthesized with Oligo(dT)$_{12\text{-}18}$ primer and SuperScript II Reverse Transcriptase (both Life Technologies). qRT-PCR was carried out with PowerUp SYBR Master Mix (Applied Biosystems) in a StepOnePlus instrument (Applied Biosystems). All primers are listed in Table 2. Relative expression of genes of interest was calculated using the comparative change-in-cycling-threshold (ΔCt) method after normalization to expression levels of the reference gene *Hprt*.

3.8 DGAT2 Protein Stain

Murine BM-derived ILC2 were seeded at 50,000 cells/well and incubated for 4 hours without cytokines. Cells were then stimulated with IL-7 alone (control) or IL-7 + IL-33 (each at 10 ng/mL). 48 hours after stimulation, cells were stained with eFluor 780 Fixable Viability Dye (eBioscience), fixed and permeabilized with FoxP3 Transcription Factor Staining Buffer Set (eBioscience) and stained with a DGAT2-specific primary antibody (Abcam; Ab102831) or a rabbit polyclonal IgG

isotype control antibody (Thermo Fisher 02-6102), followed by an anti-rabbit secondary antibody (Invitrogen A-21246). Data were acquired using a BD LSRFortessa™ flow cytometer.

3.9 Viability Assay

ILC2 were seeded at 5,000 cells/well (murine bone marrow), 3,500 cells/well (murine lung), or 1,000 cells/well (human). Cells were pre-treated with inhibitors or DMSO for 30 minutes prior to stimulation with cytokines (10 ng/mL). After 5 days, AlamarBlue™ Cell Viability Reagent (Invitrogen) was added to the culture according to manufacturer's instructions. Cells were incubated for 3 hours and fluorescence at 590 nm was measured as relative fluorescence units (RFU) using a Perkin Elmer EnSpire 2300 Multilabel reader.

3.10 ELISA

IL-5 and IL-13 in cell culture supernatants were quantified with either mouse or human IL-5 and IL-13 DuoSet ELISA kits (R&D Systems) according to manufacturer's instructions. Absorbance was measured using a Perkin Elmer EnSpire 2300 Multilabel reader.

3.11 Proliferation Assay

Cells were stained with eFluor 450 Cell Proliferation Dye (eBioscience) according to manufacturer's instructions. Cells were plated at 20,000 cells/well in complete media followed by addition of inhibitors or DMSO for 30 minutes prior to stimulation with respective cytokines (10 ng/mL). After 3 or 5 days, cells were counted and stained with eFluor 780 Fixable Viability Dye (eBioscience), fixed and permeabilized with FoxP3 Transcription Factor Staining Buffer Set

(eBioscience) and stained with anti-Ki-67 or isotype control. Data were acquired using a BD LSRFortessa™ flow cytometer.

3.12 Annexin V Staining

Cells were seeded at 50,000 cells/well in complete media followed by addition of inhibitors or DMSO for 30 minutes prior to stimulation with respective cytokines (10 ng/mL). After 18 hours, cells were stained with Annexin V Apoptosis Detection Kit (eBioscience) according to the manufacturer's protocols and eFluor 780 Fixable Viability Dye (eBioscience). Data were acquired using a BD LSRFortessa™ flow cytometer.

3.13 DGAT2 Inhibition *in vivo*

Mice were anesthetized using isofluorane and intranasally challenged with either PBS, 250 ng IL-33, or 250 ng IL-33 + 200 μg DGAT2 inhibitor (PF-06424439; PF) on three consecutive days. Mice were sacrificed 24 hours after the last treatment. Lungs were removed, weighed, minced and digested in RPMI-1640 supplemented with 5% FBS, 0.2 mg/mL Liberase™ and 100 μg/mL DNase I (both from Roche). Red blood cells were lysed using Hybri-Max™ Red Blood Cell Lysis buffer (Sigma) prior to staining. Dead cells were excluded using eFluor 780 Fixable Viability Dye (eBioscience) and cells were fixed and permeabilized with the FoxP3 Transcription Factor Staining Buffer Set (eBioscience). Data were acquired using a BD LSRFortessa™ flow cytometer.

3.14 Lipid Droplet Staining and Lipid Uptake Assays

ILC2 were seeded at 50,000 cells/well in complete media +/- IL-33 (10 ng/mL). For lipid droplet staining, cells were stained after 24 hours of stimulation with 1 μM BODIPY™ 493/503 (Thermo

Fisher). For measurement of lipid uptake, media was supplemented with BODIPY™ FL C16 (Thermo Fisher) to a final concentration of 1 μM after 12h of stimulation. After an additional 1-hour incubation, data were acquired with flow cytometry. Dead cells were excluded using 7-AAD Viability Staining Solution (eBioscience) and data were acquired on a BD LSRFortessa™ flow cytometer.

3.15 Seahorse Assay

Cells were seeded at 50,000 cells/well in complete media followed by addition of inhibitors or PBS for 30 minutes prior to stimulation with respective cytokines (10 ng/mL). 12 hours after stimulation, cells were pooled, counted, and seeded in XF96 microplate (Agilent Technologies) coated with Poly-L-lysine (Sigma) at 200,000 cells/well in XF Base DMEM (Agilent Technologies) supplemented with 2 mM L-glutamine and 11 mM D-glucose. Cells were incubated for 1 hour in a CO_2-free incubator prior to assay. Oxygen consumption rate (OCR) and extracellular acidification rate (ECR) were measured upon treatment with oligomycin (Oligo; 1.5 μM), carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazine (FCCP; 1 μM), and a mixture of rotenone (Rot; 0.5 μM) and antimycin A (AnA; 0.5 μM). ATP produced by oxidative metabolism and glycolysis were quantified as described by Mookerjee *et al.* (*501*).

3.16 Statistical Analysis

Student's *t*-test was used to determine statistical significance between two groups. One-way ANOVA followed by Tukey's or Sidak's multiple comparison test was used to determine statistical significance between three or more groups. Data are displayed as means ± standard

deviation (SD). *P* values below 0.05 were considered statistically significant with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, and $p < 0.0001$ = ****.

CHAPTER 4 – RESULTS

4.1 IL-33-induced ILC2 activation upregulates DGAT2 expression

To investigate novel genes involved in IL-33-induced ILC2 activation, we used an RNA-sequencing approach to analyze gene expression upon IL-33 stimulation of bone-marrow (BM)-derived ILC2s *in vitro*. We observed strong expression of signature type 2 cytokines, including IL-5 (*Il5*), IL-6 (*Il6*), IL-13 (*Il13*) and amphiregulin (*Areg*), as expected upon IL-33 activation **(Fig 7A)**. Although there were no changes in expression of most genes in many metabolic pathways, including lipolysis, FA synthesis and oxidation, TAG synthesis, and eicosanoid synthesis, two genes were significantly upregulated: COX2 (*Ptgs2*), which is involved in the synthesis of prostaglandins and leukotrienes; and DGAT2 (*Dgat2*), which catalyzed the final step in TAG synthesis prior to storage in LD **(Fig 7A)**. COX2 upregulation and the role of eicosanoids on ILC2 regulation have previously been described (*250, 253*); however, other pathways of lipid metabolism, including TAG synthesis and mobilization, remain poorly characterized.

To validate our RNA-sequencing results, we investigated the kinetics of *Dgat2* expression in expanded BM-derived ILC2 and freshly sorted lung ILC2 **(Fig 7B).** qRT-PCR analysis confirmed that *Dgat2* is highly and rapidly induced in ILC2 by IL-33 stimulation. Although *Dgat1* is expressed in murine ILC2, its mRNA expression levels did not markedly change by IL-33 stimulation **(Fig 7C).** After 48 hours of IL-33 stimulation a clear increase of DGAT2 protein expression was observed when compared to staining with an isotype control antibody or with a secondary antibody only **(Fig 7D).** However, DGAT2 protein expression was also observed in ILC2 that have been stimulated with IL-7 only (control), which is likely due to DGAT2 expression during the expansion process of BM-derived ILC2, and may not be seen with freshly sorted lung ILC2. Nevertheless, when comparing control and IL-33-stimulated cells, there is a significant

increase in DGAT2 expression after 48 hours of stimulation, which is in line with the observed increase of mRNA levels.

4.2 Dgat2 inhibition restricts ILC2 viability and cytokine secretion

Since mice lacking DGAT2 die shortly after birth due to lipopenia and defective skin barriers (*422*), we first investigated the effect of commercially available specific DGAT2 inhibitors PF-06424439 and JNJ DGAT2-A (referred to as PF and JNJ, respectively) on ILC2s *in vitro*. Both inhibitors significantly decreased ILC2 viability and type 2 signature cytokine (IL-5, IL-13) production in a dose-dependent manner in ILC2s isolated from mouse lungs **(Fig 8A, B).** Since the observed decrease in cytokines in the cell culture supernatant can also be reflective of a defect in IL-33-induced ILC2 proliferation, expanded BM-derived ILC2s stimulated with IL-33 for 48 hours were stained for intracellular IL-5 and IL-13. While there were fewer live cells upon DGAT2 inhibition, there was a dose-dependent decrease in the percentage of IL-5^+ and IL-13^+ live cells **(Fig 8C)**. Furthermore, DGAT2 inhibition in ILC2 rapidly led to apoptotic cell death, as determined by Annexin V staining after 16 hours IL-33 stimulation **(Fig 8D)**. When ILC2s were stained with a cell division-tracking proliferation dye prior to 72 hours IL-33 stimulation, proliferation in response to IL-33 was markedly decreased upon DGAT2 inhibition **(Fig 8E).** This proliferative defect upon DGAT2 inhibition was confirmed by staining for intracellular Ki-67, a marker of cell cycle progression.

To further investigate the potential of DGAT2 as a novel therapeutic target, data collected with murine ILC2s (**Fig 8**) were validated using peripheral human ILC2s sorted from three independent donors **(Fig 9)**. Consistent with murine ILC2 data (**Fig 8**), DGAT2 inhibition significantly reduced ILC2 viability, cytokine production, and proliferation **(Fig 9A, B).**

4.3 Inhibition of Dgat2 *in vivo* restrains IL-33-mediated allergic lung inflammation

Since DGAT2 inhibition *in vitro* resulted in impaired ILC2 function and proliferation, and ILC2s are critical early regulators of lung inflammation, we next investigated the effect of DGAT2 inhibition in a preclinical mouse model of allergic lung inflammation *in vivo*. In this model, IL-33 is administered intranasally on three consecutive days, and animals are sacrificed 24 hours after the final challenge **(Fig 10A)**. This model allows study of early chemokine production, ILC2 proliferation, and infiltration of inflammatory cells during the early allergic response, prior to IgE responses and T cell involvement. Consistent with previous observations (*210*), IL-33 administration led to a clear increase in lung mRNA expression of the chemokines TARC (*Ccl17*), eotaxin-2 (*Ccl24*), and CXCL1 *(Cxcl1*), which are important for the recruitment of inflammatory cells, such as eosinophils and neutrophils. In addition, there was an increase in the type 2 cytokine IL-5 *(Il5*), which contributes to eosinophil maturation, activation and recruitment **(Fig 10B).** Downstream of chemokine expression, there was an increase in eosinophil and neutrophil recruitment, which was also reflected in increased lung weight, total cell number, and $CD45^+$ cell count **(Fig 10C, D)**. From the direct action of IL-33 on ILC2, there is an increase in ILC2 numbers with IL-33 administration, owing to an increase in ILC2 proliferation, as demonstrated by a significant increase in Ki-67 expression by ILC2s in the lung **(Fig 10E)**. However, when the PF DGAT2 inhibitor is administered in parallel with IL-33, there is a significant decrease in chemokine expression, followed by a significant decrease in lung weight and inflammatory cell infiltration, when compared to IL-33 alone **(Fig 10B, C, D)**. There was also a marked decrease in the number of ILC2 and the percentage of ILC2 expressing the proliferation marker Ki67 upon DGAT2 inhibition in the lung **(Fig 10D).** These data support our *in vitro* findings and demonstrate

that DGAT2 function is critical for ILC2 effector functions and ILC2-mediated airway inflammation *in vivo*.

4.4 IL-33-induced ILC2 activation leads to metabolic reprogramming

To further investigate the physiological role of lipid metabolism in ILC2, we investigated the effect of IL-33 stimulation on FA uptake and storage as TAG by ILC2 *in vitro*. IL-33 stimulation of BM-derived ILC2 increased uptake of BODIPY™ FL C16, a fluorescently labeled FA **(Fig 11A).** Furthermore, staining of neutral lipids with BODIPY™ 493/503 in IL-33-stimulated ILC2 shows elevated lipid accumulation compared to unstimulated cells **(Fig 11B).** These alterations in lipid uptake and accumulation strongly suggest that lipid metabolism plays a key role in modulating ILC2 activation and function. Interestingly, upon DGAT2 inhibition, BODIPY™ 493/503 staining increases when compared to untreated cells stimulated with IL-33 **(Fig 11C).**

To better understand the metabolic requirements of ILC2, we confirmed that glycolysis is essential for ILC2 survival and function. Medium supplementation with 2-deoxyglucose (2-DG), a synthetic glucose analogue that cannot be broken down by glycolysis, significantly impaired ILC2 viability and cytokine concentration in the cell culture supernatant **(Fig 11D).** However, intracellular cytokine staining showed no decrease in cytokine production **(Fig 11E).** Thus, the decrease in cytokine concentration upon inhibition of glycolysis was likely due to a significant decrease in cell viability rather than an intrinsic defect in cytokine production.

Furthermore, Seahorse analysis revealed significant differences in mitochondrial metabolism upon IL-33-induced activation. The Seahorse XF Analyzer (Agilent Technologies) measures the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of

cell culture supernatant upon sequential treatment of cells with drugs targeting the mitochondria (*502, 503*). These data are direct readouts of oxidative metabolism and glycolysis, respectively. Oligomycin (Oligo) inhibits ATP synthase to determine the rate of ATP-linked respiration and proton leak; carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazine (FCCP) uncouples the proton gradient to determine the maximal respiratory capacity; and rotenone (Rot) and antimycin A (AnA) inhibit the electron transport chain to determine the rate of non-mitochondrial respiration (*504*) **(Fig 11F)**. These readouts can be used further to quantify ATP production from oxidative metabolism and glycolysis (*501*).

Using this method, IL-33 and IL-7 stimulation of BM-derived ILC2 induced a marked increase in basal and maximal mitochondrial respiration when compared to IL-7 stimulation alone. **(Fig 11G).** IL-7 stimulation alone was used as a control to minimize cell death during the assay. Moreover, ATP production from both oxidative metabolism and glycolytic metabolism increased with the addition of IL-33 **(Fig 11H)**. In contrast, DGAT2 inhibition during IL-33 stimulation resulted in a slight decrease in basal respiration, and a pronounced decrease in maximal respiratory capacity **(Fig 11G).** Interestingly, DGAT2 inhibition had a more pronounced effect on both oxidative and glycolytic ATP production of IL-7-stimulated cells compared to cells that also received IL-33 **(Fig 11H).** Cells stimulated with both IL-33 and PF showed a significant decrease in oxidative ATP production after DGAT2 inhibition, while glycolytic ATP remained unchanged in this group **(Fig 11H)**. This suggests that TAG formed by DGAT2 may be later broken down for FA oxidation in the mitochondria. Taken together, these data support the hypothesis that DGAT2 plays a role in metabolic reprogramming of ILC2 upon activation by IL-33, and suggest that FA storage may play a key role in downstream metabolism during sustained ILC2 activation.

However, further studies will be required to elucidate the contribution of lipid and glucose metabolism to IL-33-induced metabolic reprogramming.

CHAPTER 5 – DISCUSSION & FUTURE PERSPECTIVES

Since the first observations of a non-T, non-B lymphocyte population producing type 2 signature cytokines nearly 20 years ago (*106, 107*), ILC2s and other innate lymphocytes have been well characterized and are now considered to be a key cell type in driving early immune responses and polarizing downstream adaptive immunity (*134, 161, 320*). While the type 2 response evolved as a response to parasitic helminths and is important in tissue repair, it is also responsible for type 2 immunopathologies, such as allergies, asthma, and rhinitis (*88, 126, 320, 321, 505*). Thus, characterization of the diverse signals that modulate ILC2 activation and function is critical in understanding these immunopathologies and could lead to novel drug targets for their treatment. Using an RNA-sequencing approach, we identified DGAT2 as a novel gene that was highly upregulated in ILC2 upon IL-33 stimulation. Thus, we hypothesized that DGAT2-mediated TAG synthesis is critical in ILC2 function, and sought to investigate whether pharmacological inhibition of DGAT2 could impair ILC2 function and lung inflammation. Although the metabolic requirements of other leukocytes such as T cells and macrophages have been well characterized (*332-334*), little is currently known about ILC2 metabolism and metabolic alterations induced by IL-33 signaling.

The data presented here in this thesis demonstrate a critical role of DGAT2 in ILC2 biology and ILC2-mediated allergic airway inflammation. IL-33 stimulation of ILC2 rapidly induces DGAT2 expression, and pharmacological inhibition of DGAT2 *in vitro* results in significantly decreased ILC2 viability, proliferative potential, and type 2 cytokine production in both murine and human peripheral ILC2. In a preclinical mouse model of allergic airway inflammation, DGAT2 inhibition decreases expression of type 2 cytokines and chemokines, leukocyte infiltration into pulmonary tissue, and ILC2 proliferation. Furthermore, IL-33 stimulation of ILC2 rapidly

upregulates lipid uptake and storage in LD. In addition to restricting ILC2 proliferation and cytokine production, DGAT2 inhibition reduces oxidative metabolism in ILC2, suggesting that DGAT2 may be involved in metabolic reprogramming of ILC2 upon activation by IL-33.

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Upon IL-33 stimulation, DGAT2 mRNA is rapidly induced at levels similar to the type 2 signature cytokines **(Fig 7A, B)**, and DGAT2 protein is increased after 48 hours compared to control **(Fig 7D)**. However, DGAT2 protein is already expressed at significant levels in unstimulated ILC2s, and the increase upon IL-33 stimulation is not as striking as the increase in mRNA. However, analysis of DGAT2 protein expression was solely performed with bone marrow-derived ILC2 that have been expanded *ex vivo* using a protocol we have developed to significantly increase ILC2 cell yield and entails stimulation of ILC2 with a strong cytokine cocktail (IL-2, IL-7, IL-33, IL-25) for two weeks followed by a three-day "resting period" in IL-7 and IL-2 only. It is therefore likely that DGAT2 is upregulated during the two week ILC2 expansion phase, when the cells are stimulated with IL-33 and become strongly activated, and

DGAT2 produced during this expansion phase may not decrease significantly during the resting period of three days without IL-33. Hence, DGAT2 staining in freshly sorted ILC2 from mouse lungs may provide a more accurate readout of DGAT2 protein expression upon IL-33 stimulation. Nevertheless, these data further confirm the increase in *Dgat2* expression seen in our RNA-sequencing dataset as well as previously published datasets. A recent paper that identified upregulation of the receptor for the neuropeptide NMU through single-cell RNA sequencing also identified upregulation of *Dgat2* in murine ILC2 upon IL-33 and IL-25 stimulation (*264*). Furthermore, an RNA-sequencing analysis that contributed to the Immunological Genome Project (ImmGen) revealed high levels of *Dgat2* expression in ILC2 isolated from mouse small intestines compared to other innate lymphocyte populations (*506*).

Upon pharmacological inhibition of DGAT2 in both mouse and human ILC2s, there was a marked reduction in ILC2 viability and concentration of type 2 cytokines in the supernatant in response to IL-33 stimulation **(Fig 8A, B; Fig 9A)**. Since a reduction in cytokine concentration can be reflective of either cell death and a lack of cell proliferation, or an intrinsic defect in cytokine production, these experiments were followed up by Annexin staining, analysis of cell proliferation, and intracellular cytokine staining. Upon DGAT2 inhibition, there is a rapid increase in ILC2 apoptosis by 24 hours of stimulation **(Fig 8D)**. At 48 hours of IL-33 stimulation, there is a significant decrease in IL-5 and IL-13 expression by remaining live cells **(Fig 8C)**. By 72 hours, cells treated with DGAT2 inhibitors show no cell division or Ki-67 expression **(Fig 8E)**. Thus, the initial observation of a reduction in cytokine concentration was likely due to a combination of cell death, diminished proliferative potential, and a reduction in cytokine production. Nevertheless, the precise mechanism leading to this phenotype remains to be investigated.

Both DGAT2 inhibitors used *in vitro* have been previously described to be highly specific (*429, 493*), and the phenotypes observed *in vitro* upon treatment with either PF or JNJ DGAT2 inhibitors are similar. Regardless, the possibility of off-target effects of pharmacological inhibitors must be considered as a drawback to these studies. For example, previous descriptions of T cell and macrophage metabolism are currently under question due to recent observations of off-target effects of the drug Etomoxir, used to inhibit β-oxidation of FA, as well as discrepancies between pharmacological inhibition and genetic models of defective FA oxidation (*342, 343*). To exclude the possibility of off-targets effects of either DGAT2 inhibitor, these data should be supported by studies in ILC2s and animals lacking DGAT2. *Dgat2*$^{-/-}$ mice die shortly after birth due to metabolic defects and skin barrier abnormalities (*422, 507*); however, *Dgat2*$^{flox/flox}$ have recently been generated (*507*) which will allow ILC2-specific DGAT2-deletion in future studies to ascertain the critical role of DGAT2 in ILC2 functions.

Furthermore, lipidomics analysis of ILC2s stimulated with IL-33 in the presence or absence of DGAT2 inhibitors may provide information regarding the specificity of the used DGAT2 inhibitors. If both inhibitors are specific, an increase in DAG species would be expected upon DGAT2 inhibition. However, this does not account for the possibility that DGAT1 may compensate for a loss in DGAT2 activity, and DGAT1 inhibitors may have to be investigated in parallel to fully elucidate the effect of DGAT2 inhibition on ILC2 metabolism. We recently conducted a pilot lipidomics study that revealed significant changes in intracellular lipid species upon IL-33 stimulation of ILC2 and DGAT2 inhibition (data not shown). This analysis is currently being repeated with more replicates and controls; nevertheless, lipidomics analysis would further contribute to our knowledge of metabolic alterations in ILC2 upon activation and would help to confirm the specificity of the DGAT2 inhibitors.

In a simple preclinical mouse model in which mice are intranasally administered IL-33 to mimic tissue damage or allergen exposure, DGAT2 inhibition significantly reduced expression of type 2 cytokines and chemokines, leukocyte recruitment to the lung, and ILC2 proliferation **(Fig. 10B-D).** While these data demonstrate the potential of DGAT2 as a therapeutic target to dampen allergic inflammation in the lung, studies using a physiological allergen such as extract from the fungus *A. alternata* would further support these results. Downstream of the early allergic response, ILC2s have also been shown to be critical in orchestrating adaptive type 2 immunity (*274*, *313*). To investigate whether DGAT2 inhibition could also dampen antigen-specific B cell responses, we will intranasally administer allergen extract from the fungus *A. alternata* together with the DGAT2 inhibitor and determine *A. alternata*-specific IgG1 and IgE levels in sera and BALF of challenged animals. To analyze antigen-specific $CD4^+$ T cell responses we will intranasally challenge WT mice with IL-33 or *A. alternata* extract in combination with the immunogenic peptide 2W1S or peptide only as a control, as described previously (*317*). Antigen-specific $CD4^+$ T cell populations ($tetramer^+$) can then be tracked and further subclassified by staining with 2W1S:I-A^b MHC-II tetramer. DGAT2 inhibition in these models would contribute to our understanding of the role of DGAT2 in ILC2-driven allergic airway inflammation and would further support their use as a drug in the treatment of type 2 immunopathologies. Although DGAT2 upregulation has not been observed in other leukocytes upon activation (*506*, *508-511*), its expression is ubiquitous (*403*, *419*, *420*), and none of these models eliminate the potential of the DGAT2 inhibitor acting on other cell types involved in the type 2 immune response in the lung. Thus, animals with ILC2-specific deletion of DGAT2 could be used in these models to confirm whether the reduction in lung inflammation upon DGAT2 inhibition is mediated by suppression of ILC2 function.

Thus far, we have demonstrated that DGAT2 inhibition results in impaired ILC2 proliferation and cytokine production upon IL-33 stimulation, and a marked decrease in pulmonary inflammation upon intranasal IL-33 challenge. To further investigate the metabolic requirements of ILC2 that lead to this distinct phenotype, we investigated dynamics of lipid uptake and storage in ILC2, as well as mitochondrial and glycolytic metabolism upon IL-33 stimulation and DGAT2 inhibition. Upon activation by IL-33, ILC2s take up high amounts of the synthetic long-chain FA, BODIPY™ FL C16 **(Fig 11A)**. Since FA can freely "flip-flop" across the plasma membrane (*344, 348, 386, 394*), the rate of lipid uptake is generally determined by the concentration gradient of lipids across the plasma membrane, although the potential involvement of any of the putative FA transporters is still unclear (*351, 354, 385, 512*). Upon ILC2 activation by IL-33, FA may be more rapidly stored or channeled into metabolic pathways that decrease the effective intracellular concentration of FA, leading to increased uptake. Staining of ILC2s with BODIPY™ 493/503, a lipophilic fluorescent probe used to stain neutral lipids within LDs, shows an increased neutral lipid content upon IL-33 stimulation **(Fig 11B)**. This suggests that FA are rapidly taken up and must be transiently stored as TAG within LD, which is a well characterized protective mechanism against lipotoxicity (*397, 398, 405*). TAG can then be broken down, and the resulting FA may be channelled into metabolic pathways necessary for ILC2 survival and function. However, lipid breakdown is not occurring as rapidly as FA uptake, since there is an accumulation of neutral lipids upon IL-33 stimulation. Furthermore, our RNA-seq dataset does not show any upregulation of enzymes involved in lipolysis, such as ATGL (*Pnpla2*) **(Fig 7A).** Thus, while the rate of lipid storage by DGAT2 should increase in ILC2 with IL-33 stimulation, the rate of lipid breakdown might not change significantly. However, the rate of lipid breakdown could be determined by substrate and co-factor availability in addition to transcriptional regulation. Unexpectedly, there is

a further increase in BODIPY™ 493/503 staining upon DGAT2 inhibition **(Fig 11C)**. This does not reflect the presumed decrease in TAG upon DGAT2 inhibition; however, it may represent an accumulation of DAG species, which are also classified as neutral lipids stained by the BODIPY™ 493/503 probe. This result is also consistent with the pilot lipidomics study we conducted, which showed an increase in total DAG upon DGAT2 inhibition (data not shown). These DAG species may not be broken down as readily as TAG as a source of FA, although reasons for this are unclear.

From the data presented here, it is uncertain where FA could be channelled after TAG breakdown. However, three hypotheses seem plausible, and may be occurring simultaneously: FA may be broken down by β-oxidation for energy generation; channeled into eicosanoid synthesis to function in signaling and modulate transcriptional regulation of metabolism; or used for phospholipid generation to sustain ILC2 proliferation and vesicle formation for cytokine secretion **(Fig 12).**

In support of the hypothesis that FA are channelled into oxidative metabolism, Seahorse analysis showed clear differences in mitochondrial metabolism in ILC2s upon IL-33 stimulation and DGAT2 inhibition **(Fig 11G, H)**. Activation with IL-33 for 12 hours prior to Seahorse analysis led to a marked increase in the basal respiration rate and the maximal respiratory capacity of ILC2s, which are reflections of oxidative metabolism and mitochondrial biogenesis (*501, 502*). Mitochondrial biogenesis and fitness upon IL-33 stimulation could be further investigated using MitoTracker probes and flow cytometric analysis (*513, 514*). Upon quantification of ATP production during the Seahorse assay, there is a highly significant increase in oxidative ATP in ILC2s activation with IL-33, and DGAT2 inhibition leads to a slight but significant reduction in oxidative ATP **(Fig 11H)**. This suggests that downstream of DGAT2 activity, there is an increase in mitochondrial oxidative metabolism and supports the hypothesis that TAG synthesized by

DGAT2 are broken down for β-oxidation. This could be further investigated with metabolomics analysis pulsing cells with radiolabeled long-chain FA (e.g. palmitate) and tracking which metabolic pathways contain radiolabeled carbon after IL-33 stimulation. If FA are transiently stored in TAG prior to β-oxidation, there would be a small amount of radiolabeled carbon in TAG species. As labeled FA are broken down to acetyl CoA by β-oxidation, radiolabeled carbon would feed into the TCA cycle, which would be detected by metabolomics analysis.

Regarding glycolytic metabolism in ILC2s, there is a smaller though still significant increase in glycolytic ATP in ILC2s pre-stimulated with IL-33 **(Fig 11H)**. However, in the groups that were stimulated with IL-33, there was no change in glycolytic metabolism upon DGAT2 inhibition; this is illustrated in both the graph of ECAR and the quantification of glycolytic ATP **(Fig 11G, H)**. ILC2s that were stimulated only with IL-7 and treated with the DGAT2 inhibitor showed a decrease in glycolytic ATP, though not as marked a decrease as the decrease in oxidative ATP between these groups, and this decrease could be reflective of an overall decrease in cell fitness. When the oxidative and glycolytic ATP of all treatment groups are compared, we see that glycolysis is highly active in ILC2 and produces the majority of ATP within the cell, regardless of activation status or DGAT2 inhibition. Furthermore, inhibition of glycolysis with 2-DG, completely inhibits ILC2 viability while having no effect on intracellular type 2 cytokines in the remaining live cells **(Fig 11D, E)**. Inhibiting glycolysis not only inhibits production of glycolytic ATP, but also prevents intermediates of glycolysis to be used as building blocks for synthesizing metabolites such as TAG (*333, 335*). While metabolites from the TCA cycle can also be used for TAG synthesis (*399*), upregulating DGAT2 and TAG synthesis without continued glycolysis would likely lead to rapid depletion of the TCA, shutting down oxidative metabolism completely

and leading to rapid cell death. Thus, glycolysis is critical for ILC2 survival, regardless of activation status, and its inhibition is highly toxic.

Unlike glycolytic ATP production, oxidative metabolism is modulated by IL-33, even though it comprises a smaller percentage of the total ATP production by ILC2s **(Fig 11G, H)**. Furthermore, oxidative ATP production decreases upon DGAT2 inhibition, suggesting that transient production of TAG by DGAT2 upon IL-33-induced ILC2 activation is important for channelling metabolites to oxidative metabolism. For TAG synthesis to occur, however, there must be prior glycolysis to provide glycolytic intermediates to the Kennedy pathway of TAG synthesis (*335, 399*); thus, glycolysis does not decrease as oxidative metabolism increases in ILC2s. Altogether, these data support the hypothesis that DGAT2-mediated FA storage is an intermediate step prior to oxidative metabolism of FA, and that this pathway is critical for ILC2 activation, but not ILC2 survival at steady state. Metabolomics analysis could also be performed with after pulsing cells with radiolabeled glucose, to investigate which metabolites are built with the products of glycolysis in ILC2 at steady state and upon activation with IL-33.

To further our understanding of the role of oxidative metabolism in activated ILC2, β-oxidation can be specifically probed in a modified Seahorse assay with the addition of Etomoxir, a drug inhibiting FA uptake into the mitochondria for β-oxidation (*503*). However, as previously discussed, Etomoxir has off-target effects at high concentrations; this assay must be carefully titrated to avoid non-specific cytotoxity (*342, 343*). Nevertheless, this approach would differentiate between oxidative metabolism of lipids and glucose, indicating whether FA stored as TAG by DGAT2 are channelled into oxidative metabolism upon lipolysis. Since oxidative metabolism appears to be upregulated when DGAT2 is active, it is likely that β-oxidation of FA that were transiently stored as TAG is fueling oxidative metabolism in activated ILC2s. Thus, the

extended Searhorse assay described here in combination with metabolomics analysis using radiolabeled FA or glucose would allow for complete differentiation between oxidative metabolism of FA and glucose.

Given recent advances in the study of ILC2 and eicosanoid production, it is also possible that lipids stored by DGAT2 are channelled into eicosanoid synthesis. Positive and negative regulation of ILC2 by eicosanoids during inflammation has been well characterized (*251, 252, 256, 258-262*), and intrinsic production of PGD_2 by human ILC2s was recently described (*253*). Since PGD_2 is a known positive regulator of ILC2s (*237, 250, 252*), this suggests a feedforward mechanism in ILC2 activation. While the canonical source of arachidonic acid (AA) for PGD_2 synthesis is from the breakdown of phospholipids (*224, 226, 240*), activated ILC2s need to rapidly proliferate and produce vesicles for cytokine secretion; thus, phospholipid breakdown could be detrimental to ILC2 proliferation and function. Furthermore, TAG stored in LDs has been described to be a source of AA in mast cells, which have large lipid stores (*469, 471*). Therefore, it is plausible that TAG produced by DGAT2 in ILC2 is broken down and AA is channeled into eicosanoid synthesis to sustain ILC2 activation. Hence, future experiments will have to decipher of whether DGAT2 inhibition also impacts eicosanoid production by murine and human ILC2s. Additionally, LDs are the site of eicosanoid synthesis in other leukocytes upon infection (*477, 480, 481, 483, 485, 486, 515*); thus, an increase in LDs in ILC2s likely facilitates eicosanoid synthesis. Indeed, our RNA-sequencing dataset also shows a rapid upregulation of *Ptgs2* **(Fig 7A)**, the gene coding for COX2, which is the rate-limiting enzyme in the synthesis of PGD_2 (*241, 490*).

While this hypothesis seems plausible given the published data from human peripheral ILC2s, murine ILC2s from tissues (lung, bone marrow) do not express CRTH2, the surface receptor for PDG_2 (*237*). Thus, PDG_2 produced by murine ILC2s in our experimental setup could

not act on surrounding ILC2s. Nevertheless, eicosanoids such as PDG_2 can act intracellularly upon metabolism to endogenous ligands of the transcription factor PPAR-γ (*225, 467*). The PPAR family of transcription factors, which comprises PPARα, PPARβ/δ, and PPARγ, act as receptors for a wide array of lipid ligands, including many long-chain FA and eicosanoid metabolites (*225, 516, 517*). Target genes regulated by the PPAR family are diverse, but include many genes regulating lipid and glucose metabolism (*516*). PPAR activity can also repress the expression of pro-inflammatory genes in the context of metabolic disorders such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (*383*); however, the role of PPAR transcription factors in allergic inflammation is less clear. PPARγ in particular has been suggested to have a suppressive role in regulating airway inflammation (*518, 519*), and mice deficient in PPARγ cannot mount a pathogenic type 2 response in the lung (*520*). In ILC2s, it has been recently suggested that PPARγ engagement leads to elevated FA uptake upon activation (*521*). While the diverse roles of the PPAR family of transcription factors in ILC2 function has yet to be fully characterized, it is possible that endogenous synthesis of PGD_2 and its metabolites have an intrinsic role in modulating ILC2 function through the action of PPARγ and their target genes.

To further investigate whether eicosanoids, specifically PDG_2 and its metabolites, are regulating ILC2 function by modulating expression of PPAR target genes, many of the experiments presented here could be repeated in ILC2s or animals lacking PPARγ or other members of the PPAR family. If FA stored by DGAT2 are channeled into synthesis of PGD_2 that modulates PPARγ signaling, the ILC2 phenotype should be similar between cells deficient in PPARγ and cells deficient in DGAT2 or treated with a DGAT2 inhibitor. However, since PPARγ target genes are incredibly diverse and there are numerous PPARγ ligands that modulate its function, it may be difficult to confidently attribute changes in ILC2 phenotype upon PPARγ

deletion to a lack of intracellular PGD_2 signaling. Nevertheless, given that PPARγ is necessary for the induction of allergic airway inflammation (*520*) and may directly modulate ILC2 function (*521*), and given that eicosanoids have well characterized effects on ILC2 activation (*251, 252, 256, 258-262*), these pathways remain a promising area of study in ILC2 biology and may be active downstream of DGAT2 during ILC2 activation.

A third hypothesis that could explain the importance of DGAT2 in ILC2 function is that TAG synthesized by DGAT2 are channeled into phospholipid synthesis to sustain the increased requirement of membrane lipids necessary for ILC2 proliferation and cytokine secretion. The recent study that suggested PPARγ involvement in ILC2 activation also showed an increase in LDs and phospholipids upon ILC2 activation (*521*). However, this study only investigated phospholipid species containing palmitate, a 16-carbon FA. Our preliminary lipidomics analysis showed considerable variation in trends between activated and control ILC2s, depending on the length of the FAs incorporated into phospholipids. Since ILC2s proliferate rapidly upon activation and produce large amounts of cytokines, it seems likely that production of phospholipids would be necessary to sustain ILC2 activation. However, more extensive lipidomics analysis of lipid species in ILC2s would provide further insight into the increased requirement for phospholipids upon ILC2 activation and substrate specificity during phospholipid synthesis.

In conclusion, we demonstrate here a critical role of the TAG synthesis enzyme DGAT2 in ILC2 function and in initiation of allergic airway inflammation. We further demonstrate an increase in lipid uptake, lipid storage, and oxidative metabolism upon ILC2 activation by IL-33. Upon DGAT2 inhibition, there is a further increase in lipid storage and a decrease in oxidative metabolism. This may reflect an accumulation of DAG species and an inability to channel FA into downstream metabolic pathways, such as FA β-oxidation, eicosanoid synthesis and subsequent

modulation of PPAR activity, and phospholipid synthesis to sustain ILC2 proliferation and cytokine secretion. Given data presented here, there is not sufficient evidence favouring any one of these three hypotheses; indeed, all three metabolic pathways may be critical for sustained ILC2 activation and might be active simultaneously. Since ILC2 are critical modulators of the type 2 immune response at mucosal barrier sites, they are regarded as promising targets to dampen the excessive immune response leading to immunopathologies such as allergies and asthma. Pharmacological inhibition of DGAT2 in the treatment of non-alcoholic steatohepatitis (NASH) has thus far been well tolerated in clinical trials (*522*), and the data presented here suggests that DGAT2 inhibitors could be repurposed in the treatment of lung immunopathologies. Furthermore, characterization of the unique metabolic requirements of ILC2s will contribute to our understanding of this recently discovered cell population that is central to polarization of the type 2 immune response, and will further our knowledge within the rapidly expanding field of immunometabolism.

FIGURES AND TABLES

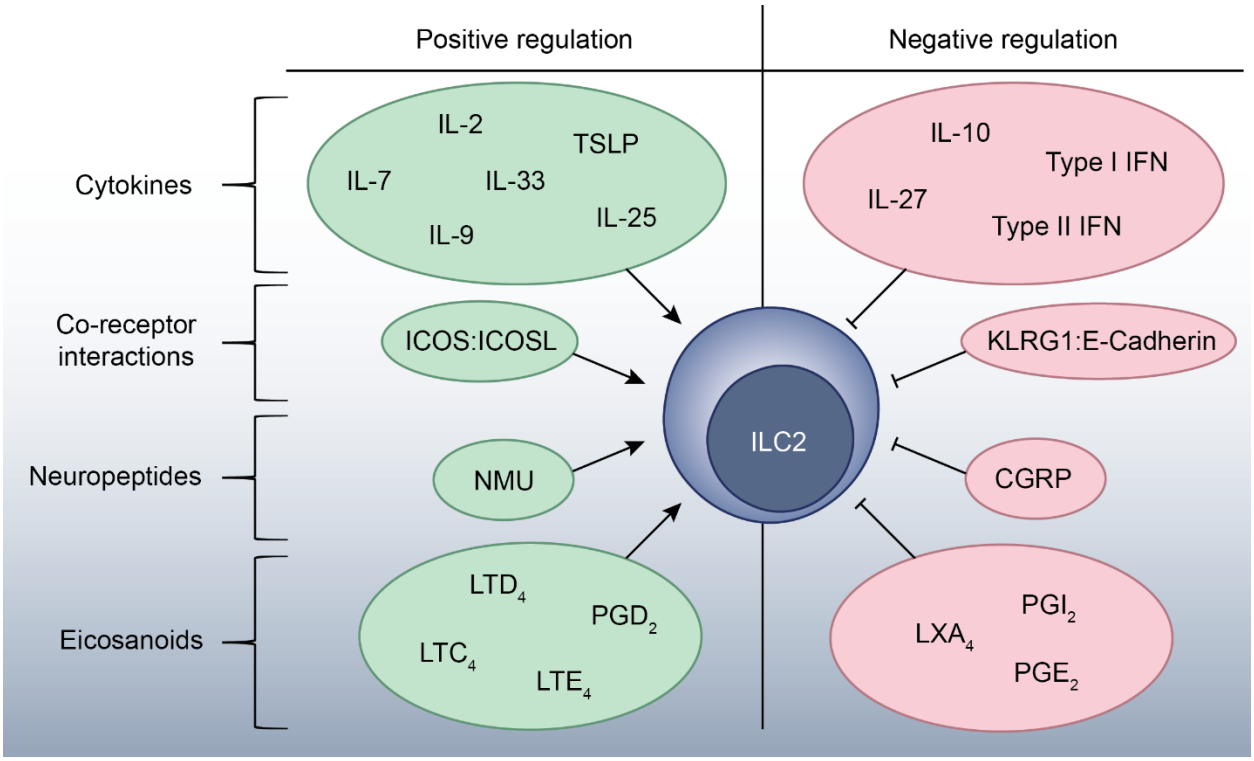

Figure 1: Positive and negative regulation of ILC2. Overview of the positive and negative regulators of ILC2s, including cytokines, surface co-receptor interactions, neuropeptides, and eicosanoids. CGRP = calcitonin gene-related peptide; ICOS = inducible T cell co-stimulator; ICOSL = ICOS ligand; IFN = interferon; IL = interleukin; ILC2 = group 2 innate lymphoid cell; KLRG1 = killer cell lectin-like receptor G1; LT = leukotriene; LX = lipoxin; NMU = neuromedin U; PG = prostaglandin; TSLP = thymic stromal lymphopoietin. β

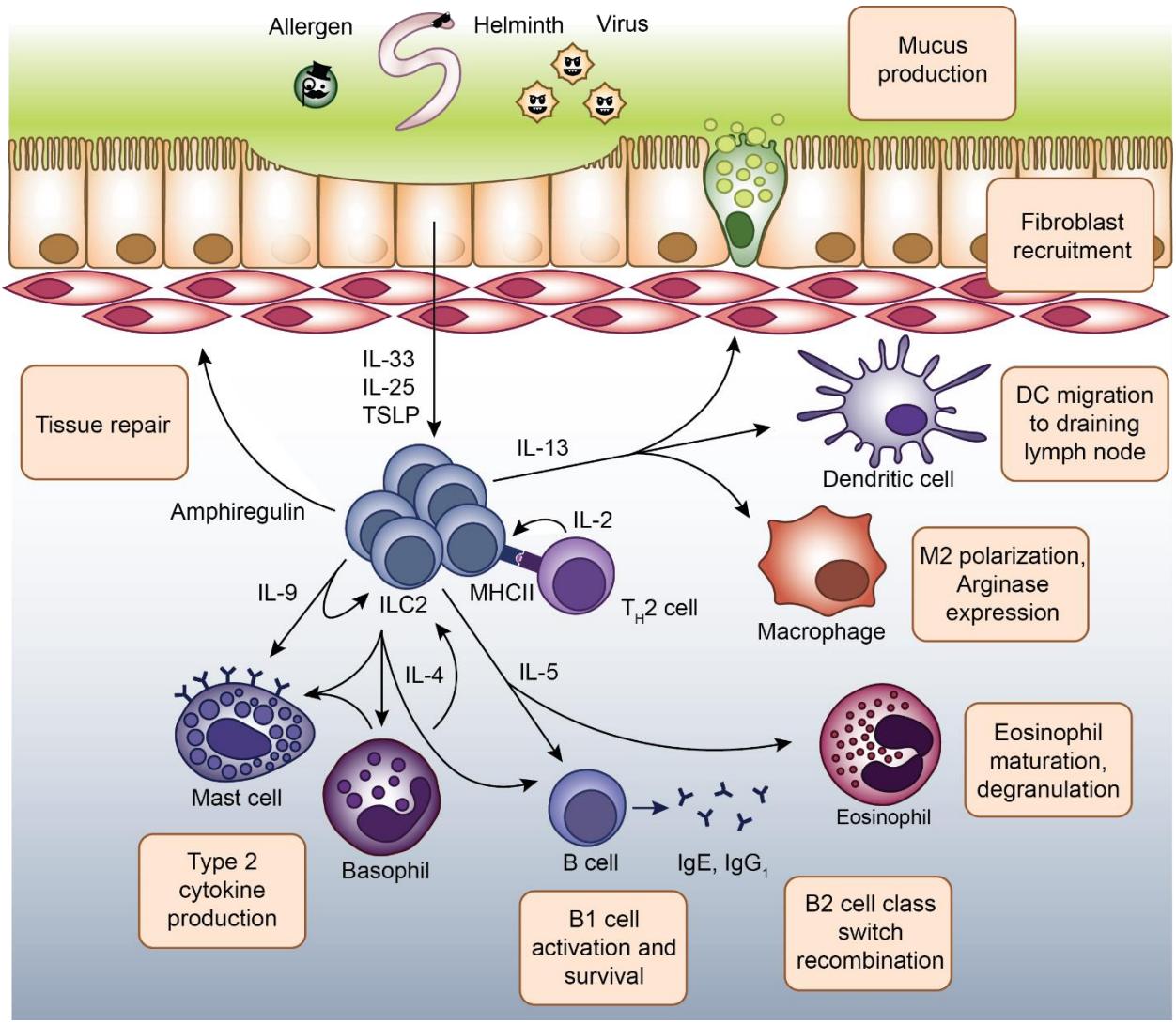

Figure 2: ILC2 effector function in the lung. Overview of the targets and functions of ILC2-derived type 2 cytokines in response to tissue damage by helminths, allergens, or viral infection. Cells within the lung epithelium release the alarmins IL-33, IL-25 and TSLP, which activate ILC2s to produce IL-4, IL-5, IL-9, and IL-13. IL = interleukin; ILC2 = group 2 innate lymphoid cell; MHCII = major histocompatibility complex class II; T_H2 = T helper cell type 2.

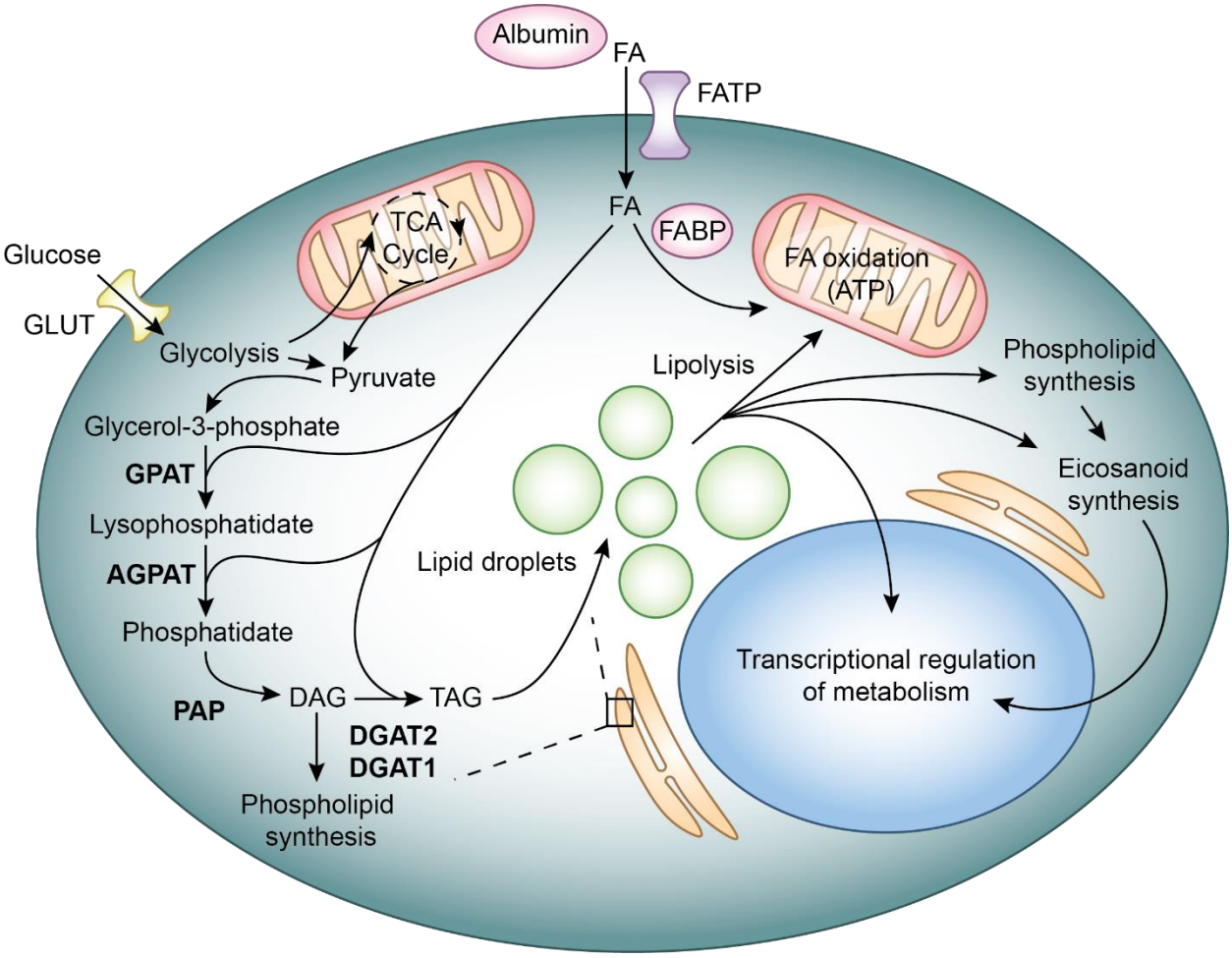

Figure 3: Major pathways in lipid metabolism. Overview of lipid mobilization between cellular compartments, and triacylglycerol synthesis, storage and breakdown. FA = fatty acid; FABP = fatty acid-binding protein; FATP = fatty acid transport protein; TCA = tricarboxylic acid; ATP = adenosine triphosphate; PPAR = peroxisome proliferator-activated receptor; GLUT = glucose transporter; DAG = diacylglycerol; TAG= triacylglycerol; GPAT = glycerol-3-phosphate acyltransferase; AGPAT = acylglycerol-3-phosphate acyltransferase; PAP = phosphatidate phosphatase (lipin-1, -2, 3-); DGAT = diacylglycerol acyltransferase.

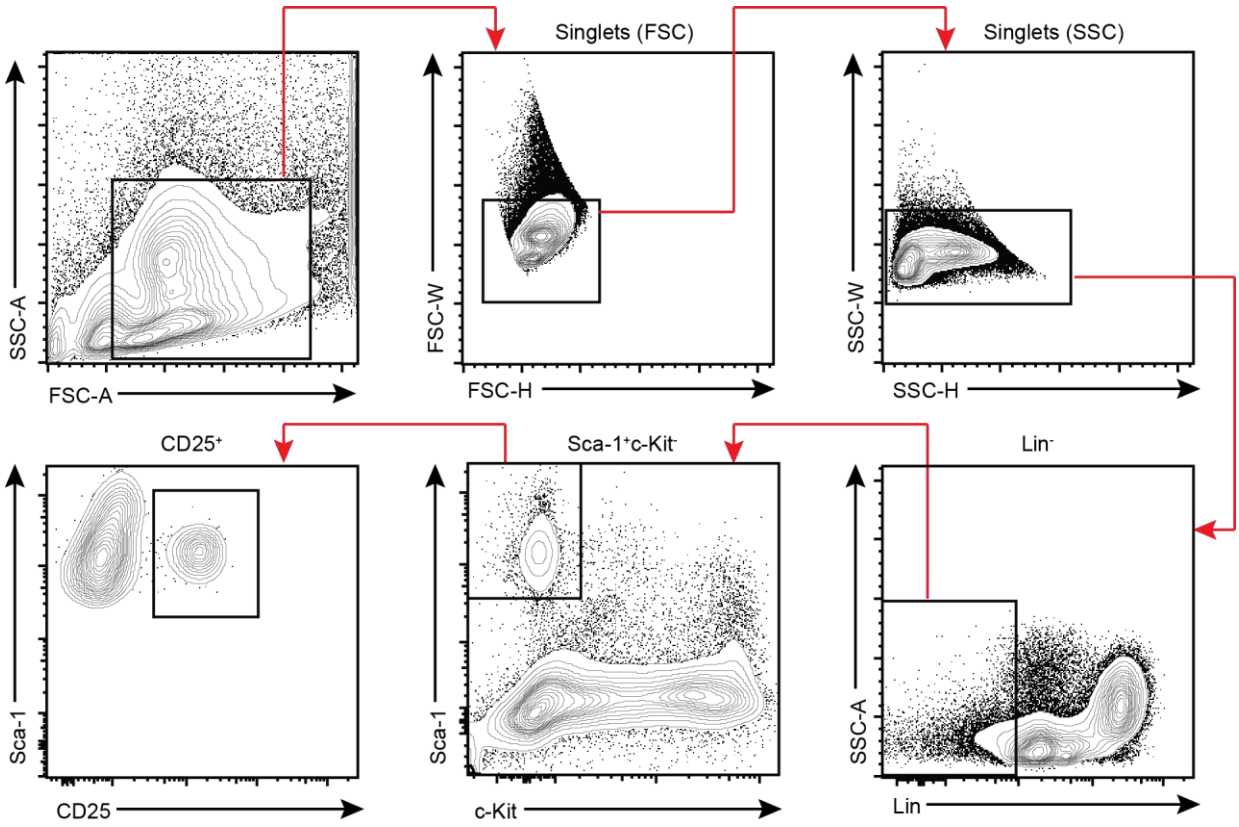

Figure 4: Gating strategy for isolation of murine bone marrow-derived ILC2. Bone marrow was isolated and red blood cells were lysed. ILC2 progenitors were sorted as Lin (B220, CD3ε, CD5, CD11b, CD11c, CD19, FcεRIα, Ly-6C/G, TCRαβ, TCRγδ, TER-119)$^{-}$Sca-1^{+}c-Kit^{-}CD25^{+} cells prior to expansion in complete media supplemented with IL-2, IL-7, IL-25, IL-33 (each at 50 ng/mL) and TSLP (20 ng/mL) for 14 days. Cells were then rested for 3 days in complete media supplemented with IL-2 and IL-7 (each at 10 ng/mL) prior to use in various experimental approaches.

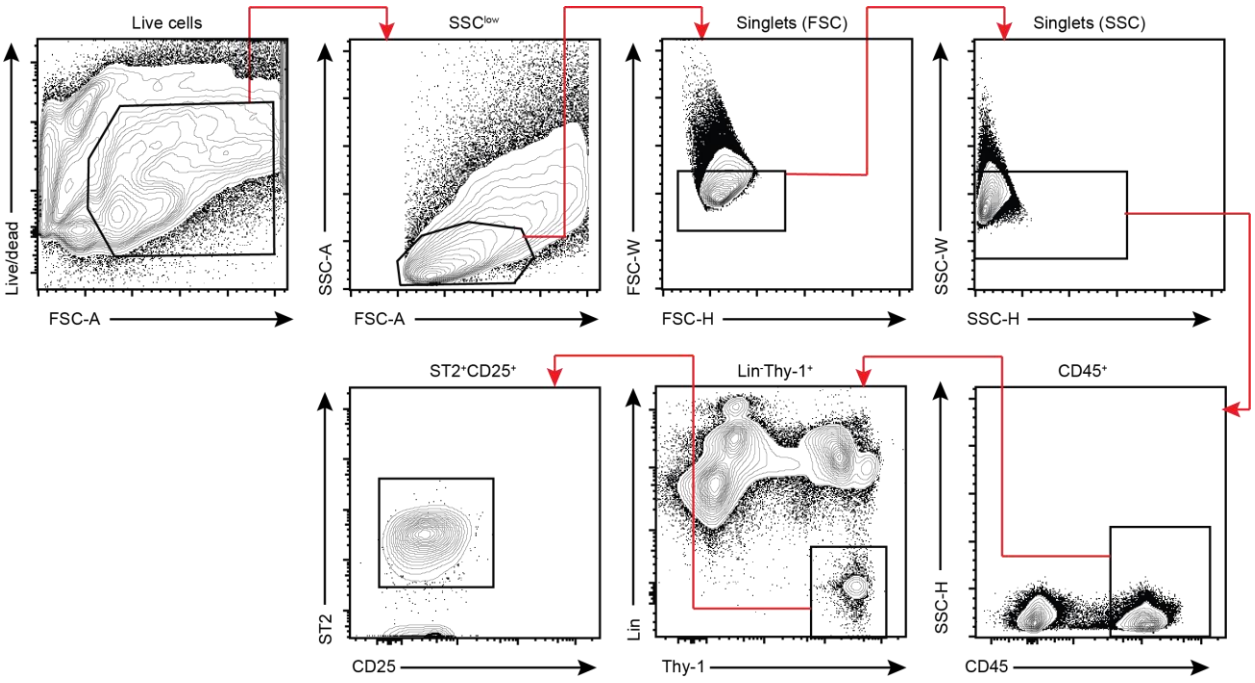

Figure 5: Gating strategy for isolation of murine lung ILC2. Lungs were minced and digested in RPMI-1640 with 5% FBS, Liberase™ (0.2 mg/mL) and DNase I (100 µg/mL). Red blood cells were lysed and ILC2s were sorted as live $CD45^+$Lin (B220, CD3ε, CD5, CD11b, CD11c, CD19, FcεRIα, Ly-6C/G, TCRαβ, TCRγδ, TER-119)$^-CD90^+ST2^+CD25^+$ cells. ILC2s were either stimulated immediately or expanded in IL-2, IL-7, IL-25, IL-33 (each at 50 ng/mL) and TSLP (20 ng/mL) and rested in IL-2 and IL-7 (10 ng/mL) prior to use in various experimental approaches.

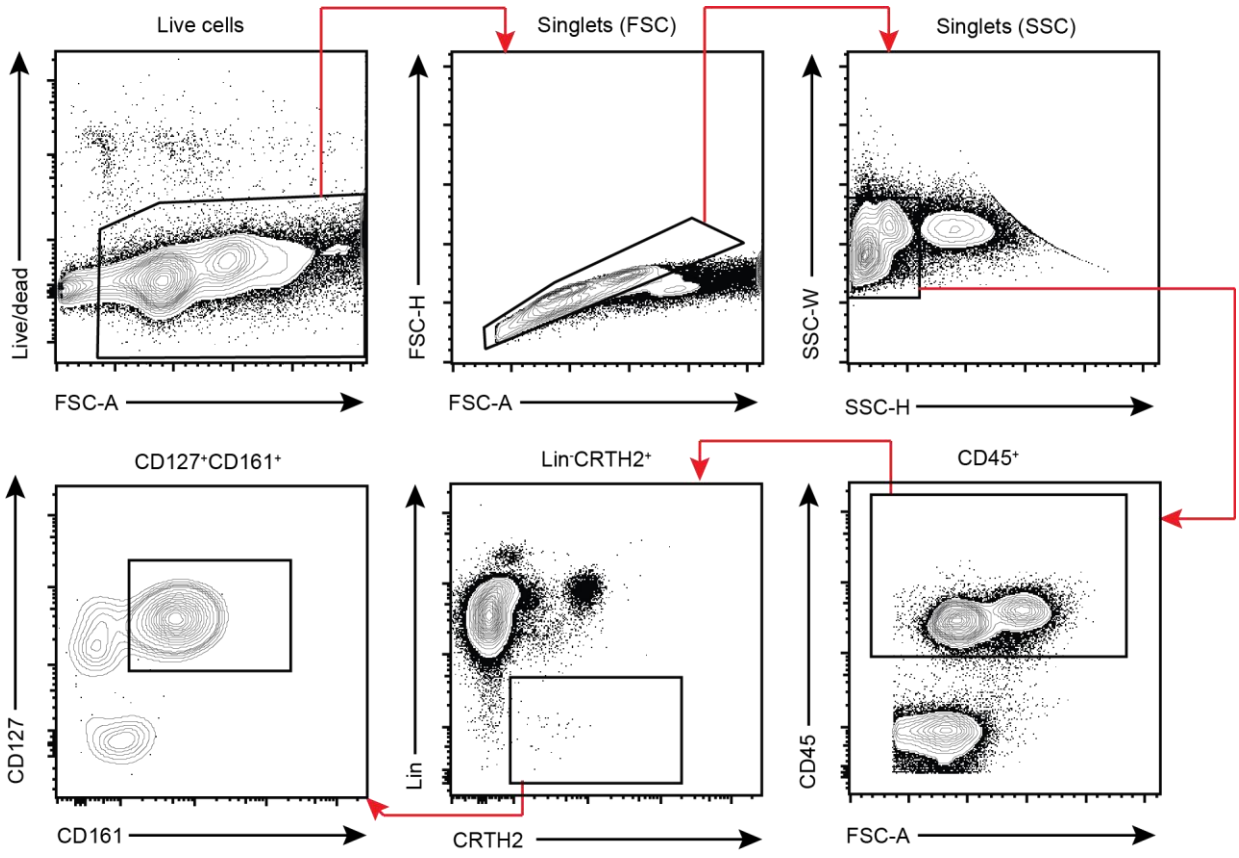

Figure 6: Gating strategy for isolation of human peripheral blood ILC2. Peripheral mononuclear cells (PBMCs) were isolated from whole blood using SepMate™ isolation tubes and lymphocyte separation medium. ILC2 were sorted as live $CD45^{+}$Lin(CD1a, CD3ε, CD11c, CD14, CD16, CD19, CD34, CD56, CD94, CD123, CD303, FcεRIα, TCRαβ, TCRγδ)$^{-}$ $CD127^{+}CD294^{+}CD161^{+}$ cells. ILC2s were either stimulated immediately or expanded in IL-2 (40 ng/mL) and rested in IL-2 (10 ng/mL) prior to use in various experimental approaches.

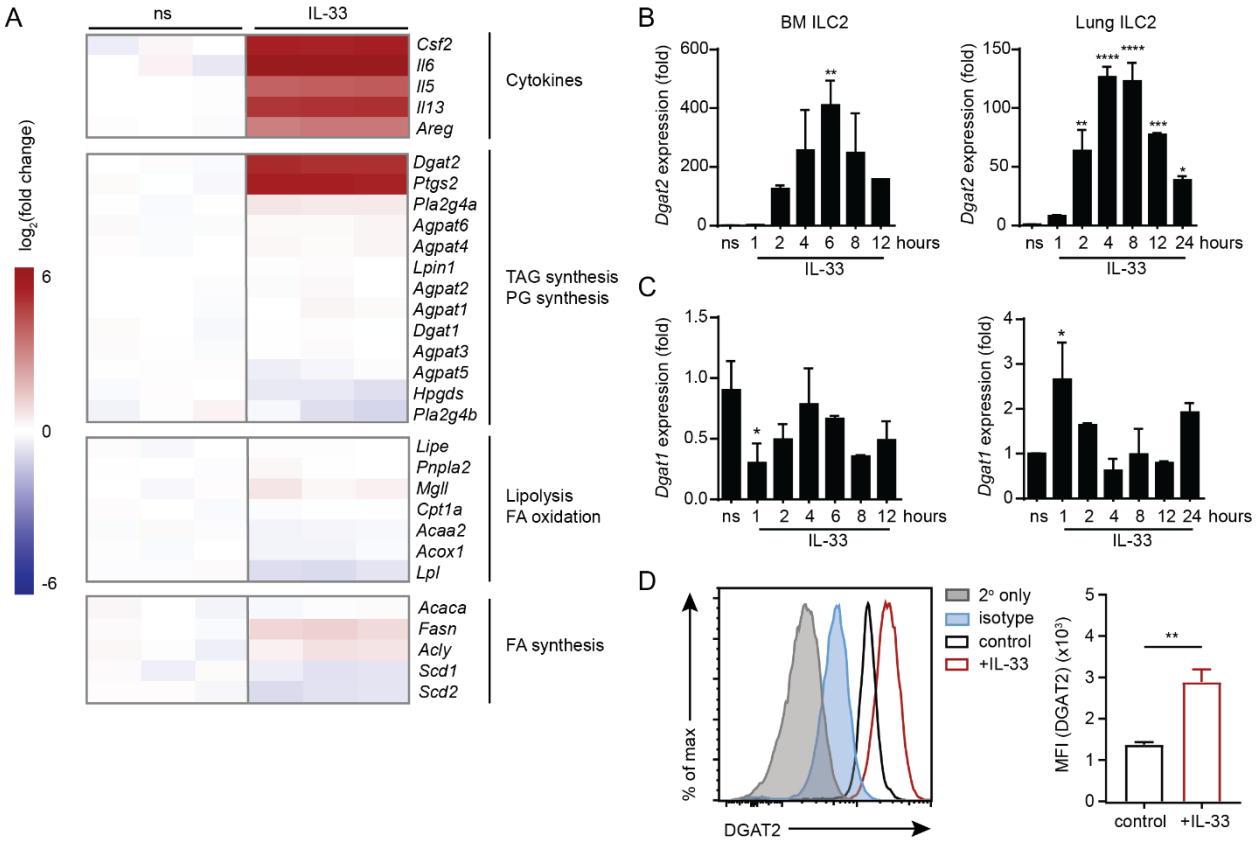

Figure 7: IL-33-induced ILC2 activation upregulates DGAT2. A, Gene expression profiles of expanded murine bone marrow (BM)-derived ILC2s left unstimulated (ns) or stimulated with IL-33 (10 ng/mL) for 4 hours. Genes were grouped arbitrarily by metabolic pathways, and clustered within groups using an expression clustering software. **B, C,** qRT-PCR analysis of *Dgat2* and *Dgat1* expression (fold change over non-stimulated) of both expanded murine bone marrow (BM)-derived ILC2 and freshly sorted murine lung ILC2s that were left non-stimulated (ns) or stimulated with IL-33 (10 ng/mL). **D,** Expanded murine BM-derived ILC2s were either stimulated with IL-7 (control) or with IL-7+IL-33 (each at 10 ng/mL). 48 hours after stimulation, DGAT2 protein was stained and quantified by flow cytometric analysis. Cells stimulated with IL-7+IL-33 and stained with a rabbit polyclonal IgG isotype control antibody or an anti-rabbit secondary antibody only are included as staining controls. Mean fluorescence intensity (MFI) of DGAT2 protein of IL-7-stimulated (control) cells and IL-7+IL-33 (+IL-33) cells is shown in a bar graph. Data are presented as means ± SD with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = *** and $p < 0.0001$ = ****. ns, no stimulus; nd, not detectable; TAG, triacylglycerol; PG, prostaglandin; FA, fatty acid.

Abbreviations in heatmap: *Acaa2* = acyl-CoA acyltransferase 2 (ACAA2); *Acly* = ATP citrate lyase (ACLY); *Acox1* = acyl-CoA oxidase 1 (ACOX1); *Agpat* = 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT); *Areg* = amphiregulin; *Cpt1a* = carnitine palmitoyl transferase 1a (CPT1a); *Csf2* = granulocyte-macrophage colony-stimulating factor (GM-CSF); *Dgat* = diacylglycerol acyltransferase (DGAT); *Fasn* = fatty acid synthase (FAS); *Hpgds* = hematopoietic prostaglandin D synthase (HPGDS); *Il* = interleukin (IL); *Lipe* = hormone-sensitive lipase (HSL); *Lpin1* = lipin 1; *Lpl* = lipoprotein lipase (LPL); *Mgll* = monoacylglycerol lipase (MGLL); *Ptgs2* = prostaglandin-endoperoxide synthase 2 (PTGS2, COX2); *Pla2* = phospholipase A2 (PLA2); *Pnpla2* = adipose triglyceride lipase (ATGL); *Scd* = stearoyl-CoA desaturase.

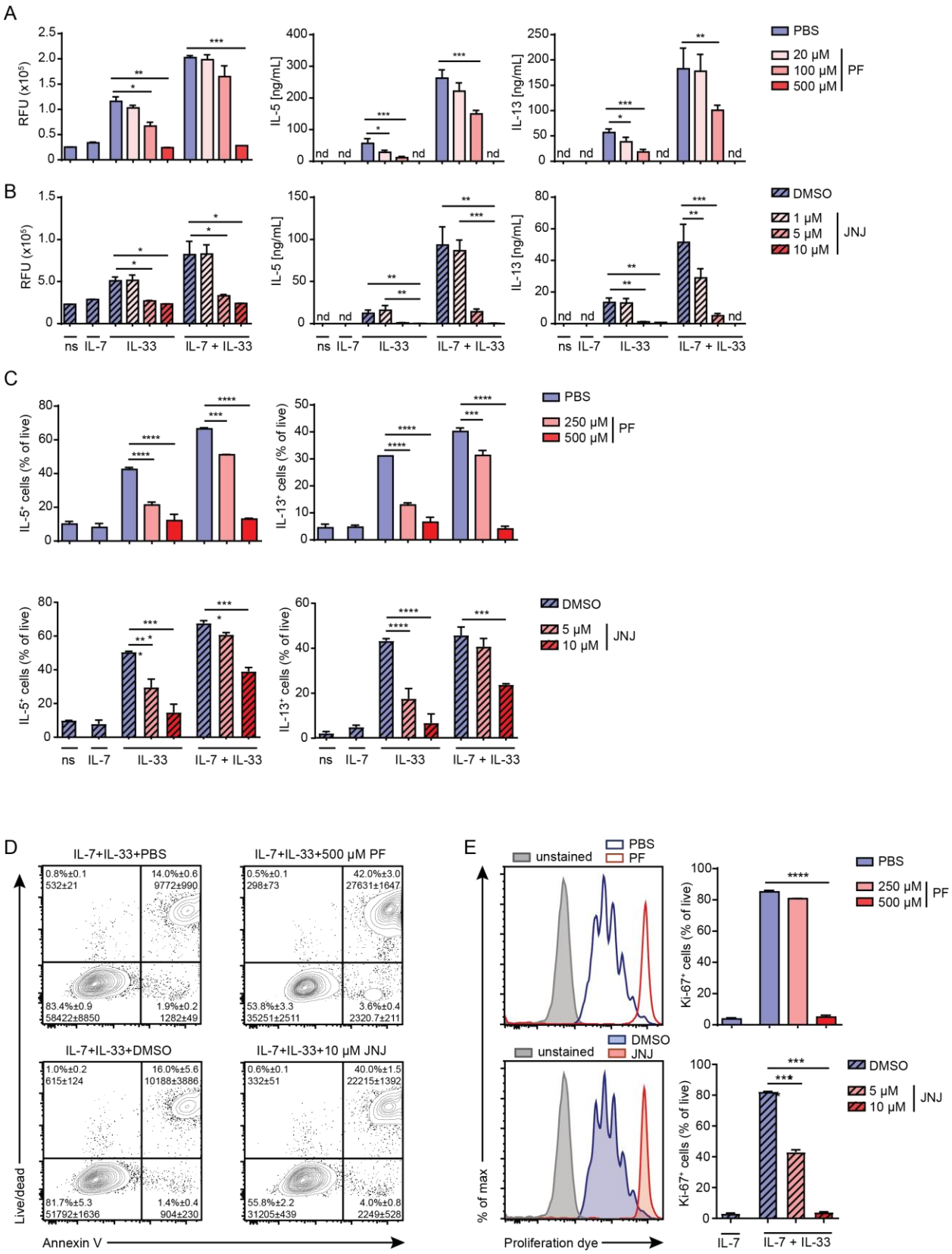
A
RFU (x10⁵)
IL-5 [ng/mL]
IL-13 [ng/mL]
nd
PBS
20 μM
100 μM
500 μM
PF
B
DMSO
1 μM
5 μM
10 μM
JNJ
ns
IL-7
IL-33
IL-7 + IL-33
C
IL-5⁺ cells (% of live)
IL-13⁺ cells (% of live)
250 μM
500 μM
D
IL-7+IL-33+PBS
IL-7+IL-33+500 μM PF
IL-7+IL-33+DMSO
IL-7+IL-33+10 μM JNJ
0.8%±0.1
532±21
14.0%±0.6
9772±990
83.4%±0.9
58422±8850
1.9%±0.2
1282±49
0.5%±0.1
298±73
42.0%±3.0
27631±1647
53.8%±3.3
35251±2511
3.6%±0.4
2320.7±211
1.0%±0.2
615±124
16.0%±5.6
10188±3886
81.7%±5.3
51792±1636
1.4%±0.4
904±230
0.6%±0.1
332±51
40.0%±1.5
22215±1392
55.8%±2.2
31205±439
4.0%±0.8
2249±528
Live/dead
Annexin V
E
unstained
PBS
PF
DMSO
JNJ
% of max
Proliferation dye
Ki-67⁺ cells (% of live)
IL-7
IL-7 + IL-33

Figure 8: DGAT2 inhibition restricts ILC2 viability and cytokine production in murine ILC2. A, Freshly sorted murine lung ILC2s were left non-stimulated (ns) or stimulated with IL-7, IL-33, or IL-7 + IL-33 (all at 10 ng/mL) in the absence or presence of the DGAT2 inhibitor PF-06424439 (PF; 20 μM, 100 μM, 500 μM). Five days after stimulation, viability (RFU) was determined using AlamarBlue cell viability reagent, and cytokine contents in culture supernatants were quantified by ELISA. **B,** Freshly sorted murine lung ILC2s were stimulated as described in A in the absence or presence of the DGAT2 inhibitor JNJ DGAT2-A (JNJ; 1 μM, 5 μM, 10 μM) and analyzed five days after stimulation. **C,** Expanded BM-derived ILC2 were stimulated as described in A**.** 48 hours after stimulation, intracellular cytokines were stained and quantified with flow cytometric analysis. **D,** Expanded BM-derived ILC2 were stimulated as described in A. 18 hours after stimulation, cells were stained for Annexin V and with a viability dye and analyzed with flow cytometry. Percentages and absolute cell numbers are noted in each quadrant. **E,** Expanded murine BM-derived ILC2 were stained with eFluor 450 proliferation tracking dye and stimulated as described in A. Three days after stimulation, cells were stained for intracellular Ki-67 and analyzed by flow cytometry. Data are presented as means ± SD with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = *** and $p < 0.0001$ = ****. ns, no stimulus; nd, not detectable.

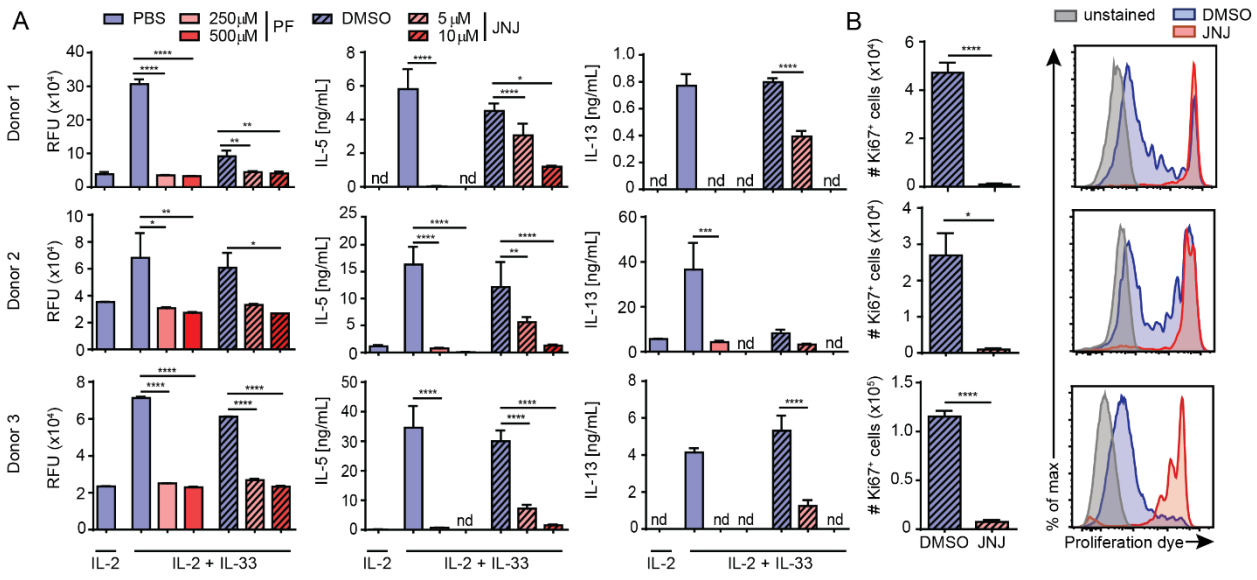

Figure 9: DGAT2 inhibition restricts ILC2 viability and cytokine production in human peripheral ILC2. A, Human ILC2s were sorted from peripheral blood from three independent donors and were stimulated with IL-2 only or IL-2+IL-33 (both at 10 ng/mL) in the absence or presence of the DGAT2 inhibitors PF-06424439 (PF; 250 µM, 500 µM) or JNJ DGAT2-A (JNJ; 5 µM, 10 µM). Five days after stimulation, viability (RFU) was determined using AlamarBlue cell viability reagent, and cytokine contents in culture supernatants were quantified by ELISA**. B,** Human ILC2s were isolated as described in A and expanded in IL-2 prior to stimulation. Cells were stained with eFluor 450 proliferation tracking dye and stimulated with IL-2+IL-33 in the absence or presence of the DGAT2 inhibitor JNJ DGAT2-A (JNJ; 10 µM). Five days after stimulation, cells were stained for intracellular Ki-67 and analyzed by flow cytometry. Data are presented as means ± SD with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = *** and $p < 0.0001$ = ****. ns, no stimulus; nd, not detectable.

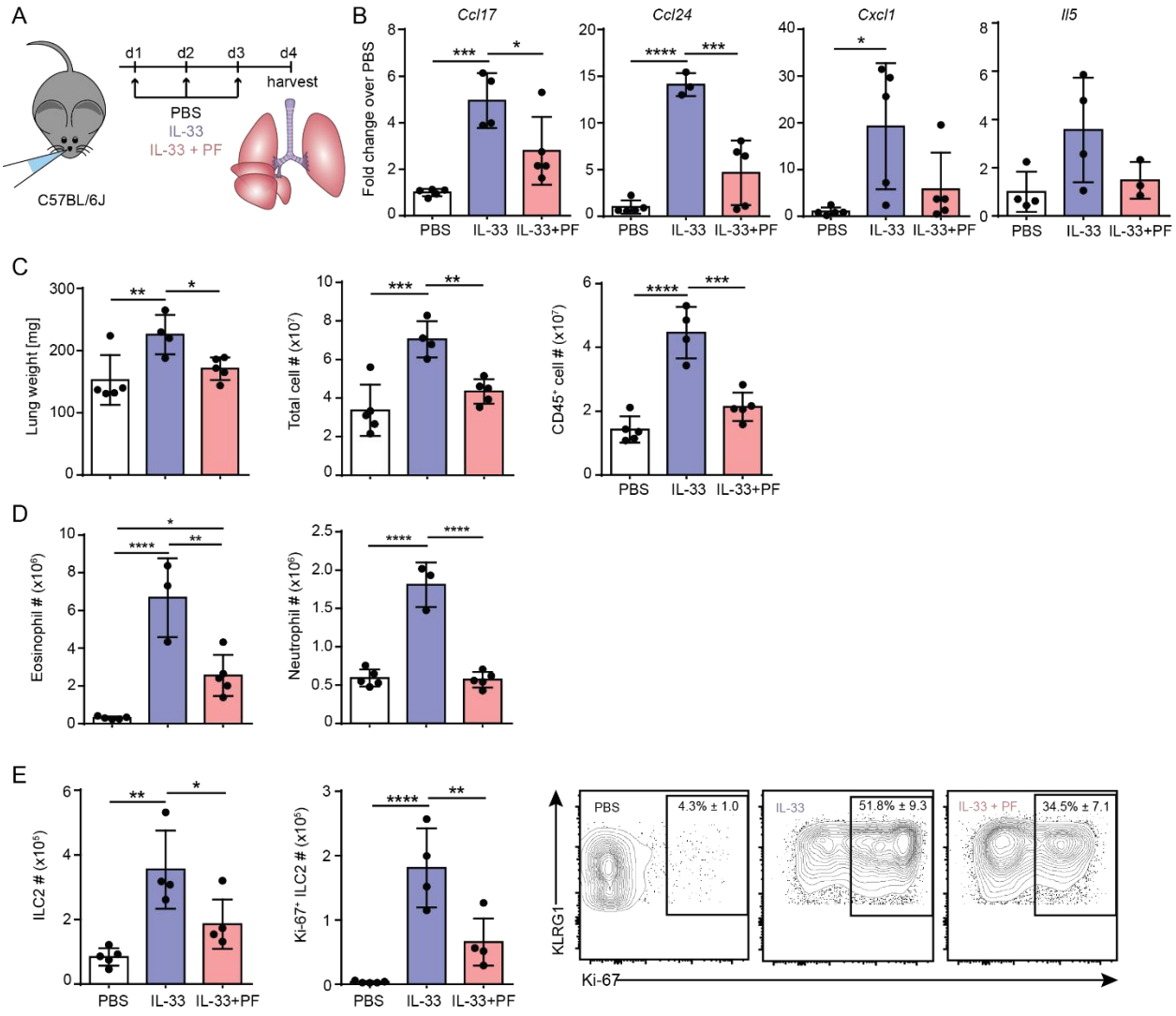

Figure 10: Inhibition of DGAT2 *in vivo* restrains IL-33-mediated allergic lung inflammation. **A,** Wild-type mice were challenged intranasally for three consecutive days with PBS as control, IL-33 or IL-33 plus the DGAT2 inhibitor PF-06424439 (PF). Lung tissues were obtained 24 hours after the final treatment. **B,** mRNA was extracted from whole lung tissue and expression levels for *Ccl17*, *Ccl24*, *Cxcl1*, and *Il5* were determined by qRT-PCR. **C,** Weight, total cell counts, and $CD45^+$ cell counts of lungs were determined. **D,** Total numbers of eosinophils and neutrophils of lungs were determined by flow cytometry. **E,** Total ILC2 numbers and Ki-67^+ ILC2s were determined by flow cytometry. Data are presented as means ± SD with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ** and $p < 0.0001$ = ****.

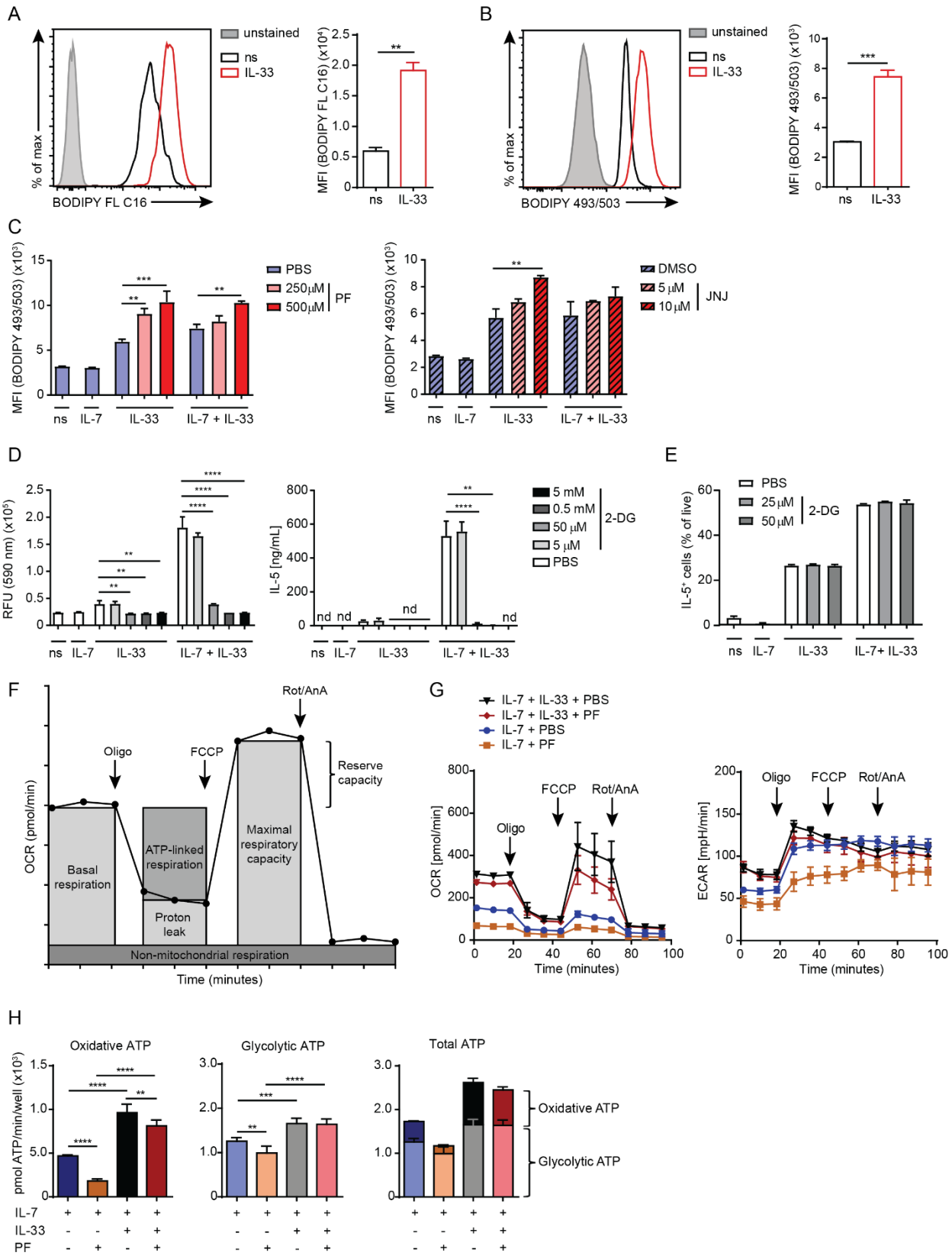
A
unstained
ns
IL-33
% of max
BODIPY FL C16
MFI (BODIPY FL C16) (x10⁴)
B
BODIPY 493/503
MFI (BODIPY 493/503) (x10³)
C
PBS
250μM
500μM
PF
DMSO
5 μM
10 μM
JNJ
ns
IL-7
IL-33
IL-7 + IL-33
D
RFU (590 nm) (x10⁵)
IL-5 [ng/mL]
5 mM
0.5 mM
50 μM
5 μM
PBS
2-DG
nd
E
IL-5⁺ cells (% of live)
25μM
50μM
IL-7+ IL-33
F
Rot/AnA
Oligo
FCCP
Reserve capacity
OCR (pmol/min)
Basal respiration
ATP-linked respiration
Proton leak
Maximal respiratory capacity
Non-mitochondrial respiration
Time (minutes)
G
IL-7 + IL-33 + PBS
IL-7 + IL-33 + PF
IL-7 + PBS
IL-7 + PF
OCR [pmol/min]
ECAR [mpH/min]
H
Oxidative ATP
Glycolytic ATP
Total ATP
pmol ATP/min/well (x10³)
IL-7
IL-33
PF

Figure 11: IL-33 induces lipid uptake and alters the metabolic profile of ILC2. A, Expanded murine BM-derived ILC2s were left non-stimulated (ns) or stimulated with IL-33 (10 ng/mL). After 23 hours, medium was supplemented with BODIPY™ FL C16. After 1 hour, BODIPY FL C16 uptake was quantified by flow cytometric analysis. **B,** Expanded murine BM-derived ILC2s were either left non-stimulated (ns) or treated with IL-33 (10 ng/mL) for 24 hours, and neutral lipid content was quantified by staining with BODIPY™ 493/503 and flow cytometric analysis. **C,** The experimental setup in **B** was repeated in the presence of DGAT2 inhibitors PF-06424439 (PF-2) or JNJ DGAT2-A (JNJ) or controls. **D, E,** Expanded murine BM-derived ILC2s were either left non-stimulated (ns) or with IL-7, IL-33, or IL-7+IL-33 (all at 10 ng/mL) in the absence or presence of the glucose analogue 2-deoxyglucose (2-DG) to inhibit glycolysis. **D,** Five days after stimulation, viability (RFU) was determined using AlamarBlue cell viability reagent, and cytokine concentration in culture supernatants were quantified by ELISA. **E,** 48 hours after stimulation, intracellular cytokines were stained and quantified with flow cytometric analysis. **F,** Schematic of Seahorse analysis. **G,** Expanded murine bone marrow ILC2 were stimulated with IL-7 or IL-7+IL-33 in the presence or absence of PF-06424439 (PF; 250 μM) for 12 hours. Cells were then seeded into a Seahorse XF96 plate in XF base minimal DMEM supplemented with 2 g/L D-glucose and 2 mM L-glutamine. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured upon treatment with oligomycin (Oligo; 1.5 μM), carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazine (FCCP; 1 μM), and a mixture of rotenone (Rot; 0.5 μM) and antimycin A (AA; 0.5 μM). **H,** ATP produced by oxidative metabolism and glycolysis were quantified as described by Mookerjee *et al.* (J Bio Chem, 2017). Data are presented as means ± SD with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ** and $p < 0.0001$ = ****. Ns, no stimulus; nd, not detectable.

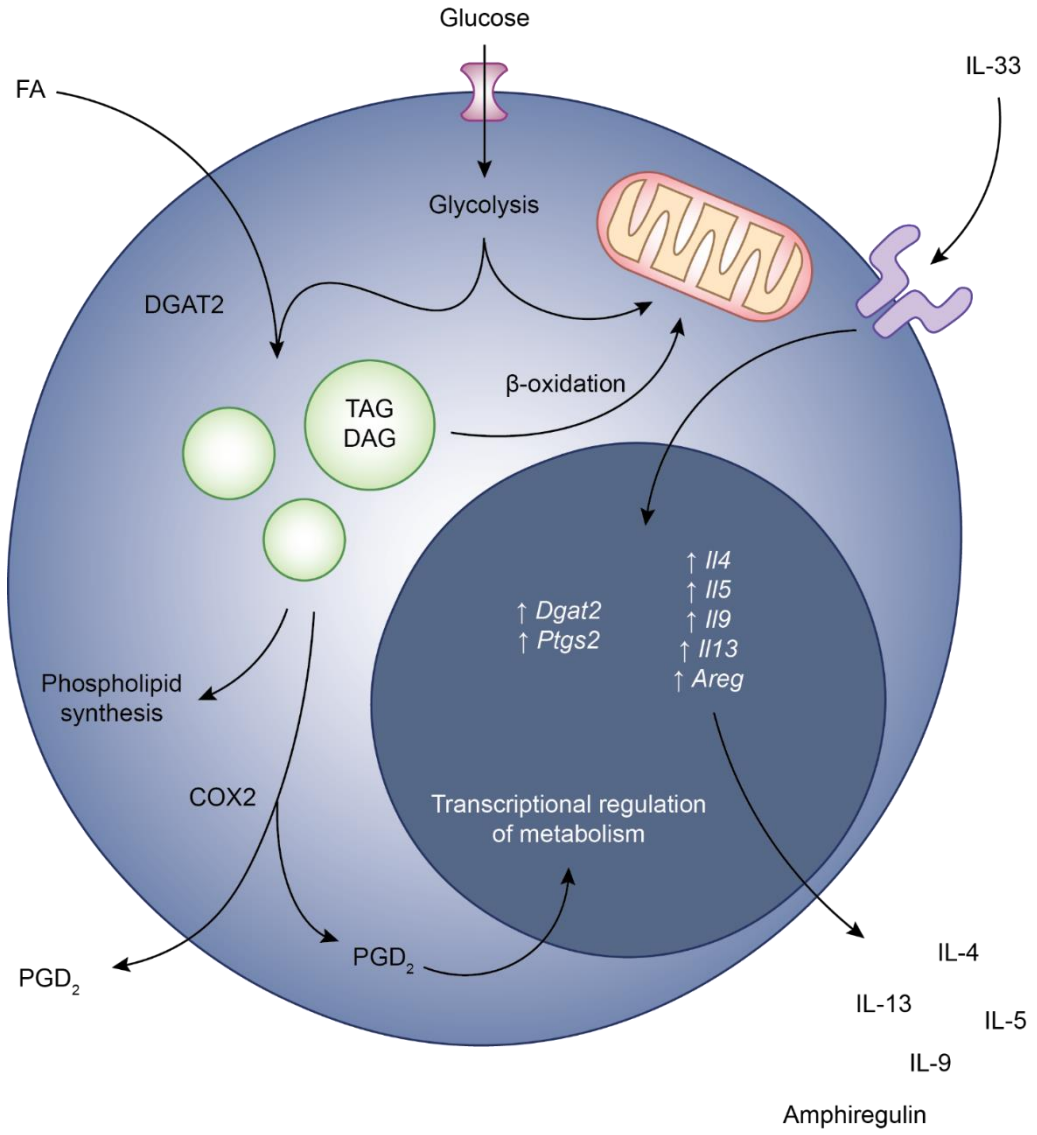

Figure 12: Proposed working model of DGAT2 and lipid metabolism in ILC2 function. DGAT2 (*Dgat2*) and COX2 (*Ptgs2*) are upregulated upon IL-33 stimulation of ILC2s. FA are stored as TAG by DGAT2, and FA uptake increases. TAG are subsequently broken down to feed into oxidative metabolism (β-oxidation) in the mitochondria, phospholipid synthesis to sustain ILC2 proliferation, and PGD_2 production to modulate PPAR function and transcriptional regulation of metabolism. Upon DGAT2 inhibition, DAG accumulate and FA cannot be channelled into these downstream metabolic pathways. COX2 = cyclooxygenase 2; DAG = diacylglycerol; DGAT2 = diacylglycerol acyltransferase 2; FA = fatty acid; IL = interleukin; PGD_2 = prostaglandin D_2; TAG = triacylglycerol.

Table 1: Flow cytometry antibodies

Supplier	Clone	Target	Conjugate
BioLegend	104	CD45.2	Brilliant violet 421
BioLegend	HP-3G10	Human CD161	Brilliant violet 421
BioLegend	53-2.1	CD90.2 (Thy-1)	Brilliant violet 510
BioLegend	M1/70	CD11b	Brilliant violet 650
BioLegend	30-F11	CD45.2	Brilliant violet 785
eBioscience	PC61.5	CD25	eFluor 450
eBioscience	SolA15	Ki-67	eFluor 450
eBioscience	PC61.4	CD25	Alexa Fluor 488
BioLegend	HI149	Human CD1α	FITC
BioLegend	CD3	Human CD3ε	FITC
BioLegend	3.9	Human CD11c	FITC
BioLegend	HCD14	Human CD14	FITC
BioLegend	3G8	Human CD16	FITC
BioLegend	H1B19	Human CD19	FITC
BioLegend	581	Human CD34	FITC
BioLegend	6H6	Human CD123	FITC
BioLegend	201A	Human CD303	FITC
BioLegend	AER-37 (CRA-1)	Human FcεRIα	FITC
BioLegend	IP26	Human TCRα/β	FITC
BioLegend	B1	Human TCRγ/δ	FITC
BD Horizon	B159	Human CD56	BB515
eBioscience	RMST2-2	ST2	PerCP-eFluor 710
eBioscience	PC61.5	CD25	PerCP-Cy5.5
BD Pharmingen	1A8	Ly6G	PerCP-Cy5.5
eBioscience	145-2C11	CD3ε	PE
eBioscience	M1/70	CD11b	PE
eBioscience	N418	CD11c	PE
eBioscience	eBIo1D3	CD19	PE
eBioscience	MAR-1	FcεRI	PE
eBioscience	RB6-8C5	Ly6G	PE
eBioscience	PK136	NK1.1	PE
eBioscience	H57-597	TCRβ	PE
eBioscience	eBIoGL3	TCRγ/δ	PE
eBioscience	RB6-8C5	Gr1	PE
eBioscience	TER-119	Ter119	PE
eBioscience	PA3-6B2	B220	PE
eBioscience	53-7.3	CD5	PE

BioLegend	A019D5	Human CD127	PE
BD Pharmingen	E50-2440	Siglec-F	PE
eBioscience	2F1	KLRG1	PE-Cy7
BioLegend	BM16	Human CD294 (CRTH2)	Alexa Fluor 647
BioLegend	HI30	Human CD45	Alexa Fluor 700
eBioscience	2B8	c-Kit	APC
eBioscience	SolA15	Ki-67	eFluor 660
eBioscience	50-9966	GATA3	eFluor 660
eBioscience	A7R34	CD127	APC-eFluor 780
eBioscience	N418	CD11c	APC-eFluor 780

Table 2: qRT-PCR primers

Gene	Forward primer (sense)	Reverse primer (antisense)
Ccl17	GGAAGTTGGTGAGCTGGTATAA	GATGGCCTTCTTCACATGTTTG
Ccl24	CTTGCTGCACGTCCTTTATTTC	ACAGATCTTATGGCCCTTCTTG
Cxcl1	CACCTCAAGAACATCCAGAGC	CTTGAGTGTGGCTATGACTTCG
Dgat1	CAGCTCAGACAGTGGTTTCA	ACCTTGCATTACTCAGGATCAG
Dgat2	ACCCTGAAGAACCGCAAAG	CCTCAAAGATCACCTGCTTGTA
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Il5	GCTTCCTGTCCCTACTCATAAA	CCCACGGACAGTTTGATTCT

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