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Department of Oncology

Characterization of the interaction between circulating human Innate Lymphoid Cell Progenitors and endothelial cells

Doctoral thesis ès Life Sciences (PhD)

presented to

the Faculty of Biology and Medicine of the University of Lausanne

by

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Characterization of the interaction between circulating human Innate Lymphoid Cell Progenitors and endothelial cells

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pour le Doyen
de la Faculté de biologie et de médecine

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Directeur de l'Ecole Doctorale

Summary (English)

Besides the well-established role of NK cells in mediating innate anti-tumor immune responses in different tumor types, the recently described helper Innate Lymphoid Cells (ILCs) are also emerging as novel players in tumor immunity. However, due to their heterogeneous tissue-distribution and to their high plastic potential, both pro- and anti-tumor activities have been observed. However, whether and how human ILCs could have anti-tumor effects through the interaction with the vascular endothelium, remains unknown.

During my PhD, I focused on the isolation, expansion and characterization of circulating human ILC subsets (ILC1s, ILC2s and ILCPs) to be employed in co-culture experiments with primary endothelial cells, to assess their capacity to induce adhesion molecules expression on endothelial cells *in vitro*. I was able to show that, following *in vitro* expansion, ILCPs increase the adhesive properties of endothelial cells, by significantly upregulating E-Selectin, ICAM-1 and VCAM-1 expression on endothelial cell surface. This interaction was primarily dependent on the direct contact between the two cell types and on the engagement of NF-kB in endothelial cells via surface TNF and RANKL on ILCPs. Moreover, ILCPs acquired a more activated and ILC3-/LTi-like phenotype, given the upregulation of CD69, the key transcription factor for ILC3 development RORγt and two known markers of LTi cells, i.e., CCR6 and CXCR5. The ILCP-mediated activation of endothelial cells resulted to be functional, by allowing the adhesion of T, B, NK cells and monocytes freshly isolated from the peripheral blood of healthy donors. Interestingly, pre-exposure of ILCPs to bladder and colon cancer cells impaired their endothelial cell-activating capacity, defining a potential alternative strategy of tumor cells to escape an anti-tumor immune response, but also an intriguing target to develop novel immunotherapeutical strategies to treat cancer patients.

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Département d'Oncologie

La caractérisation de l'interaction entre les Progéniteurs des Cellules Lymphoïdes Innées humaines circulantes et les cellules endothéliales

Thèse de doctorat ès sciences de la vie (PhD)

présentée à

la Faculté de Biologie et de Médicine de l'Université de Lausanne

par

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Lausanne 2021

Résumé (Français)

Outre le rôle bien établi des cellules NK dans la médiation des réponses immunitaires anti-tumorales innées dans différents types de tumeurs, les cellules lymphoïdes innées (ILCs), récemment décrites, apparaissent également comme de nouveaux acteurs dans l'immunité tumorale. Toutefois, en raison de leur distribution tissulaire hétérogène et de leur plasticité, des activités tant pro-tumorales qu'anti-tumorales ont été observées. À ce jour, on ignore encore comment ces cellules pourraient avoir des effets anti-tumoraux via leur interaction avec l'endothélium vasculaire.

Au cours de mon doctorat, je me suis concentrée sur l'isolation, l'expansion et la caractérisation des sous-ensembles de ILC humaines circulantes (ILC1s, ILC2s et ILCPs), que j'ai utilisé dans des expériences de co-culture avec des cellules endothéliales primaires, afin d'évaluer leur capacité à induire l'expression de molécules d'adhésion sur les cellules endothéliales *in vitro*. J'ai en effet pu montrer qu'après une expansion *in vitro*, les ILCPs augmentent les propriétés d'adhésion des cellules endothéliales, en augmentant de manière significative l'expression de E-Selectin, ICAM-1 et VCAM-1 à leur surface. Cette interaction dépend principalement du contact direct entre les deux types de cellules et de l'engagement de NF-kB dans les cellules endothéliales via le TNF de surface et le RANKL sur les ILCPs. De plus, les ILCPs ont acquis un phénotype plus activé et plus proche de celui des ILC3/LTi, étant donné la haute expression de CD69, de RORγt, le facteur de transcription clé pour le développement des ILC3s, de CCR6 et CXCR5, deux marqueurs connus des cellules LTi. L'activation des cellules endothéliales par les ILCPs s'est avérée fonctionnelle, en permettant l'adhésion de cellules T, B, NK et des monocytes fraîchement isolés du sang périphérique des donneurs sains. Il est également intéressant de noter que la pré-exposition des ILCPs aux cellules de cancer de la vessie et du côlon a altéré leur capacité d'activer les cellules endothéliales, définissant une stratégie alternative potentielle des cellules tumorales pour échapper à une réponse immunitaire anti-tumorale, mais aussi une cible intéressante pour développer de nouvelles stratégies immuno-thérapeutiques pour traiter les patients atteints de cancer.

Résumé large public (Français)

Le système immunitaire détecte et détruit tout ce qui est étranger à notre organisme, comme les virus et les bactéries. Du fait des nombreuses mutations qui s'accumulent lors de la croissance tumorale, les cellules cancéreuses peuvent être considérées comme étrangères et vont donc pouvoir être reconnues par le système immunitaire. En temps normal, le système immunitaire œuvre pour éliminer ces pathogènes ou ces cellules cancéreuses grâce à l'activation de cellules spécifiques que l'on appelle les lymphocytes T, qui vont rechercher puis éliminer ces potentielles menaces. C'est pour cette raison que les recherches actuelles se focalisent essentiellement sur la compréhension des interactions entre lymphocytes T et cellules cancéreuses, afin de développer des immunothérapies, c'est-à-dire des thérapies basées sur l'exploitation des défenses immunitaires du patient afin d'attaquer le cancer. Aujourd'hui, certaines immunothérapies consistent à prélever directement chez les patients des lymphocytes T reconnaissant spécifiquement les cellules cancéreuses grâce à une prise de sang. Une fois ces cellules spécifiques isolées, elles sont multipliées et activées en laboratoire, avant d'être réinjectées chez ces mêmes patients.

Malheureusement, les cellules cancéreuses sont capables d'évoluer afin d'échapper aux lymphocytes T et donc d'échapper à une réponse immunitaire anti-tumorale. C'est pour cette raison également, qu'il est très important de comprendre comment se comportent les autres cellules immunitaires, et en particulier les cellules du système immunitaire innée, qui constituent la première ligne de défense contre tous pathogènes. Récemment, des études ont montré que les cellules du système immunitaire innés, et en particulier les cellules lymphoïdes innées (ILCs), sont souvent altérées dans leur fonction chez les patients atteints de cancer, et pourraient donc contribuer à la progression de la tumeur.

Étant donné que les lymphocytes doivent abandonner la circulation sanguine et traverser les vaisseaux sanguins pour pouvoir accéder à la tumeur et déployer leur fonctions anti-tumorales, le projet de ma thèse a pour but de comprendre si les ILCs circulantes peuvent faciliter l'adhésion aux vaisseaux sanguins des lymphocytes adaptatives et d'autres leucocytes, et donc de favoriser leur infiltration dans la tumeur, ce mécanisme étant souvent altéré dans le microenvironnement tumoral.

Grace à l'utilisation d'anticorps, des molécules qui reconnaissent des protéines présentes à la surface des cellules et qui sont couplés à une molécule fluorescente, nous avons pu isoler les ILCs à partir du sang de donneurs sains et nous avons pu les cultiver en présence de cellules de vaisseaux sanguins, que l'on appelle les cellules endothéliales. De plus, les progéniteurs des ILCs ont permis l'augmentation des propriétés adhésives des cellules endothéliales. Cette interaction était fonctionnelle, puisqu'elle a provoqué l'adhésion d'un grand nombre de cellules immunitaires aux cellules endothéliales. Chez les patients avec

une tumeur de la vessie, on a observé que la fréquence des progéniteurs des ILCs était réduite par rapport aux individus sains. Étonnamment, les cellules tumorales de la vessie ont impacté la capacité des progeniteurs des ILCs d'interagir de façon fonctionnelle avec les cellules endothéliales.

Nous espérons que ce travail permettra un meilleur suivi ainsi que l'utilisation des cellules lymphoïdes innées dans les futures thérapies visant les patients atteints de cancer.

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Abbreviations

AAM	alternatively-activated macrophage
AAV	ANCA-associated vasculitis
AD	atopic dermatitis
ADC	antibody drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
AHR	aryl hydrocarbon receptor
AIRE	autoimmune regulator
ALCL	anaplastic large cell lymphoma
AML	acute myeloid leukemia
ANCA	acute-phase anti-neutrophil cytoplasmic antibody
ANG2	angiopoietin 2
AOM/DSS	azoxymethane/dextran sodium sulfate
APC	antigen-presenting cell
APL	acute promyelocytic leukemia
AR	allergic rhinitis
AREG	amphiregulin
Arg-1	arginase-1
AS	ankylosing spondylitis
BCG	Bacillus Calmette-Guerin
BCR	B-cell receptor
bFGF	basic FGF
BM	bone marrow
CAR	chimeric antigen receptor
CCR	chemokine receptor
CD	cluster of differentiation
CGRP	calcitonin gene-related peptide

CHILP	common innate helper lymphoid progenitor
CILP	common innate lymphoid progenitor
c-Kit	tyrosine-protein kinase Kit
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
COPD	chronic obstructive pulmonary disease
CRC	colorectal cancer patients
CRS	chronic rhinosinusitis
CRTH2	chemoattractant receptor-homologous molecule expressed on T-helper type 2
CSF-3	colony-stimulating factor 3
CTL	cytotoxic T lymphocytes
CysLT	cysteinyl leukotrienes
DAMP	damage-associated molecular pattern
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell
ECM	extracellular matrix
ET	endothelin
ETR	endothelin receptor
FasL	Fas ligand
FGF	fibroblast growth factor
GDNF	glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCC	hepatocellular carcinoma
HDM	house dust mite
HEV	high endothelial venules
HLA	human leukocyte antigen
IBD	intestinal bowel disease

IC	immune checkpoint
ICI	immune checkpoint inhibitor
ICAM-1	intercellular adhesion molecule 1
IDO	indoleamine 2,3-dioxygenase
ie	intraepithelial
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-7Rα	IL-7Rα chain
ILC	innate lymphoid cell
ILCP	ILC progenitor
iNKT	invariant NKT cell
ITIM	immunoreceptor tyrosine-based inhibition motif
LEC	lymphatic endothelial cells
LFA-1	lymphocyte function-associated antigen 1
lncRNAs	long noncoding RNAs
LPS	lypopolisaccharide
LT	lymphotoxin
LTi	lymphoid tissue-inducer cell
LTβR	lymphotoxin beta receptor
MadCAM-1	mucosal vascular addressin cell adhesion molecule 1
MAIT	mucosal-associated invariant T cell
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MIBC	muscle-invasive bladder cancer
MLN	mesenteric lymphnode
M-MDSC	monocytic myeloid-derived suppressor cell

MMP9	matrix metallopeptidase-9
MNP	mononuclear phagocyte
MSC	mesenchymal stromal cell
MS	multiple sclerosis
MSS	microsatellite stable
NCAM-1	neural cell adhesion molecule-1
NCR	natural cytotoxicity receptor
NGS	next generation sequencing
NK	natural killer
NKP	NK precursor
NKT	natural killer T
NMIBC	non-muscle-invasive bladder cancer
NMU	neuromedin U
NMUR1	neuromedin U receptor 1
NSCLC	non-small-cell lung cancer
PAMP	pathogen-associated molecular pattern
PD-1	programmed cell death 1
PDGF	plateled-derived growth factor
PD-L1	programmed cell death ligand 1
PECAM-1	platelet endothelial cell adhesion molecule
PGD2	prostaglandin D2
PlGF	placental growth factor
PLZF	promyelocytic leukemia zinc finger
PP	Peyer's patch
PRR	pattern recognition receptor
RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
RET	rearranged during transfection tyrosine kinase receptor
ROI	reactive oxygen intermediates

RORα	retinoid-related orphan receptor α
RORγt	retinoid-related orphan receptor γt
ROS	reactive oxygen species
SDF-1	stroma cell-derived factor 1
siLP	small intestine lamina propria
siRNA	short interfering RNA
SLO	secondary lymphoid organ
SSc	system sclerosis
TAA	tumor-associated antigen
TAM	tumor associated macrophage
TANK	tumor-associated NK
TBX21	T-box transcription factor
TCR	T-cell receptor
TEM	transendothelial migration
TGF-β	transforming growth factor β
Th	T helper
TIL	tumor-infiltrating lymphocyte
TILN	tumor-infiltrated lymph nodes
TINK	tumor-infiltrating NK
TLS	tertiary lymphoid structure
TME	tumor microenvironment
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory
TSLP	thymic stromal lymphopoietin
VAT	visceral adipose tissue
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal polypeptide

VIPR2	VIP receptor 2
VLA-4	very late antigen 4
VTP	vascular targeting peptide
β2-AR	β2 adrenergic receptor
γc	γ chain

Table of contents

Introduction

1. The Innate Immunity

Historically, the term *immunity* derived from the Latin word *immunitas*, which consisted in the protection of Roman senators from legal prosecution during their administration. In biology, *immunity* refers to the ability of multicellular organisms to resist against potentially harmful microorganisms. The cells and molecules that coordinate the *immune response*, i.e., the reaction against the introduction of a foreign agent into the body, constitute the *immune system*. To establish an infection, the pathogen must first overcome numerous surface barriers, such as skin, hair, enzymes and mucus that either are directly antimicrobial or inhibit the attachment of the microbe. Any organism that breaks through these first barriers encounters the two further levels of defense, the *innate* and *adaptive immune systems* (1).

The *innate immune system* is the oldest evolutionary defense strategy that consists of numerous cellular and biochemical defense mechanisms that are already in place, ready to respond rapidly and in a non-pathogen-specific manner (Figure 1). For this reason, it is believed that innate responses remain unchanged irrespective of how often the antigen (Ag) is encountered. However, a growing body of literature suggests that also innate immune cells can show immunological memory, in light of the enhanced responsiveness that confers greater protection against reinfection, reported for example in plants and invertebrates (that lack and adaptive system) (2). This property has been defined “trained immunity” and it consists in epigenetic and metabolic modifications of the cells of the innate system, conferring them the ability to adjust the responses to future stimulations with the same pathogen (2).

In contrast, the adaptive immune system is slower in responding to an infection but will then involve cells that are highly specialized and are able to specifically recognize pathogens (3). A fraction of these cells will then be capable of forming a memory to a specific pathogen in order to reduce the response time to the same pathogen and to generate a stronger response upon a second encounter (3). The capacity of B and T cells to recognize specific Ags is ensured via the high frequency of gene rearrangement during the development of the B and T cell receptors (BCR and TCR) in these cell populations (3).

The principal components of the innate immunity are:

1. physical and chemical barriers (epithelia and anti-microbial chemicals produced at epithelial surfaces);

2. phagocytic cells (neutrophils, macrophages), dendritic cells (DCs), natural killer (NK) cells, innate lymphoid cells (ILCs), eosinophils, basophils and mast cells;
3. blood proteins (the complement system and other mediators of inflammation), cytokines and chemokines, that regulate and coordinate the trafficking and the functions of cells of both the innate and adaptive immunity.

To discriminate between self and non-self, the innate immune cells are able to recognize evolutionarily conserved structures on pathogens, named pathogen-associated molecular patterns (PAMPs) (4) that include carbohydrates, cell wall lipids, proteins and nucleic acid of bacterial and viral origin. Moreover, the innate immune system is able to recognize endogenous danger molecules that are released from damaged/dying cells, called damage-associated molecular patterns (DAMPs) (5), such as nuclear proteins, crystals and stress-induced proteins (1). These structures are recognized through a limited number of non-clonal pattern recognition receptors (PRRs) that are independent of immunological memory (6). The activation of innate immune cells via PRRs results in the triggering of signaling cascades that provoke the induction of genes involved in antimicrobial host defense, mainly leading to pro-inflammatory cytokine and type I interferon (IFNs) production (6).

Innate immune responses are effective at controlling and even eradicating infections. However, many pathogenic microbes evolved to resist to innate immunity. Hence, defense against these pathogens requires the stronger and specialized mechanisms of the *adaptive immunity*, which prevents them from invading and replicating in the host's cells and tissues (1).

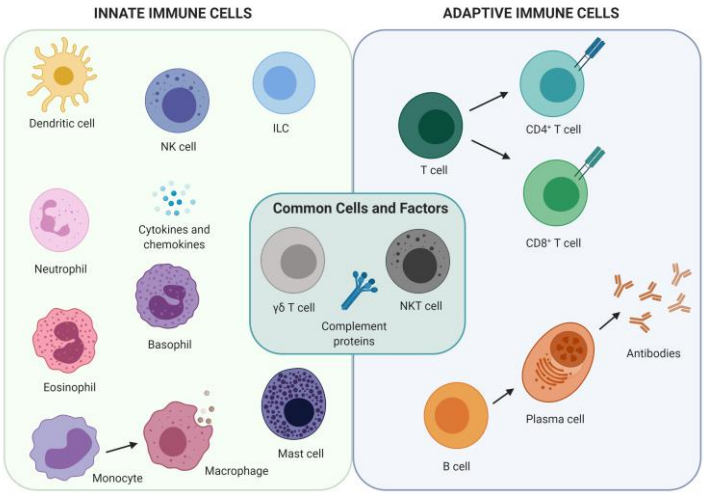

Figure 1: The components of the innate and adaptive immune systems. The innate immunity provides the rapid initial defense against infections. Adaptive immune responses develop later and consist of activation of Ag-specific lymphocytes. Adapted from (1).

The primary cells involved in the innate immune response are found in close proximity to surface barriers, like the skin, gut and respiratory tracts. They include surveillance cells – macrophages, DCs and ILCs, and the effector cells, such as NK cells and granulocytes, recruited during inflammatory processes (7). T and B lymphocytes are then added to the immune cell arsenal to develop the adaptive immune response (7).

Macrophages, or "the big eaters", derive from circulating monocytes that rapidly migrate to sites of infection (8), where they uptake and degrade dead cells, cellular debris, and non-self material to coordinate inflammatory processes (9). The tissue microenvironment regulates differentiation of precursor monocytes into two main subtypes of tissue-resident macrophages (10). During viral and bacterial infections, IFN-γ and/or lipopolysaccharide (LPS) favor the generation of the *classically activated* M1 macrophages, that produce high levels of pro-inflammatory cytokines (such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-12 and IL-23), microbicidal products and reactive oxygen intermediates (ROIs) (11). In contrast, the *alternatively activated* M2 macrophages arise in response to IL-4, IL-13, or fungal and helminth infections and produce IL-10, to limit T cell proliferation and IFN-γ production, and arginase-1 (Arg-1) to promote tissue repair (12).
However, macrophages are unable to initiate a primary immune response, which is the main role of the DCs, professional Ag-presenting cells (APCs) (1).

DCs are mostly bone marrow (BM)-derived leukocytes capable of detecting microbial molecules in their host's environment and they act as a critical link between the innate and adaptive arms of immunity (13). First, immature circulating DCs are attracted to the site of danger, transiently increase in Ag uptake capabilities (a change in phenotype indicated by the expression of costimulatory molecules) and change in expression of homing and chemokine receptors (CCRs), directing migration of DCs to lymphoid tissues (14). A second regulatory phase, which occurs as the DCs enter the lymph nodes, is associated with the presentation of pathogen-derived Ags in combination with other signals to T and B lymphocytes, and culminates with the initiation of the adaptive immune response (14).

Granulocytes (neutrophils, eosinophils, and basophils) are short-lived effector cells of the innate immunity that mature in the BM and, once fully differentiated, migrate to the circulation (15). They play an important role during the early phases of inflammation: in response to chemoattractants released by damaged and/or infected cells, such as IL-8 and eotaxin, they extravasate and infiltrate into the site of infection (15).

Here, they start to release cytotoxic granules containing lytic enzymes and ROIs with anti-microbial potential (16).

Mast cells are granular immune cells that systemically migrate from the BM to accumulate at inflammatory sites, in particular the ones in direct contact with the external environment (such as skin, mucosal surfaces and gastrointestinal tracts) (17). Mast cells are key players during allergic and anaphylactic inflammatory reactions: they promote vasodilation and vascular permeability, to favor recruitment of other immune cell types and facilitate the downstream adaptive responses for pathogen elimination (17).

The complement system is an ancient member of the immune system that primarily activates in response to acute-phase proteins synthetized in the liver (18). It consists of a wide array of soluble proteins with important protective roles, including recognition and clearance of foreign Ags and apoptotic cells (19), stimulation of phagocytosis of covalently bound (opsonized) targets (20) and, similarly to DCs, it is considered at the interface between the innate and the adaptive Immune systems, given its ability to promote and modulate both humoral and cellular immune responses (18).

Finally, there are three cell types which display both innate and adaptive features: mucosal-associated invariant T (MAIT) cells, natural killer T (NKT) cells and $\gamma\delta$ T cells, able to bridge innate and adaptive immune responses.

MAIT cells are abundant cells that are involved in various infectious and non-infectious diseases. MAIT cell activation can be TCR-dependent or not and exhibit fast innate-like effector responses (21). Their TCR has a particular affinity for the microbial riboflavin-derivative Ags which are presented by the major histocompatibility complex (MHC) class I–like protein MR1, a β2 microglobulin-associated Ag-presenting molecule (21). MAIT cell role in immunity is still to this date unclear.

NKT cells are a subset of innate-like T-cells that express a semi-variant TCR, able to recognize lipid and glycolipid Ags presented by the MHC-like molecule cluster of differentiation (CD) 1d (22). These adaptive characteristics go along with the innate ability of NKTs to promptly produce cytokines upon activation (23), through which they modulate the recruitment of other innate immune cells and favor DC and B-cell maturation (24). In humans, NKT cells can be divided in two groups, according to their TCR: Type I NKT cells, also termed invariant NKT (iNKT) cells, have a distinct invariant TCR α-chain with restricted TCR β-chain repertoires; on the contrary type II NKT cells can express different TCR chain combinations (25).

γδ T cells are defined as mostly CD4 and CD8 negative unconventional T lymphocytes that rapidly expand upon reinfection with a previously encountered pathogen, similarly to adaptive immune lymphocytes (26). They recognize phosphorylated metabolites through their TCR, that shows very restricted diversity (27). Moreover, γδ T cells express the NK cell marker NKG2D, an activating receptor that recognizes stress-inducible MHC class I-related molecules that are frequently upregulated on malignant and stressed cells (28). γδ T cells participate in wound healing processes, in the clearance of distressed and transformed cells and in the regulation of excessive inflammation (29).

2. The big family of Innate lymphoid cells

The term "innate lymphoid cells" identifies a heterogeneous group of innate immune cells, both in mice and humans, including three macro populations: NK cells, Lymphoid Tissue-inducer (LTi) cells and non-cytotoxic helper ILCs (30).

NK cells are the most well described innate granular lymphocytes, and constitute the 7-15% of total circulating lymphocytes in healthy individuals (31) and can also be found in healthy skin and gut, in the intestinal mucosa, in Peyer's patches (PPs) and mesenteric lymph nodes, as well as in the liver, in the lungs, and in the uterus during pregnancy (32). Besides, NK cells can also be found in kidney, joints and breast under pathological conditions (32). NK cells are known for their ability to recognize and lyse virus-infected and malignant cells, but also orchestrate the subsequent immune responses (33). The differentiation of NK cells depends on the transcription factors Tox, NFIL3, Id2, Ets1, T-bet and Eomes (Figure 2) (23). $CD3\varepsilon^{-}CD7^{+}CD127^{-}$ cells mark the earliest stage of committed NK precursors (NKPs) (34). Mature NK cells are defined as $CD3^{-}CD56^{+}CD94^{+}$ cells (35). The development and maintenance of human NK cells relies on IL-15 signaling through IL-2Rβ (CD122) and the cytokine receptor common γ chain (γc) (36). In humans, they are classically divided into two major subsets, based on the expression of CD16 (the low affinity Fc gamma receptor 3A) that mediates antibody-dependent cellular cytotoxicity (ADCC) (31) and CD56 (the neural cell adhesion molecule-1 NCAM-1). Despite being poorly represented in the circulation (5% of total NKs) and with little cytolytic potential, $CD56^{bright}CD16^{lo/-}$ NKs are primarily found in the liver and in the lymphnodes for their ability to produce enormous amounts of immunomodulatory cytokines, especially IFN-γ (31) upon stimulation with monocyte-, DCs- and T-cell-derived IL-1β, IL-2, IL-12, IL-15, and/or IL-18 (37,38). On the contrary, $CD56^{dim}CD16^{+}$ NK cells account for the 95% of the total circulating NK cell pool and can efficiently mediate direct cytotoxicity of the target cells via perforin and granzyme secretion, but produce significantly lower amounts of IFN-γ upon stimulation (31). NK cells can lyse any target cells that

lacks self MHC class I molecule expression (the “missing-self hypothesis”) (39). However, regulation of NK cell activation and function is a complex fine-tuning of the integration between activating and inhibitory signals, received after encountering a target cell (40).

For example, the best-characterized NK cell activating receptor is NKG2D (40). This receptor recognizes “induced-self” ligands (i.e., molecules that are not expressed or are expressed at very low levels on the majority of normal cells, but which are upregulated on stressed, infected or malignant cells) (41). NKG2D recognition of target cells is the major approach of natural killing of tumor cells (42). NK cells are also regulated by inhibitory receptors that mainly recognize MHC class I molecules on target self-cells, causing the activation of signaling pathways that stop the cytolytic activity of NK cells (40). In mice, the Ly49 receptors directly recognize MHC class Ia molecules. Differently, human NK cells recognize MHC class Ia via a specific set of receptors called killer cell Ig-like receptors (KIRs), absent in mice, that can have either activating or inhibitory functions (40). Both mice and humans also express the heterodimeric inhibitory receptor CD94/NKG2A heterodimeric inhibitory receptor, that engages non-classical MHC I molecule (human leukocyte antigen (HLA)-E in humans and Qa-1 in mice) (40).

LTi cells were the first population of ILCs to be discovered, with important functions in secondary lymphoid organ (SLO) formation during the fetal stage and in the regulation of T cell central tolerance to self-Ags in the thymus (43). In the adult stage, a subset of phenotypically related but functionally different LTi cells has been described in secondary lymphoid tissues, and named LTi-like cells (44,45). Although LTi/LTi-like cells express the ILC3s master regulator retinoid-related orphan receptor γt (RORγt) and produce the effector cytokines IL-22, IL-17A and IL-17F, their development diverged from all other innate lymphoid cell family members (Figure 2) (46).

Given their abundance in the intestinal lamina propria, LTi-like cells are crucial in the maintenance of gut homeostasis by repressing the activation of commensal Ag-specific $CD4^+$ T effector cells (47). LTi/LTi-like cells are also closely linked to the adaptive immune responses: they facilitate the central tolerance during T cell generation, promote the survival of $CD4^+$ memory T cells and sustain the production of T-dependent or T-independent antibodies in lymphoid or mucosal tissues (45).

Non-cytotoxic helper-like ILCs are the most recently identified members of the lymphoid lineage (48). ILCs are characterized by the constitutive expression of the IL-7 receptor α chain (IL-7Rα, or CD127) but lack the expression of commonly known lineage markers and re-arranged Ag-specific receptors (see Table 1) (48,49). Mainly enriched at surface barriers, ILCs evolved to rapidly respond to tissue- and cell-derived

stress signals by rapidly producing high quantities of effector cytokines, which orchestrate subsequent immune responses (50). However, it is possible to find ILCs also in the human peripheral circulation, where they account for the 0.2-0.3% of the total pool of lymphocytes (51).

Interestingly, increasing reports have highlighted tissue-specific functions and features of ILCs. In humans, circulating ILCs and SLO-resident ILCs have more a quiescent and migratory state, similarly to naïve T cells (52). By contrast, ILCs in peripheral organs display a tissue-specific activation state. For example, the ability of mouse ILC1s to produce tissue type-dependent quantities of IFN-γ, as well as their cytotoxic potential, seems to be regulated by the cytokines present in the tissue (52). In humans, liver and intraepithelial (ie) ILC1s express CD49a, whereas circulating ILC1s are negative for this marker, but express CD127 (52). ILC2s can sense and respond differently according to the cytokines, the neurotransmitters and the lipids present in the microenvironment they are exposed to (52). In the circulation, in the lung and in the tonsils, ILC2s express the IL-1R1 and IL-17RB, the CCR6 in the adenoid and CCR4 in the blood, highlighting the potential of responding differently to the cytokines and chemokines present in the microenvironment and to home differently at distant sites (52). Finally, the abilities of ILC3s to be retained in the tissue, to respond to chemokines and cytokines and to regulate T cell activity, seem to depend not only on the organ they populate, but also on the presence of anatomical substructures (e.g., intestinal lymphoid follicles, harboring LTi cells) (52). For example, circulating ILC3s show a more naïve phenotype, given the expression of CD45RA, whereas skin and lung ILC3s display CD25 expression and a more activated phenotype is observed in almost all other tissue-resident ILC3s; in addition, IL-23R expression seems to be exclusive of tonsillar and intestinal ILC3s, although its expression at mRNA level is also detectable in circulating, splenic and lymph node ILC3s (52). Overall, the high phenotypical diversity of ILCs across tissues supports the different ILC behaviors that are observed.

2.1. Development of non-cytotoxic helper ILCs

Like all lymphocytes, ILCs primarily develop in the BM. They derive from the inhibitor of DNA-binding 2 (Id-2)-dependent $Lin^{-}CD34^{+}CD45RA^{+}CD10^{+}$ common lymphoid progenitor (CLP) (53), that antagonizes the activity of E-proteins, important for B and T cell development (50) and relies on IL-7 for its development (Figure 2) (48). Subsequently, the common innate lymphoid progenitor (CILP) can either maintain the expression of Id2 to become a NKP cell, or downregulate it while increasing GATA3 levels to differentiate into the common innate helper lymphoid progenitor (CHILP) (35). The two distinct precursors give rise to NK cells and ILCs respectively, while the transcription factor promyelocytic leukemia zinc finger (PLZF)

further divides the progeny of the ILC progenitor into the PLZF-dependent ILC1, ILC2 and ILC3 subsets (54). ILCs populate various tissues from mid to late stages of fetal development (55). However, human ILCs are under-characterized if compared to mouse ILCs, where developmental studies are more accessible. It was reported that mouse ILCs might also generate and maturate in extramedullary organs. For example, NK/ILC1-like cells and $CD4^+$ LTi-like cells have been identified in the mouse thymus, although it is not clear whether these cells develop or migrate there from distant sites (56,57). $CD34^+ROR\gamma t^+$ ILC3 progenitors have been found in human tonsils and intestinal lamina propria, but they are absent in the circulation and in the BM, suggesting that a subset of human ILCs might mature at barrier sites (58).

The group of Rudensky elegantly show that ILCs, in both lymphoid and non-lymphoid organs, are locally maintained but expand as tissue-resident cells in response to acute environmental challenges (59). Moreover, they identified donor-derived lymphoid and ILC progenitor cells in parabiotic BM, raising the possibility that ILC progenitors can seed tissues also in adult mice, under physiological conditions. Overall, the tissue-residency feature of ILCs is consistent with their innate role as local keepers of tissue functions.

2.2. Classification of ILCs

Besides LTi and conventional NK cells, ILCs are categorized into three main groups: T-box transcription factor (TBX21, also known as T-bet)-dependent ILC1s, GATA3 and retinoid-related orphan receptor α (RORα)-expressing ILC2s and RORγt-dependent ILC3s (51). The latter includes the LTi-like cells, therefore it constitutes the second most heterogeneous, after ILC1s, among all ILC subsets (45).

ILCs are defined as lineage negative cells that constitutively express CD127 (Table 1). The markers that should be considered to exclude lineage contaminating cells in human ILCs are: CD3, CD4 and CD8 (to exclude T and NKT cells), CD14 (to exclude monocytes), CD15 (to exclude neutrophils), CD16 (to exclude NK cells, myeloid DCs, monocytes and macrophages), CD19 and CD20 (to exclude B cells), CD33 and CD34 (to exclude myeloid cells and hematopoietic stem cells and precursors), CD203c (to exclude basophils and mast cells) and FcεRI (to exclude eosinophils and mast cells) (60,61).

However, different groups have developed their own lineage marker composition. For example, some include CD56 and HLA-DR in the lineage “mix”, but since these markers are expressed on subsets of ILC3s, they should not be included (62). CD1c and CD123 are two additional marker often found in the lineage mix composition, used to detect DCs (63). However, in our hands, we did not detect neither $CD1c^+$ or $CD123^+$ cells in the lineage negative $CD127^+$ total ILC gate, and therefore decided not to include them in the lineage mix (60). Finally, we decided to not include CD161 and CD7 in the ILC inclusion marker list

(together with CD127, tyrosine-protein kinase KIT (c-Kit) and chemoattractant receptor-homologous molecule expressed on T-helper type 2 (CRTH2)), since CD161 is not always expressed by all ILC subsets (in particular, by group 1 ILCs and LTi cells) (60) and CCR10^{+}c-Kit^{+} ILC2s showed reduced levels of both markers in comparison to other ILC2 populations (64).

According to the differential expression of surface markers, the three major ILC subsets can be easily discriminated: ILC1s are defined as c-Kit and CRTH2 double negative cells. ILC2s are CRTH2^{+} cells with different levels of expression of c-Kit. Finally, ILC3s are c-Kit expressing CRTH2^{-} cells (51). The distribution of ILCs in human healthy tissues is very heterogeneous and shows tissue-specific transcriptional gene signatures, suggesting that different sites can directly impact the composition of ILC subsets and, possibly, their effector functions (65,66).

		Group 1 ILCs		Group 2 ILCs	Group 3 ILCs
		NK cells	ILC1s	ILC2s	ILC3s
		Hu	Hu	Hu	Hu
TFs	Eomes	+	–	–	–
	T-bet	+	+	–	–
	GATA3	Low	Low	+	Low
	RORα	–	+	–	–
	RORγt	–	–	–/Low	+
Surface proteins	Lineage	+/– (CD16)	–	–	–
	IL-7Rα (CD127)	–/Low	+	+	+
	IL-2R (γc)	+	+	+	+
	IL-12RB1	+	+	ND	+
	IL-17RB (IL-25R subunit)	–	–	+	–
	IL-23R	–	–	–	+
	CRTH2 (CD294)	–	–	+	–
	c-Kit (CD117)	–	–	+/–	+
	NCAM-1 (CD56)	+	+/–	–	+/–
	NKG2 (CD94)	+	+/–	–	–
	NKp46 (NCR1)	+	–	–	+/-
	NKp44 (NCR2)	+/–	–	–	+/–
	NKp30 (NCR3)	+	–	–	+/–
	KLRB1 (CD161)	Low	+/–	+	+
	CD11c	+/–	–	–	+/–
	MHC class II	–	–	+/-	+

Table 1: Phenotypical markers for circulating human innate lymphoid cells.
Adapted from (49,60,61,64). TFs, transcription factors; +, positive; –, negative; +/–, heterogeneous; ND, not determined.

Although ILCs are considered the phenocopy of $CD4^+$ T helper (Th) subsets (67), our group recently reported that circulating human ILCs and CD4 T cell subsets actually display opposite transcriptomic profiles (68). In particular, we observed significant differences in CCR expression, in activation state and inhibitory functions in ILCs compared to CD4 Th cells, and we reported for the first time the distinct expression of long noncoding RNAs (lncRNAs) suggesting that lncRNA might influence human ILC biology. Similarly, Li *et al.* reported one of the first whole-genome gene expression studies, performed on circulating ILC subsets, although lacking the comparison with $CD4^+$ T cell subsets (69).

Transcriptomic analyses on tonsil- and mucosal-associated lymphoid tissue-derived human ILC1s, ILC3s, Th1 and Th17 subsets were also performed by Koues *and colleagues*. ILCs and Th cells showed to employ a set of overlapping and divergent enhancers to express shared genes and different activating signatures, important for mediating innate and adaptive unique functions (70). In addition, genome-wide chromatin accessibility studies were performed in mouse ILCs and Th cell subsets, isolated from different organs. This study revealed that each ILC subset owns a unique epigenetic landscape, defining them as distinct lineages (71). Despite the fact that ILC regulomes are acquired developmentally and in an Ag-independent manner, whereas the chromatin accessibility in T cells is shaped following activation (72), these regions of open chromatin, in both cell types, deeply overlap. Interestingly, in homeostatic conditions, the chromatin accessibility of distinct human and mouse ILC and T cell subsets seems to diverge (70,71). However, during infection with *Nippostrongylus brasiliensis,* mouse Th2 regulomes were found to converge with those of ILC2s (71). Similarly, after human cytomegalovirus infection, the DNA-methylation profile of NK cells mirrors the one of cytotoxic/effector $CD8^+$ T cells . Besides confirming the close relationship between ILCs and Th cells, these studies also highlight the profound complexity of ILC biology and, at the same time, identify previously unknown gene signatures, which might be translated into novel ILC biomarkers. The increased sensitivity of next generation sequencing (NGS) technologies will enable to continue elucidating the functions of REs that contribute to identity, development and functions of ILCs.

2.2.1. Differentiation and development of group 1 ILCs

Group 1 ILCs are defined by the production of type-1 cytokines, where IFN-γ and TNF constitute the signature cytokines. The prototypical members of this group are NK cells.

Helper-like ILC1s produce IFN-γ and display many overlapping markers with conventional NKs (including NKp46, the natural cytotoxiticy receptor 1 (NCR1) and CD161) (36). In mice, ILC1s are defined as Lin^- $Thy1.1^+$ $NK1.1^+NKp46^+$T-bet-expressing cells and display very different phenotypes and activation states across different tissues (52).

In humans, ILC1s are defined as T-bet-dependent $Lin^{-}CD127^{+}c\text{-}Kit^{-}CRTH2^{-}$ cells that rapidly respond to type 1 cytokines (i.e., IL-12, IL-15 and IL-18) by mainly producing IFN-γ and TNF (48). Despite being described for their non-cytotoxic phenotype, three subsets of cytotoxic non-NK helper-like ILC1s have been recently described. The first consist of ieILC1s, found in the intestine with a similar phenotype as NK cells (73). Here, ieILC1s express the TNF-related apoptosis-inducing ligand (TRAIL) and CD49a (the α1 integrin chain) (35). Moreover, ILC1s are negative for the expression of NKp80, a NK cell marker (74), but can express NKp44 and NKp46, markers shared with NK and tissue-resident ILC3s (35).

Another subset of unconventional cytotoxic $CD127^{+}$ $CD56^{+}$ $CD117^{-}$ ILCs was identified by our group and named $CD56^{+}$ ILC1-like cells, given their expression of cytotoxic molecules, like perforin and granzymes (75). These cells partially express CD94, NKp30, NKp44 and NKp46 and NKp80, the latter being widely described as a marker of human mature NK cells, putting these cells more in relation to the NK cell lineage (74,75). The third and last subset of cytotoxic CD94/NKG2A co-expressing $CD127^{+}$ ILC1s was recently described and identified in the tonsils (76). The transcriptional profile of these cells resembles the one of conventional helper ILCs. However, these cells are not committed towards an NK-like phenotype, since they did not produce IFN-γ, but the ILC3-related cytokine IL-22, in response to IL-15 *in vitro* (76).
There is evidence that IFN-γ-producing ILCs can also develop under the influence of IL-12 from a subset of RORγt-expressing ILCs (which are classified as group 3 ILCs, see below). The development of ILC1s from ILC3s was shown to be accompanied by the downregulation of RORγt expression and a strong increase in T-bet expression, which constitute important regulators of the plasticity of ILC subsets (77).

2.2.2. Differentiation and development of group 2 ILCs

Group 2 ILCs produce type-2 cytokines (including IL-4, IL-5, IL-9 and IL-13) and amphiregulin (AREG) in response to stimulation with IL-25, IL-33, thymic stromal lymphopoietin (TSLP), prostaglandin D2 (PGD2) as well as lipids and neuropeptides (49). Similar to Th2 cells, ILC2s depend on the transcription factors GATA3 but also RORα for their development and function (3). At steady state, mouse and human ILC2s are phenotypically similar and express CD127, suppression of tumorigenicity 2 (ST2, i.e., the IL-33R), the α and γ chains of IL-2 receptors (IL-2Rα and IL-2Rγ, also known as CD25 and CD132, respectively) and IL-17RB (a subunit of the IL-25 receptor) (33). In addition, human ILC2s are also characterized by the expression of CRTH2 (the receptor for PGD2) and CD161 (C-type lectin receptor) (78–80). In humans, besides being present in the circulation, ILC2s are mainly enriched in the skin, the lung and the gut (33). Plasticity events are also reported for ILC2s: they can convert to ILC1s under the influence of IL-1 and IL-12, especially in

the context of inflammatory diseases (81,82). Moreover, it has been recently proposed that circulating human ILC2s consist of two phenotypically yet functionally distinct subpopulations, according to the expression of c-Kit (defined as c-Kit^{lo} and c-Kit^{hi} ILC2s, respectively) and to their cytokine secreting potential upon different types of stimulation *in vitro* (83). In particular, c-Kit^{hi} ILC2s resulted to be more committed towards an ILC3-like phenotype, given their ability to convert into RORγt-expressing IL-17-producing cells in response to the ILC3-activating factors IL-1β and IL-23, but also to transforming growth factor β (TGF-β), *in vitro* (84). Conversely, c-Kit^{lo} ILC2s produced more type 2 cytokines and showed a more mature and ILC2-committed phenotype, if compared to c-Kit^{hi} ILC2s. These findings also correlated with the increased frequencies of IL-17 expressing RORγt^{+} ILC2s in skin lesions of psoriatic patients with the concomitant reduction of ILC2 frequencies, thus supporting the plastic nature of human ILC subsets.

2.2.3. Differentiation and development of group 3 ILCs

Aryl hydrocarbon receptor (AHR) and RORγt-expressing group 3 ILCs are defined by their capacity to produce Th17-related cytokines in response to tissue signals, such as IL-1β and IL-23 (85) and by the expression of c-Kit (also known as CD117) (Figure 2) (49). In tissues, human ILC3s can express NKp44 (i.e., NCR2) and produce lymphotoxin (LT), granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF, IL-17A and IL-22 (85). Besides the expression of NKp44, human ILC3s can express other NCRs, i.e., NKp46 (NCR1) and NKp30 (NCR3). Human ILC3s primarily reside in secondary lymphoid organs, in the small intestine lamina propria (siLP), in intestinal cryptic patches, in lymphoid follicles and in PPs (55). In human tissues, the majority of ILCs is mainly terminally differentiated, while a population of circulating Lin^{-} CD127^{+}CD117^{+}CRTH2^{-}NKp44^{-} ILCs, able to differentiate both *in vitro* and *in vivo* into all mature ILC subsets, has been recently identified and named ILC precursor, or ILCPs (86). Similarly to NK cells (87), ILCPs are characterized by the expression of CD62L, that drives their migration to the lymph nodes (88). Moreover, a recent publication showed that CD117^{+} CRTH2^{-} human ILCPs actually consist of two main subpopulations: NKp46^{+} ILCPs and KLRG1^{+} ILCPs, with an ILC3- and ILC2-committment respectively, emphasizing the multipotent capacity of these cells (89).

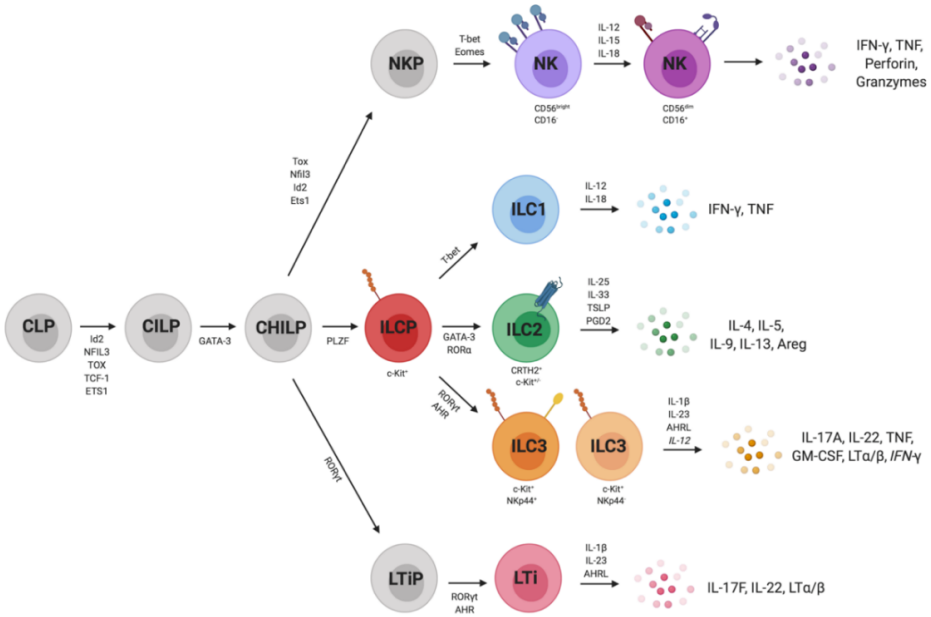

Figure 2: **The development of ILCs.** The development of ILCs from CLCR requires Id2-mediated suppression of alternative lymphoid cell fates that generate B and T cells. Distinct precursors give rise to NK cells, LTi cells and ILCs, where the transcription factor PLZF is crucial for the commitment of the ILC precursor (ILCP) into the differentiation of the three ILC subsets ILC1, ILC2 and ILC3 (Adapted from (85,86,90)).

2.3. Physiological roles of ILCs

The strategic localization of ILCs across tissues allows them to become locally activated and rapidly expand following the encounter with potential pathogens, and to secrete a wide range of soluble mediators to initiate the immune responses (91). In addition, ILCs can mediate wound healing and tissue repair, whereas in other circumstances they can promote inflammation and tumor progression/control (48,92). Recent findings implicated ILCs, and in particular ILC3s, as critical immune cells that orchestrate some of the host-commensal bacteria interactions that can impact immunity, inflammation, and tissue homeostasis in the intestine (93,94). Noteworthy, ILC2s are associated with visceral adipose tissue (VAT) where they produce IL-5 and IL-13, thus leading to the recruitment of eosinophils and the generation of alternatively-activated macrophages (AAMs) that protect the organism from fat-induced ILC3-mediated inflammatory diseases (95,96). It is still unclear how fat tissue regulates the activation of ILCs, however it may involve arachidonic acid metabolites, such as prostaglandins and lipoxins, which constitute respectively activators and inhibitors of ILC2s (97) .

2.3.1. ILC functions during immune responses

Within adult tissues, ILCs constitutively enrich surface barriers which represent common sites of colonization or invasion by pathogens (98). ILCs are recruited to barrier tissues during the embryonic development and further migration of ILCs likely occurs during inflammation, upon stimulation with host-derived cytokines and alarmins during viral, bacterial, fungal and parasitic infections (67). IL-12 and IL-18 activate NK cells and ILC1s, whereas IL-25, IL-33 and TSLP activate ILC2s. By contrast, IL-1β and IL-23 stimulate ILC3 responses (67).

Under physiological conditions, NK cells circulate in a resting state. However, during infections, they become active and migrate to the infected tissue, in a CD62L-dependent manner (99). NK-cell activation is largely regulated by the balance between stimulatory and inhibitory signals sensed via their surface receptors. Once the activation threshold is exceeded, a response is triggered, resulting in the specific lysis of the target cell, often an infected cell, via release of perforin and granzyme, or IFN-γ secretion (98). Perforin is a pore-forming molecule, that causes the rupture of the cell wall of the target cell (100). Granzymes are proteases that induce apoptosis via different mechanisms, including cleavage of caspase 3 (100). NK-derived IFN-γ is important for activating antimicrobial functions in macrophages, increasing Ag presentation and Ig class switching (98).

In the context of immunity to intracellular bacteria and parasites, ILC1s and ILC3s exert important roles in host defense. For example, IFN-γ-producing ILC1s contribute to resistance to *Salmonella enterica* and *Toxoplasma gondii* infection in the intestine (87,101). Moreover, ILC1s protect from *Toxoplasma gondii* infections by recruiting myeloid cells (87) and establish defense mechanism against *Clostridium difficile* infections, since mice lacking ILC1s or IFN-γ are more susceptible to lethal infections with this pathogen (102).

In addition, before the development of adaptive immune responses, innate immunity to the extracellular Gram-negative bacterium *Citrobacter rodentium* is critically dependent on ILC3-derived IL-22 (103,104) which has an important role in stimulating the expression of antimicrobial peptides and the maintenance of intestinal epithelial barrier functions. Moreover, ILC3 expression of $LT\alpha_1\beta_2$ promotes the epithelial-derived CXCL2-dependent recruitment of neutrophils, to support bacterial clearance (105). Finally, ILC3s can produce GM-CSF, that was reported to be key in modulating T regulatory (Treg) cell activity (33,106).

While ILC1s and ILC3s are key players in the innate immunity to viruses, intracellular bacteria and parasites, ILC2s promote type-2 inflammation in the airway mucosa and in the skin during extracellular parasitic

infections (54). The ILC2-driven type-2 inflammatory response is characterized by the production of IL-4, IL-5, IL-9 and IL-13 to regulate the alternative activation of macrophages, granulocyte responses, goblet cell hyperplasia and smooth muscle contractility to promote parasite expulsion, and AREG to promote tissue-repair processes (107). Recent findings show that ILC2s can also interact with the adaptive immune system to promote protective type-2 immune responses: ILC2s express MHC class II molecules and can activate T cells (although to a lower extent if compared to DCs) to induce IL-2 production, which leads to ILC2 proliferation and production of Th2-associated cytokines that in turn favor worm expulsion (108).

ILC functions need to be tightly regulated, as uncontrolled activation and proliferation can contribute to severe inflammation and damage in gut, lung, skin, and liver (51).

2.3.2. ILC interaction with the microbiota

In contrast to their role in supporting antimicrobial responses to pathogens, ILCs also orchestrate the interaction between the host and the array of commensal bacteria that constitute the microbiota, by regulating non-hematopoietic and hematopoietic cell functions, to limit inappropriate immune responses to commensal bacteria (109). Although commensal bacteria are not essential for the ILC development (93), commensal bacteria-derived signals might be crucial for ILC functions (93). In particular, it was shown that ILC3s can produce IL-22 following stimulation via TLR2 (110). ILC3-derived IL-22 acts on IL-22R-expressing gut epithelial cells to promote an immunologically tolerogenic state in the intestine, limiting potentially damaging T-cell responses against commensal bacteria (47).

Commensal bacteria can also regulate ILC functions in an indirect way, i.e., through the modulation of myeloid cell or epithelial cell responses (93). For example, commensal bacteria can regulate mononuclear phagocytes through Myd88, Trif and epigenetic pathways to promote IL-6, IL-12, IL-15, TNF and type 1 IFN production to promote optimal NK cell responses (111). Moreover, commensal bacteria can also influence RORγt$^+$ ILC responses through regulation of IL-1β and IL-23 production by myeloid cells (112). In addition to direct and indirect effects of commensal bacteria on ILC populations, ILCs can reciprocally influence commensal bacterial communities through a number of distinct mechanisms. Cytokines produced by ILCs can dynamically regulate the composition or anatomical location of commensal bacteria. For example, T-bet$^+$ ILCs are critical sources of IFN-γ and TNF, which have been shown to increase the permeability and translocation of commensal bacteria across monolayers of human intestinal epithelial cells (113). Intestinal ILC3s were also shown to be crucial in the prevention of the dissemination of intestinal commensals to

peripheral tissues, since RORγt-deficient mice showed increased titers of intestinal commensal-specific IgG in the serum (114).

While ILC functions are actively influenced by commensal bacteria, ILCs themselves, and in particular ILC3s, can regulate T and B adaptive immune cell responses to help maintaining the host-microbial mutualism intact. The $CCR6^+$ subset of ILC3s present in the colonic lamina propria and mesenteric lymph nodes expresses MHC class II molecules and can act as APCs to negatively select commensal bacteria-specific $CD4^+$ T-cells (115). The elimination of microbiota-specific T-cells was very important to prevent low-grade systemic and spontaneous intestinal inflammation. Finally, ILC3s are also found in the mesenteric lymph nodes (MLN) at the interface between the T- and B-cell zones, where Ag presentation by ILC3s resulted in reduced immunoglobulin A $(IgA)^+$ B-cell responses to commensals, fostering the host-microbiota symbiotic relationship (116).

Likewise, ILC2s can also influence $CD4^+$ T cell functions and, in particular, Th2 cell responses in the gut. Since ILC2s can also present Ags to T-cells via MCH-II molecules, they could also affect the generation of intestinal IgA in a IL-5-dependent manner, as well as the B-cell proliferation via IL-6 production (108,117,118).

2.3.3. ILC function in tissue development and repair

Tissue-resident ILCs also contribute to tissue development, homeostasis and repair (119). In the fetal period, LTi cells play an essential role in the formation of lymph nodes and PPs (120). $CXCR5^+$ LTi cells are recruited and cluster with mesenchymal stromal cells (MSCs) at the site where the lymph node will form (121). Both lymph node and Peyer's patch development rely on the interaction between $LT\alpha_1\beta_2$, expressed by LTi cells, and the LT-β receptor (LTβR), expressed on stromal organizer cells. Signaling through LTβR promotes the production of chemokines (such as CXCL13, CCL19 and CCL21) and adhesion molecules (i.e., vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1)) to attract, retain and support the survival of hematopoietic cells recruited to the developing lymphoid tissue (121).

In mice, similar functions are exerted in the intestine by $CCR6^+$ ILC3s, the adult counterpart of LTi cells (85). ILC3s are attracted to the cryptopatches via the chemokine CCL20, where they interact with myeloid cells via the $LT\alpha_1\beta_2$-LTβR axis to stimulate secretion of IL-23, resulting in increased IL-22 secretion by ILCs during infection with *Citrobacter rodentium*, and enhanced production of antibodies in cryptopatches (122,123).

IL-22 production is also stimulated by glial cell-derived neurotrophic factor (GDNF), produced by glial cells that surround the nerve fibers associated to the cryptopatches. IL-22 was also reported to have a protective role during *Citrobacter rodentium* infection and dextran sulfate sodium- (DSS-) induced colitis (124). Moreover, receptor activator of NF-κB (RANK)-dependent signals from ILC3s support the maturation of autoimmune regulator (AIRE)$^+$ medullary thymic epithelial cells (125).

In addition to their role in fetal lymphoid tissue formation, group 3 ILCs has been implicated in the repair of lymphoid tissues damaged after an injury, for example following acute viral infection. They are also involved in regeneration processes of the inflamed intestine (47). Similarly, LTi cells were shown to promote lymphoid tissue architecture regeneration via the $LT\alpha_1\beta_2$-dependent interaction with stromal cells (126). Moreover, ILC3-derived IL-22 was shown to promote regeneration of thymic epithelial cells following radiation-induced damage (57) and to stimulate epithelial stem cell regeneration to restore mucosal barrier functions and non-lymphoid tissue architectures following ablation therapy in mice (127). IL-22 signaling triggers the STAT3-dependent proliferative response in $Lgr5^+$ stem cells, that promotes tissue repair (128). A similar effect is also observed in human inflammatory bowel disease (IBD), where ILC3-derived IL-22 correlates with increased mucosal healing (129).

Type 2 cytokines can also promote tissue repair, e.g., by acting directly on non-myeloid parenchymal cells to facilitate muscle regeneration (130). Moreover, ILC2-derived IL-13 was shown to promote catenin pathway–dependent renewal in intestinal stem cells (131). In addition to classical type 2 cytokine production, ILC2s produce AREG in response to IL-33, that was shown to support bronchial epithelium regeneration that has been damaged following infection with influenza virus (132). Following helminth infection in the lung, autocrine production of IL-9 amplifies IL-5, IL-13, and AREG production, promoting tissue repair mechanism in the recovery phase of the infection (133).

2.3.4. ILC plasticity

Similarly to Th cells, ILCs are highly plastic cells, i.e., they have the capacity of changing their commitment during development but also to adapt their phenotype, according to the alterations in their microenvironment (Figure 3) (77). For example, studies on chromatin accessibility revealed that the majority of circulating human $CD127^+$c-Kit^+ are biased towards a specific ILC subset (in particular, either ILC2s or ILC3s), but their pre-established destiny can change (89). *In vitro*, ILC2s and ILC3s can convert into ILC1s in response to IL-1β and IL-12, given the upregulation of the ILC1 master regulator T-bet (77). *In vivo*

conversion of ILC2s and ILC3s into IFN-γ-producing ILC1s was observed in the lungs of mice during influenza virus infection and in the mouse intestine during infection with *Salmonella enterica* (82,134). ILC2-to-ILC1 conversion was also observed in patients with chronic obstructive pulmonary disease (COPD) (82). Moreover, NK-to-ILC1-like cell plasticity events were also described in tumor-bearing mice, under the influence of TGF-β present in the tumor microenvironment (TME) (135). Conversely, an *in vitro* ILC-to-NK plastic event was described by Caligiuri *and colleagues*: by antagonizing AHR pathway in tonsillar IL-1R1^{hi} human ILC3s, transdifferentiation to CD56^{bright}CD94^{+} cytolytic NK cells can occur (136). Similarly, ILC1s and ILC2s can differentiate into ILC3s under the influence of IL-1β and IL-23 both in *in vitro* and *in vivo* settings (84,137,138). Interestingly, the ILC2-to-ILC1 and the ILC2-to-ILC3 conversion can be reversed by IL-4, resulting in GATA3 induction and CRTH2 expression by ILC2s (139).

Until recently, the innate counterpart of Treg cells has never been described, but different groups identified IL-10^{+} ILCs both in humans and mice, arguing for the existence of a regulatory ILC population. *In vitro*, IL-10^{+} human ILCs were shown to derive from retinoic acid-stimulated ILC2s, which displayed CD25 expression together with downregulation of CRTH2 and type 2 cytokine production (140), whereas in mice IL-10-producing ILC2s that lack GATA3 were generated following stimulation with IL-33 (141). However, these cells were barely detectable in healthy humans and mice, but they increased in patients with chronic rhinosinusitis and in the lungs of mice challenged with house dust-mite (HDM) and papain (140,141). Moreover, IL-10-secreting ILCs that suppress ILC1s and ILC3s have been recently identified in the gut of mice with intestinal inflammation and named ILCreg (142). However, these findings were not reproduced by the group of Colonna, despite the individuation of IL-10-producing cells, i.e., activated ILC2s, in response to IL-2, IL-4, IL-10, IL-27 and neuromedin U (NMU)(143). Overall, the lack of the expression of the key Treg transcription factor Foxp3 and the fact that these cells only appear upon “alternative” activation suggest that ILCs, and in particular ILC2s, can be primed to transiently produce IL-10, rather that defining a separate innate counterpart of Treg cells. Unravelling the cellular programs and signaling cascades that regulate ILC plasticity will be key to better understand the contribution of ILCs in different immunological disorders.

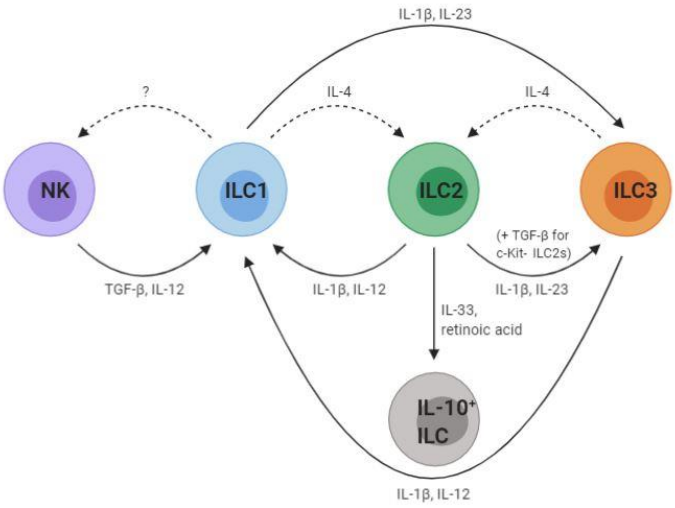

Figure 3: Plasticity of human ILCs. ILC2s and ILC3s can transdifferentiate into ILC1s in response to IL-1β and IL-12, whereas IL-1β and IL-23 can drive the plasticity of ILC1s and ILC2s towards ILC3s. The transdifferentiation of ILC2s into ILC1s or ILC3s can be reversed by IL-4. ILC2s can also produce IL-10 in response to IL-33 and retinoic acid. TGF-β and IL-12 can induce NK cells to acquire an ILC1-like phenotype. The c-Kit$^-$ ILC2s requires TGF-β, in addition to IL-1β and IL-23, to differentiate into ILC3s, compared to c-Kit$^+$ ILC2s. The solid lines indicate a proven plastic event, whereas the dashed lines indicate less well defined plasticity events. Adapted from (77).

2.3.5. Neuro-ILC immune crosstalk

It is very well known that the peripheral nervous system, via neurotransmitters and neuropeptides, and the hypothalamic-pituitary-adrenal and gonadal axis are actively involved in the maintenance of homeostasis and in the regulation of inflammatory processes (144). Increasing evidences also support the idea that the nervous system can actively regulate the immune responses (144). Interestingly, recent studies showed that ILCs anatomically co-localize with nerve terminals in certain tissues, suggesting that neuro-ILC interactions might occur (144).

One of the clearest examples of nervous cells and ILCs crosstalk is the rearranged during transfection (RET) tyrosine kinase receptor that is activated by GDNF and other ligands of the GDNF family (GFLs) (144). In mice, enteric ILC3s express high levels of the rearranged during transfection tyrosine kinase receptor (RET) and are found in close proximity to neurotrophic factor-expressing glial cell projections in cryptopatches that, during infection, control IL-22 production in ILC3s (124). This glial cell-ILC3 pathway defines an important way to regulate intestinal defense, since ILC3-specific *Ret* ablation led to decreased IL-22 production and to increased susceptibility to infection with *Citrobacter rodentium*. In the mouse respiratory tract, a portion of ST2$^+$ ILC2s was found to exclusively express the neuropeptide receptor NMUR1, both at steady state and after induction of airway inflammation (145). NMUR1 resulted to bind

to NMU produced in the lungs by cholinergic neurons, and NMUR1-NMU signaling in ILC2s strongly amplified allergic inflammation.

In addition to NMUR1, recent studies have also showed that ILC2s express receptors for the vasoactive intestinal polypeptide (VIP) and for the calcitonin gene-related peptide (CGRP) (144). In particular, VIP production by Nav1.8^{+} nociceptors was shown to be crucial in generating and maintaining OVA-induced type 2 inflammation in the respiratory tract (146). Interestingly, VIP acted on both ILC2s and CD4^{+} T cells to induce type 2 cytokine production while suppressing type 1 responses, creating a positive feedback loop to amplify the immune response. *In vivo*, VIP stimulates ILC2s to produce IL-5 in the peripheral tissues during homeostasis, e.g., in response to circadian and food intake. Therefore, VIP is an essential regulator of ILC2-driven eosinophil accumulation in peripheral tissues (96).

Besides regulating ILC2 function, VIP was found to control the production of IL-22 by intestinal ILC3s to maintain tissue homeostasis (147). In the gut, ILC3s express high levels of the VIP receptor 2 (VIPR2), and their activation relied on the food-induced expression of VIP by enteric neurons, thus defining a temporal regulation mechanism of ILC3 function to maintain physiological protection in the gut, which is coordinated with food intake.

CGRP is also a regulator of ILC2 cytokine production and function (144). In combination with IL-7, IL-25 and IL-33, CGRP induced the production of IL-5 by mouse lung ILC2s *in vitro*, but genetic deletion of CGRP receptor in ILC2s resulted in reduced immune cell infiltration in the lungs in HDM-induced airway inflammation (148). A negative regulatory function of CGRP in controlling ILC2 activity was also described in the intestine, where CGRP was found to limit the magnitude of type 2 innate immune responses following infection with *Nippostrongylus brasiliensis* (149).

Finally, another mechanism of neuronal-dependent regulation of ILC function might rely of the activity of adrenergic nerves, that directly innervate lymphoid organs (150). It is known that lymphocytes primarily express the β2-adrenergic receptors (β2-ARs): for example, circulating human CD56^{dim} NK show higher expression of the β2-AR if compared to CD56^{bright}, suggesting that CD56^{dim} NK cells could respond more rapidly and vigorously to adrenergic signaling (151).

β2-AR signaling is involved in retention of adaptive lymphocytes in the lymph nodes during inflammatory conditions (152), whereas it was shown to favor the increase of circulating NK cells in a model of acute stress (153).

As for ILCs, it was shown in mice that ILC2s express higher levels of the β2-AR gene (*Adrb2*) in the small intestine and in the lungs, but lower levels in the mesenteric adipose tissue, if compared to other ILC subsets. *Adrb2* expression in human ILCs was also detected in ILC2s isolated from the blood and the lungs (154). However, in contrast with what was shown for NK cells, β2-AR signaling impaired ILC2 responses during lung and intestine inflammation, thus defining a cell-intrinsic negative regulator of ILC2 activity, by limiting their proliferation and effector functions (154).

Despite the majority of evidences for the interplay between ILCs and the neuroendocrine system have been collected with the use of mouse models, all these findings suggest that the immune and the nervous systems are complementary and evolved together to cooperate in responding to environmental changes, to restore and efficiently maintain the homeostatic balance.

2.4. Pathological roles of ILCs

Despite their protective involvement during immune responses, chronically-activated ILCs contribute to pathology onset in a wide variety of inflammatory disorders (49).

2.4.1. ILCs and inflammatory diseases

In humans, IFN-γ producing ILC1s are involved in pathological inflammatory processes. In this regard, the number of ILC1s and their IFN-γ production increases during hepatitis B virus infection, and this upregulation is significantly associated with liver damage in patients with chronic hepatitis B (155). ILC1s are also expanded in the inflamed intestine of patients with Crohn's disease and in the bronchi of patients with COPD (156,157). Similarly, increased ILC1 numbers, at the expenses of ILC2s and ILC3s, are observed in acute-phase anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (158). Higher ILC1 frequencies have also been described in the joints of spondyloarthritis patients and in the peripheral blood of patients with systemic sclerosis (SSc), together with ILC3s (159,160).

IL-17- and IL-22-producing ILC3s have been also associated with the inflammatory skin disease psoriasis vulgaris (31), since their number was found to be increased both in lesional and non-lesional skin and blood of psoriatic patients (161–163).

Similarly, during chronic intestinal infections, resident mononuclear phagocytes (MNP) produce IL-23 and favor the accumulation of pro-inflammatory IL-17A and IFN-γ-producing ILCs, that also express RORγt (164). It is known that T-bet expression in ILC3s favors their conversion into ex-ILC3s, i.e., ILC1-like cells

able to produce IFN-γ (101). In human IBD, while an initial increase in ILC3 production of IL-22 correlates with mucosal healing (129), chronic colitis may reflect a transition from tissue-repairing ILC3s to inflammatory ex-RORγt^{+} IFN-γ-producing ILC1s (156,165). Moreover, higher numbers of RORγt^{-}T-bet^{+}NKp44^{+} ILC3s that produced IL-17 and IL-22 were found in the gut, in the synovial fluid and in the BM of ankylosing spondylitis (AS) patients, an autoimmune disease that causes chronic inflammation in the joints of the spine (166). Of note, a significant reduction in intestinal and circulating ILC3 numbers was observed in AS patients after anti-TNF treatment, suggesting that intestinal ILC3s potentially contribute to AS development and induction of inflammation in the joints (166).

A similar dual role can be observed in ILC2s, the primary producers of type 2 effector cytokines in asthma and pulmonary fibrosis (167). Through the production of IL-5 and IL-13, ILC2s play a central role in recruiting eosinophils to the lung during allergic inflammatory responses and in promoting mucus production and smooth muscle contraction. In addition, an increase in the number of peripheral-blood ILC2s is observed in patients with allergic rhinitis (AR) during pollen season, indicating the contribution of ILC2s to inflammatory responses in AR (168).

However, sustained production of IL-13 can also lead to increased collagen accumulation, resulting in chronic tissue damage (169,170). Profibrotic functions of ILC2s are also described in the skin (171) and in the kidney (172), and increased circulating ILC2s have been reported in patients with rheumatoid arthritis and in asthmatic individuals (173,174). ILC2s are enriched in the skin of atopic dermatitis (AD) patients (175) and in nasal polyps of patients with chronic rhinosinusitis (CRS), where they secrete significant amounts of type 2 cytokines in response to TSLP, IL-33, and cysteinyl leukotrienes (CysLT), also found to be upregulated in patients with CRS (139,176).

Therefore, the roles of ILCs in various inflammatory and autoimmune disorders, as already discussed, has raised considerable interest in developing strategies to target and modify ILC functions/polarities to treat these diseases.

2.4.2. ILCs and cancer

It is well established that the immune system is involved in the suppression of cancer initiation and development (177). In particular, the innate immune system is essential for establishing and maintaining adaptive immune responses, and fully integrates the cancer–immunity cycle (178) . The roles of ILCs in cancer are going to be discussed in detail below and are summarized in Figure 4 towards the end of this section.

2.4.2.1. The role of group 1 ILCs in cancer

The role of NK cells in tumors is the most well characterized. NK cells are equipped with a panel of activating receptors, such as NKG2D and the NCRs NKp30, NKp44 and NKp46 that can detect cell-surface and soluble markers of stressed cells, such as tumor cells (178). For example, following DNA damage, dying tumor cells can generate NK ligands on their cell surface (179). However, NK cell activity can be obstructed by the engagement of inhibitory cell surface receptors, e.g., killer cell Ig-like receptors (KIRs) or NKG2A, which contain intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) and recognize MHC-I molecules expressed on the tumor cell surface (180). Through a combination of direct cytotoxic activities and IFN-γ secretion, NK cells contribute to anti-tumor immunity and promote the establishment of downstream adaptive responses (180).

As mentioned above, NK cells rely on the balance between activating and inhibitory receptors to exert their killing effects, and therefore NK cells can also show pro-tumor activities. The liver is the organ with the highest content of NK cells (36), whose numbers and anti-tumor functions were found to be reduced in patients with hepatocellular carcinoma (HCC), thus favoring tumor progression (181). Similarly, ILC1s favored the establishment of a detrimental IFN-γ/p-STAT1 axis, which supported tumorigenesis of hepatitis B-infected mice in hepatitis B-virus infected hepatocellular carcinoma tumor-bearing mice (182). Recently, the group of Ohashi described a $CD56^{+}CD3^{-}$ population of cells able to inhibit tumor-infiltrating lymphocyte (TIL) expansion in high-grade serous ovarian cancer via surface NKp46 (183). Despite being defined as ILC3-like cells, the expression of CD56, NKG2D, NKp30 and NKp46 but the lack of CD16 and RORγt suggest that these cells are probably more committed towards an NK-like phenotype. Moreover, tumor-associated NK (TANK) and tumor-infiltrating NK (TINK) cells showed decreased expression of the activating marker NKG2D, impaired degranulation activity but produced angiogenin and invasion-associated enzymes MPP2/9 and TIMP in colorectal cancer (CRC) patients (184). Proangiogenic functions

in NK cells were also observed in non-small cell lung cancer patients (185). Whereas the proangiogenic functions of NK cell support tumor progression, remains to be determined.

Apart from NK cells, the involvement of other ILC subsets in cancer progression or resistance is still unclear and contentious, since current studies indicate that the roles of ILCs in cancer depend on their phenotype, the type of tumor and also the TME (177).
For example, ILCs were shown to be significantly dysregulated in terms of frequency, subtype composition and function in CRC patients and chronic lymphocytic leukemia (CLL) patients (186,187). Moreover the ILC compartment was also shown to be impaired in treatment naïve acute myeloid leukemia (AML) patients at diagnosis, but partially restored in patients responsive to therapy, suggesting that ILC dysregulation is associated with the disease itself and is not just a consequence of the chemotherapy (188).

Given their ability to produce high quantities of IFN-γ, ILC1s might represent the ILC subset more prone to exert a protective role against tumors. Indeed, IFN-γ is known to play anti-proliferative, anti-angiogenic and pro-apoptotic effects against cancer cells and also to increase immunogenicity of tumor cells for a better recognition and elimination by cytotoxic T lymphocytes (CTLs) (189). Moreover, IFN-γ promotes the polarization of Th1 cells, inhibiting Th2 cell development and it can also activate macrophages and induce production of chemokines, which can recruit specific effector cells to the cancer region (189). However, although IFN-γ has long been associated with anti-tumor functions, it can induce the upregulation of the inhibitory enzyme indoleamine 2,3-dioxygenase (IDO) in both tumor cells and DCs. IDO upregulation may interfere with T cell proliferation favoring the development of Tregs, causing suppression of T cell responses (189). In addition, the non-classical MHC class I molecules (e.g., HLA-G and HLA-E) and PD-1 ligand (PD-L1) are regulated by IFN-γ and are implicated in immune escape in a variety of cancers by mediating resistance to CTL- and NK cell-mediated responses (189,190).

ILC1-like cells were found to be expanded in breast and prostate tumor-bearing mice, and exhibited high cytotoxic potential in response to IL-15, that could limit tumor growth (191). Another potential anti-tumor role of ILC1s was described by our group: melanoma patients have higher frequencies of functionally impaired ILC1s in the peripheral blood and in tumor-infiltrated lymph nodes (TILNs) (Ercolano *et al.*, Annex III) (192). In that work, the impairment of ILC1 function was associated with immunosuppressive mediators that are present in the TME. These findings were recapitulated with the use of an *in vivo* melanoma model. In gastric cancer, a predominant group 1 ILC phenotype was also detected in the malignant tissues compared with healthy adjacent tissues (193). Whether ILC1s can actively contribute to gastric cancer

growth and/or progression remains to be fully elucidated. Our group also identified a $CD56^+$ ILC population with NK cell properties that is impaired in its cytotoxic capacity in AML patients, suggesting that these cells are actively involved in tumor immunosurvelliance (75).

2.4.2.2. The role of group 2 ILCs in cancer

Group 2 ILCs can be considered the ILC subset with more pro-tumorigenic activity (revised in Ercolano *et al.*, Annex I); the production of type-2 cytokines, mainly IL-5 and IL-13, promotes tumor formation, progression and metastasis since they can activate growth and angiogenic factors-producing M2 macrophages. IL-13 is critical for the recruitment and activation of myeloid-derived suppressor cells (MDSCs) that are considered potent inhibitors of anti-cancer immune responses (194). In addition to type-2 cytokines, ILC2s produce AREG, which can further inhibit anti-tumor immune responses by improving the activities of Treg cells (195,196).
In addition, IL-13-producing ILC2s also show pro-tumor immunity and are associated with a negative outcome in cancer (197).

Cell stress or damage causes the release of the alarmin IL-33, a potent ILC2 activator that showed ambiguous effects of this subset during immune responses in the cancer setting (198). It was recently published that IL- 33-dependent ILC2 activation in the lung promoted metastasis formation through the inhibition of NK cell activation and anti-metastatic functions in a melanoma mouse model (199). Tumor-derived IL-33 also promoted the ILC2 secretion of CXCR2 ligands (i.e., CXCL1 and CXCL2) that bind to CXCR2-expressing lymphoma cells, reinforcing the tumor cell-specific apoptosis, that was independent of adaptive immunity (200). Of note, the IL-33/ILC2/IL-13 axis promoted hepatic fibrosis and cholangiocyte hyperplasia in a murine biliary injury model, which induced cholangiocarcinoma with liver metastases (201,202).

A study by Jovanovic *et al.* indicated that IL-33 accelerated cancer progression and lung metastases formation in a 4T1 mouse breast cancer model, by facilitating the intratumoral accumulation and expansion of immunosuppressive IL-13-producing ILC2s (203). Similarly, the Halim group showed that IL-33-activated ILC2s indirectly suppressed the anti-tumor and cytotoxic functions of NK cells in a lung metastasis model (199). By producing IL-5, ILC2s induced lung eosinophilia, which ultimately led to impairment of the metabolic fitness of NK cells.

Studies on ILCs in patients with gastric cancer are rare. However, it is known that the accumulation of Th2 cell phenotypes can be associated with gastric cancerogenesis and poor prognosis of affected patients

(204). Bie *et al.* reported that the expression levels of ILC2-associated genes (RORα, GATA3 and PGD2R) and molecules (IL-4, IL-5 and IL-13) were higher in the peripheral blood mononuclear cells (PBMCs) of gastric cancer patients (205). The increase of ILC2 frequencies correlated with an enrichment of circulating monocytic MDSCs (M-MDSCs) and M2 macrophages, suggesting a role for ILC2s in favoring an immunosuppressive microenvironment (205).

Similarly, starting from the observation that ILC2s were also expanded in the peripheral blood of acute promyelocytic leukemia (APL) patients, Trabanelli *et al.* described a pro-tumor function of ILC2s that establish an immunosuppressive axis via the engagement of CRTH2 and NKp30 on their cell surface with tumor-derived PGD2 and B7H6, expressed on APL blasts. (206). This axis activated ILC2s to produce IL-13 and to recruit M-MDSCs, with important roles in cancer immunosuppression. Similar observations were done in prostate cancer (206) and in patients with non-muscle-invasive bladder cancer (NMIBC), which showed lower recurrence-free survival when the local T cell/MDSC ratio, which is modulated by ILC2s, was low (207).

Wang *and colleagues* profiled tumor-infiltrating ILCs in a azoxymethane/dextran sodium sulfate (AOM/DSS)-induced colitis-associated CRC model and identified 3 clusters of ILC2s (named ILC2-A, -B and -C) (208). Interestingly, ILC2-C but not ILC2-A nor ILC2-B showed higher expression of programmed cell death 1 (PD-1), and when engrafted in a tumor-bearing recipient mouse, favored tumor progression. Conversion of tumor-infiltrating ILC2-C into an anti-tumor ILC subset might represent a novel strategy to treat CRC patients.

Despite the IL-33-driven pro-tumor effect described so far, Moral *et al.* recently reported that tumor-infiltrating ILC2s express high levels of PD-1; following activation with IL-33, they showed that ILC2s amplify the response to programmed cell death protein (PD)-1 checkpoint blockade in pancreatic ductal adenocarcinoma model (209). Given the shared expression of immune modulatory molecules between T cells and activated ILC2s, the authors suggest that ILCs might constitute a co-target during immune checkpoint (IC)-based therapies, to amplify their beneficial effects. Antibody-mediated PD-1 blockade relieves ILC2 cell-intrinsic PD-1 inhibition to expand tumor ILC2s (TILC2s), augment anti-tumor immunity, and enhance tumor control, identifying activated TILC2s as targets of anti-PD-1 immunotherapy.

Moreover, IL-33 is also able to stimulate ILC2s to produce high amounts of IL-5, a potent eosinophil chemoattractant which was reported to drive the selective expansion of eosinophils, eliciting both blood

and tissue eosinophilia (210). In humans, eosinophilia is frequently observed following immunotherapy with IL-2 and upon tumor vaccination (211).

In a murine model with metastatic melanoma, a population of $CD3^{neg}CD90^{+}CD127^{+}CD25^{+}$ and $ST2^{+}$ (a marker for bona fide murine ILC2s) was shown to produce IL-5 and to recruit eosinophils to the tumor site, favoring tumor regression and reducing lung metastases formation (212). Similarly, Saranchova *and colleagues* reported anti-tumor function of ILC2-derived IL-5 and IL-13 in shaping effector functions of CTLs in a metastatic lung cancer model, by directly acting on DCs (213).

2.4.2.3. The role of group 3 ILCs in cancer

Group 3 ILCs have been well characterized for their involvement in chronic inflammation induced by IL-23 (214). Although IL-23 has always been studied for its role in host defense, autoimmunity and chronic inflammatory diseases, high levels of this cytokine and its receptor have also been identified in different human cancers, including those of the colon, ovaries, lung, breast, stomach, skin, liver, and head and neck (215–217).

IL-23 receptor signaling is associated with the pathogenesis of IBD, which can consequently promote tumor growth in the gut (197). In this context, one study showed that ILC3s accumulated in the inflamed intestine of patients with Chron's disease in response to IL-23, where they contributed to intestinal inflammation through the secretion of IL-17, IL-22 and IFN-γ and the recruitment of other immune cells (165). Other studies have reported that the IL-23/IL-23 receptor (IL-23R) expressing ILC3s/IL-17 axis may favor the establishment of a long-term inflammatory and pro-tumorigenic state in the gut (218–220). Similarly, Chan *et al.* suggested that IL-23 may be sufficient to induce intestinal adenomas formation in mice via IL-17-producing $Thy1^{+}IL\text{-}23R^{+}$ ILC3s, which occurred prior to inflammatory cell infiltration and independently of pre-existing carcinogens (221) . However, the mechanisms that link gut inflammation with ILC3-dependent cancer promotion need further investigations.

Carrega *et al.* reported that the frequency of infiltrating total ILCs is dramatically reduced in specimens from colorectal cancer patients, if compared to normal adjacent mucosa. In particular, the ILC1/ILC3 proportions were altered, with increased numbers of ILC1s at the expenses of ILC3s, which represent the most abundant ILC subset in the normal colon. This could be partially dependent on ILC3 plasticity, that

might convert into IFN-γ-producing ILC1s under the influence of ILC1-converting cytokines, such as IL-1β and IL-12, that might be present in the CRC microenvironment (222).

ILC3s can produce large amounts of IL-22, that despite the role in epithelial tissue repairing processes, appears to be closely related to tumorigenesis (223). In particular, a negative effect in a mouse model of colorectal cancer has recently been proposed for ILC3-derived IL-22, which would act on epithelial cells and induce Stat3 phosphorylation and cell proliferation (224). The study also showed that in human CRC, tumor tissue expressed more IL-22 than normal colon tissues, supporting the idea that ILC3s have a pro-carcinogenic role in the human intestinal tract (224).

Inversely, ILC3s NCR^+ have been recently shown to be present in pleural effusions of primary and metastatic tumors (225) and in non-small-cell lung cancer (NSCLC) patients, where they mainly localize at the edge of tumor-associated tertiary lymphoid structures (TLS), and their presence correlates with a more favorable clinical outcome (226). A protective role of group 3 ILCs in tumors is further supported in a melanoma mouse model, where tumor rejection is promoted by NKp46-expressing and RORγt-depending cells, that were shown to be able to infiltrate into the tumor site, where they induce the expression of ICAM-1 and VCAM-1 on tumor vascular endothelial cell (EC) surface, favoring the infiltration of tumor-specific T cells and, ultimately, tumor growth control (227).

An interesting publication suggested that anaplastic large cell lymphoma (ALCL), a rare type of non-Hodgkin's lymphoma, may actually originate from ILC3s since ALCL cell lines consistently expressed ILC3-associated genes (i.e., RORγt, AHR, IL-22, IL-26 and IL-23R) while lacking B- or T-cell receptor gene expression (228). This study suggests for the first time that ILCs can themselves give rise to blood malignancies.

Furthermore, in patients with breast tumors, Irshad *et al.* reported an absolute increase in ILC3s in tumor tissue, which was associated with an increased likelihood of lymph node metastasis (229). These findings were evaluated with the use of a breast cancer model and it resulted that CCL21-recruited ILC3s induced the production of CXCL13 in stromal cells present in the TME, which resulted in ILC3–stromal interactions and in the production of RANK ligand (RANKL) that ultimately favored the formation of metastasis in the lymph nodes.

Overall, these data show that the role of ILCs in cancer really depends not only on the tumor type, the ILC subset involved but also on the microenvironment.

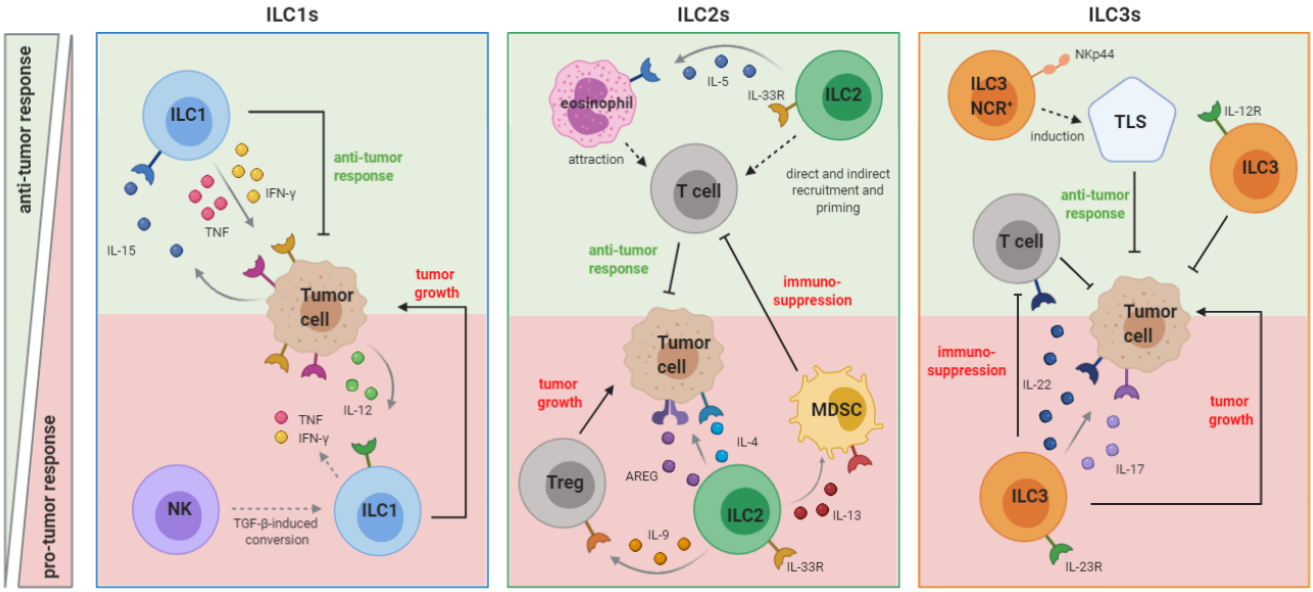

Figure 4: Anti- and pro- tumor roles of human ILCs. When activated by tumor-derived IL-15, ILC1s produce IFN-γ and TNF that can promote the apoptosis of tumor cells. In the presence of IL-12, ILC1 functions are impaired and facilitate tumor growth. TGF-β-induced NK-to-ILC1 plasticity may also promote tumor growth. IL-33-activated ILC2s can produce IL-5, leading to eosinophilia and T cell recruitment, to generate and adaptive anti-tumor response. However, ILC2-derived IL-13 recruits MDSCs, thus having an immunosuppressive effect on T cells. ILC2s can also produce IL-9, which activates Treg cells, AREG and IL-4, which promote tumor growth and tumor cell migration. In the presence of IL-12, ILC3s can promote an anti-tumor response. NCR^+ ILC3s might favor the formation and/or maintenance of TLS at the tumor sites, and these structures are often associated with a better prognosis. Still, IL-23-activated ILC3s that produce IL17 and IL-22 can also have pro-tumorigenic effects, in particular by limiting T cell activity. In the figure, only the cytokines produced by ILCs are shown. Adapted from (230).

3. The vascular endothelium

3.1. The vascular endothelium in homeostasis and physiological angiogenesis

The vascular endothelium is a thin layer of mesodermic origin made of squamous cells called *vascular ECs*, which form the lining of the blood vessels (231). Besides the endothelium, blood vessels are composed of connective tissue and smooth muscle, in varying amounts according to the vessel's diameter and function, which are separated from the endothelium by a basal lamina (232). Smaller blood vessels (i.e., the capillaries) are also surrounded by a few scattered perycytes, that are cells of the connective-tissue family related to vascular smooth muscle cells (232).

Originally considered as a passive interface between the blood stream and the vessel wall, the healthy endothelium is actually involved in many aspects of vascular health and biology (231), including:

- barrier function: the endothelium acts as a semi-selective barrier between the vessel lumen and the surrounding tissues, to regulate the passage of macromolecules between the vascular lumen and the vascular smooth muscle (e.g., the hematoencephalic barrier, that separates the circulating blood from the brain extracellular fluid in the central nervous system) (233);
- prevention of platelets' aggregation and thus prevention of blood clotting (234);
- control of the blood pressure by secreting vasoactive factors that control vasoconstriction and vasodilation (233);
- angiogenesis: the process of formation of new vessel structures from pre-existing ones (235);
- leukocyte transendothelial migration: movement of leukocytes out of the blood stream and towards the site of tissue damage and/or infection (236);

Blood vessels are dynamic structures: new vessels are formed when needed (e.g., during wound healing and every month in the female reproductive tract – in the ovaries and in the uterus) through a process that is called *angiogenesis* (235). Physiological angiogenesis is a tightly controlled, self-regulating and reversible process that depends on angiogenic factors (including vascular endothelial growth factors (VEGFs), extra-cellular matrix proteins, adhesion receptors and proteolytic enzymes), which stimulate ECs to migrate, proliferate and differentiate to form new vessel lumens (235). The VEGF family includes VEGF-A (important for the different steps of angiogenesis), VEGF-B (important for embryonic angiogenesis), PlGF (placental growth factor, important for vasculogenesis but also angiogenesis during embrionyc development), VEGF-C and VEGF-D (mainly involved in lymphangionenic processes) (237).

Angiogenesis is characterized by the dissolution of the extracellular matrix (ECM) and the proliferation and sprouting of ECs. During ECM remodeling, the products that are generated can inhibit EC proliferation and migration, thus fine-tuning the formation of new vascular structures.

3.2. Leukocyte transendothelial migration

Leukocyte transendothelial migration (TEM), or diapedesis, is the outward passage of leukocytes through intact vessel walls into tissues where a damage or an infection occurred (238); it is a vital physiological process that occurs during both the adaptive and innate immune responses and during routine immune surveillance and homing (236). As the primary physical barrier between blood and tissue compartments within the body, blood vessel ECs and integrity of the connecting-cell junctions must be carefully regulated to support leukocyte transendothelial migration only when necessary (231). Inflammation triggers the upregulation of adhesion molecule expression in ECs, promoting the accumulation of leukocytes and their adhesion to blood vessel walls. This phenomenon is mediated by inflammatory cytokines, such as TNF and IL-1β, through the activation of NF-kB signaling pathways.

Leukocyte TEM has been described as a sequential set of events, each of which facilitates progression to the next stage. The original leukocyte adhesion multistep model includes five steps (236) (Figure 5):

1. *chemoattraction*: upon recognition of and activation by pathogens, injured/infected tissue-resident macrophages release pro-inflammatory cytokines, such as TNF and IL-1β, that stimulate the production of chemokines by ECs to orchestrate the circulating leukocyte attraction towards the site of injury and/or infection. In parallel, ECs start to upregulate adhesion molecules (selectins) to initiate the second step of leukocyte TEM;
2. *rolling adhesion*: during the initial rolling adhesion step, leukocytes become loosely tethered to the blood vessel wall due to transient and weak interactions between E-selectin on activated ECs and leukocyte carbohydrate ligands (e.g., sialyl Lewis glycoproteins). This step slows down leukocyte passage through the blood vessel, allowing the binding to ECs and the exposure to a local environment that facilitates the progression to the third step, the activation;
3. *activation*: the local production of cytokines, such as TNF by leukocytes, initiates a positive feedback loop, resulting in further production of more cytokines, causing the upregulated expression of EC-leukocyte adhesion molecules, such as ICAM-1 and VCAM-1 on the EC surface (i.e., endothelial activation). Furthermore, production of specific chemokines like IL-8, monocyte chemotattractant protein 1 (MCP-1) and stroma cell-derived factor 1 (SDF-1) by ECs attracts more

leukocytes and causes their migration towards the site of infection;

4. *firm adhesion/arrest*: this step is promoted by the increased affinity of leukocyte integrins, such as α4β1 (VLA-4, very late Ag-4) and αLβ2 (LFA-1, lymphocyte function-associated Ag-1), for EC Ig-family adhesion molecules such as the previously mentioned VCAM-1 and ICAM-1. EC adhesion molecules also associate with actin-binding adaptor proteins such as cortactin, α-actinin and filamin; this secures anchorage to the EC cortical cytoskeleton, resulting in the formation of a force-transduction platform (also known as a diapedesis synapse), which promotes strong adhesive interactions with leukocytes and their immobilization, despite the shear forces of the blood flow;
5. *transendothelial migration* is the fifth and last step, concluding a complete inflammation cascade. The cytoskeleton of the leukocytes is re-organised in such a way that the leukocytes are spread out over the ECs. Leukocytes can use two independent routes to cross the endothelium: the paracellular route, i.e., the migration in between adjacent endothelial cells that requires transient junctional disruption. Transmigration occurs as platelet EC adhesion molecule (PECAM-1, or CD31) proteins, constitutively expressed on the endothelial cell surface, interact and pull the cells through the endothelium (Figure 5). The transcellular route, instead, occurs directly through an individual EC body, likely requiring the formation of a channel or a pore. Formally, this process could be driven by the leukocyte, by the endothelium, or by contributions from both.

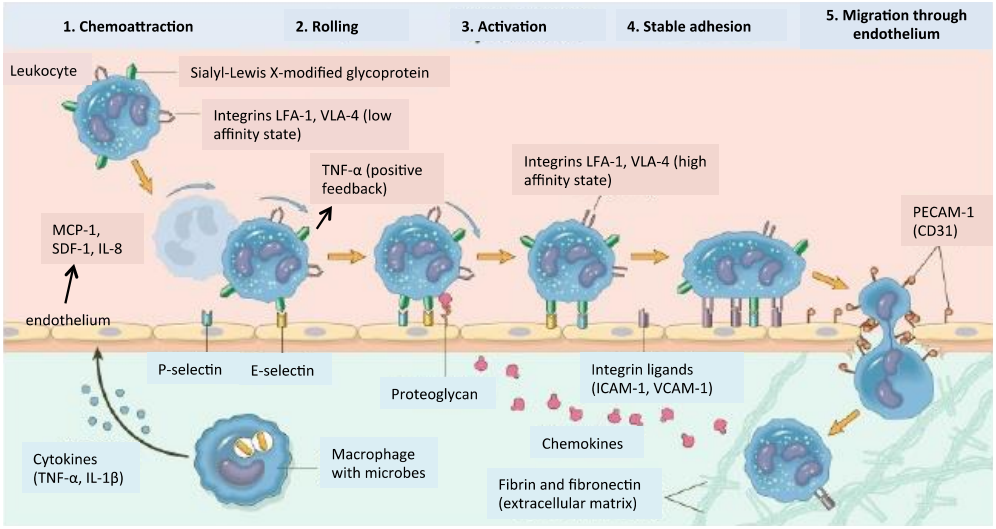

Figure 5: The multistep process of leukocyte migration through blood vessels. By an inflammatory stimulus [1], leukocytes initially loosely adhere on the vascular ECs, rolling along the blood vessel wall via transient selectin-mediated interactions [2]. During the activation stage, both ECs and leukocytes begin to upregulate expression and/or activity of adhesion receptors on the cell surface [3], and this is required for initiating the firm adhesion stage [4]. Finally, leukocytes exit the bloodstream, crossing the endothelium by the process known as transendothelial migration or diapedesis [5].Adapted from (236).

3.3. Endothelial dysfunction in cancer

Besides playing a role in the pathogenesis of cardiovascular diseases (239), endothelial dysfunction also plays a role in several processes that contribute to cancer-associated mortality. Compared to healthy vessels under physiological conditions, the tumor vasculature is frequently poorly functional and the hierarchical organization is often lost and replaced by a chaotic vascular system, with disturbed blood flow and aberrant morphology (240,241). Poor vascular function leads to intermittent or chronic hypoxia, which mediates the infiltration of cells of the immune system (like macrophages and neutrophils) into hypoxic tumor areas: upon arrival, the hypoxic TME stimulates macrophages to produce growth factors (PlGF, fibroblast-derived growth factor (FGF), plateled-derived growth factor (PDGF), macrophage colony-stimulating factor (M-CSF), and TGF-β) and cytokines (IL-1, IL-8, and TNF) which further contribute to tumor angiogenesis and to chronic endothelium activation (242). Sustained angiogenic signaling in the TME often coincides with the transition from a benign to a malignant stage of the tumor and with an angiogenic switch, i.e., the transition to a tumor vasculature that is highly infiltrative and promotes the dissemination of cancer cells to form metastases at distant sites (241,242).

4. Interactions between immune and endothelial cells in the cancer setting

Immune cells in the circulation are dependent on the vascular network to reach the tumor and kill malignant cells (243). However, tumor cells can alter the characteristics of the endothelium and, therefore, the interaction between circulating immune cells and ECs (240). Structural and functional abnormalities of tumor blood vessels, which are often poorly activated due to constitutive pro-angiogenic signaling, represent difficult hurdles for leukocyte recruitment and for the establishment of effective anti-tumor immune response (243).

4.1. Cancer promotion/metastasis via immune cell-endothelium interactions

In cancer patients, high numbers of tumor-infiltrating macrophages often correlate with poor prognosis and with highly vascularized tumors, suggesting that tumor-associated macrophages (TAMs) mainly exert tumor-promoting effects, resulting from their M2 in situ polarization (242,244). TAMs can actively interact with ECs and promote tumor angiogenesis via the secretion of inflammatory cytokines and growth factors, since they are an important source of VEGF-A in the TME (245). By increasing vascular permeability, TAM-derived VEGF-A supports the entry of cancer cell into the blood stream, and metastasis formation (246). Besides VEGF-A, TAMs also produce the pro-angiogenic factors PlGF, FGF-2, VEGF-C, TNF, IL-1β, IL-6 and IL-8 (242).

Neutrophils also show pro-angiogenic features in the TME. In humans, neutrophils release VEGF-A and BV8 (i.e., prokineticin 2) in response to TNF and to colony-stimulating factor 3 (CSF-3), respectively, and support tumor-associated angiogenesis (242).

The ability of mast cells to release pro-angiogenic factors and to produce proteases and, therefore, to support tumor angiogenesis by interacting with ECs has been well documented in various genetically engineered mouse models (247–249). In these settings, mast cells either surrounded or directly infiltrated the pre-malignant lesions, while their inactivation with the use of mast cell inhibitors impaired the angiogenic switch and the tumor progression.

B cells could also stimulate tumor angiogenesis, either directly by producing VEGF-A, FGF-2 and matrix metallopeptidase-9 (MMP-9) or indirectly via IgG secretion and by macrophage polarization (250).
Not only B cells, but also T cells can support tumor angiogenesis. For example, by secreting IL-4, $CD4^+$ Th2 cells favor the M2-like activation of immunosuppressive and pro-angiogenic TAMs (251).

Interestingly, the tumor vessels may block the activation of T cells that are recruited to the tumor tissue by expressing inhibitory molecules, such as programmed death-ligand 1 (PD-L1) and IDO or directly induce T cell apoptosis by expressing death-receptor family members including TRAIL or Fas ligand (FasL) (252). Another mechanism through which the tumor endothelium can suppress T cell adhesion and homing to tumoral sites has been reported in ovarian cancer patients. It relies on the overexpression by tumor ECs of the endothelin-B receptor (ET_BR). The paracrine interaction with its ligand endothelin-1 (ET-1), known to be overexpressed by cancer cells, supports tumor angiogenesis by promoting EC survival and proliferation (253). Therefore, the tumor vasculature constitutes an attractive therapeutic target to treat cancer patients, as it not only supports tumor progression but it also provides an escape route for metastatic dissemination (254).

4.2. Cancer clearance via immune cell-endothelium interactions

Besides supporting cancer progression, some reports suggest a correlation between high levels of macrophage infiltration and positive prognosis, for example in patients with osteosarcoma, where the presence of high numbers of tumor-infiltrating macrophages correlated with vascular density (255). The tumor-suppressing effects might be mediated by pro-inflammatory macrophages (M1 macrophages) that can produce reactive oxygen species (ROS), together with IL-1β and IL-6, acting on vascular ECs to favor cytotoxic T lymphocyte recruitment to the TME, which can suppress tumor growth (255). However, a clear correlation between the anti-tumor activity of macrophages and their interaction with ECs remains to be clarified.

A study in melanoma and fibrosarcoma tumor-bearing mice showed that endogenous IFN-β signaling in the TME limits the pro-angiogenic function of neutrophils, by inhibiting their VEGF-A production (256). Activated neutrophils can also release proteases (like elastases and the matrix metallopeptidase P9 – MMP9) to free angiostatin from plasminogen, leading to VEGF-A and FGF2 degradation and to reduced IL-8-dependent recruitment of neutrophils to the TME (257).

Regarding T cells, it was reported that $CD4^+$ Th1 cells can inhibit tumor angiogenesis by repressing the proliferation of ECs via IFN-γ, which restrains EC proliferation (258). Moreover, $CD4^+$ Th1 cell- and $CD8^+$ CTLs- derived IFN-γ may stimulate TAMs to produce CXCL9, an angiostatic cytokine, CXCL10 and CXCL11 (259,260).

The involvement of NK cells in tumor angiogenesis is poorly understood (242). In a lymphoma mouse model, the inactivation of Stat5, which is necessary for cancer immune-surveillance by NK cells, provoked increased levels of VEGF-A in NK cells and enhanced tumor angiogenesis (261). VEGF-A-producing NK cells have been described also in different human tumor types, arguing for a potential association between NK cells and tumor angiogenesis during cancer progression (184).

As for ILCs in the vasculature, IL-12-responsive NKp46^{+} ILCs were described to be recruited to the tumor site in a subcutaneous melanoma mouse model, where they supported a massive leukocyte infiltration through the upregulation of adhesion molecules in the tumor vasculature, supporting the establishment of anti-tumor immune responses (227). This effect in cancer patients has not been investigated yet. In humans, NKp44^{+} ILC3s were found at early stage in NSCLC patients (226) and correlated with a more favorable prognosis, possibly promoting intratumoral TLS formation (262).

4.3. Immunotherapy targeting immune cell-endothelium interactions

Starting from the simple idea that it might be possible to block tumor progression by blocking tumor angiogenesis, depriving the tumor cells of oxygen and nutrients, intense research efforts and several clinical trials were set up (243). To date, many anti-angiogenic drugs have been approved, including antibodies or small tyrosine kinase inhibitors that target VEGF/VEGFR signaling (263). However, VEGF targeting in cancer patients was not that successful, as patients often relapsed and tumor regrowth was often more aggressive than before anti-angiogenic therapy (264). Tumors can rapidly escape current anti-angiogenic therapies by means of alternative pro-angiogenic signals as well as by the co-option of non-angiogenic vessels and VEGF-independent pathways of neovascularization, resulting in only a modest increase in the patients' overall survival (265).

However, recent publications show that it is possible to rescue an anti-tumor immune response by the dual blockade of VEGF-A and angiopoietin-2 (ANG2) with the use of a bispecific antibody (A2V) in genetically engineered and transplanted tumor mouse models, by favoring vessel normalization, rather than blocking tumor-associated angiogenesis (266). Moreover, the use of an antibody-drug conjugate (ADC) for the dual targeting of the cell surface protein B7H3 (CD276), which is abundantly expressed by different tumor types but also by tumor-associated ECs, resulted to be a promising strategy in pre-clinical studies and largely improved long-term overall survival (267). The combinatorial targeting of these two

cell compartments at the same time could be useful to improve current cancer therapies and in particular to treat late-stage metastatic diseases.

It is more and more accepted that the promotion of vascular normalization, rather than the inhibition of angiogenic processes, is crucial for the onset of an anti-tumor response. As shown by Tian *et al.*, impaired vessel normalization by means of pericyte coverage leads to reduced effector T lymphocyte infiltration in a syngeneic breast cancer mouse model, where a key role was played by Th1 IFN-γ producing $CD4^+$ T-cells (268). Since the success of immunotherapy depends on the ability of effector T cells to infiltrate tumors, the identification of factors promoting T cell recruitment into the hostile TME is crucial in fostering tumor eradication.

Another emerging concept is that the combination of vascular targeting with IC inhibitors (ICIs) may promote anti-tumor immune responses by inducing high-endothelial venule (HEV) formation. HEVs are specialized vessels found in SLOs that are adapted for lymphocyte trafficking (269), but also in TLS that are often observed to spontaneously form in some solid tumors (270). For example, the combination of anti-VEGFR2 with anti-PD-L1 blocking antibodies induced the formation of HEVs in breast cancer and pancreatic neuroendocrine tumor models, improving T cell infiltration in the tumor site (271). Formation of HEVs was also observed in glioblastoma models, although they required additional stimulation using a LTβR agonistic antibody, resulting in enhanced T cell infiltration and reduced tumor growth (271).

Furthermore, the promotion of vessel normalization in combination with a carboxy-terminal vascular targeting peptide (VTP) coupled to LIGHT, an LTβR ligand, induced HEVs and TLS formation in pancreatic neuroendocrine tumors. Interestingly, this therapeutic strategy sensitized these tumors to anti-PD-1 and anti-cytotoxic T-Lymphocyte Ag-4 (CTLA-4) antibody treatment (272). These studies indicate that beyond normalizing vessels, converting tumor vessels into HEVs can be beneficial for enhancing the response to cancer immunotherapy. Furthermore, HEVs may promote formation of TLSs which have been associated with a beneficial response to cancer immunotherapy in several types of cancer (269,273).

To date, little is known about the interaction between ILCs and the endothelium and, in particular, whether circulating human ILCs could have an impact on tumor growth and/or tumor spread through the interaction with the endothelium.

5. Aims of my PhD project

The first aim of my PhD project was to isolate and characterize circulating ILCs from the peripheral blood of healthy volunteers and to perform an in-depth characterization of the interaction between circulating human ILC subsets and primary blood ECs *in vitro.*

The second aim was to understand whether/how, through this interaction, ILCs might exert an active role in controlling tumor growth, either by infiltrating and/or by facilitating the infiltration of other immune cells into the tumor bed *in vitro*.

This work is accepted for publication in the journal *eLife* (February 2021). For this study, I performed the *ex vivo* isolation and *in vitro* characterization of circulating human ILCPs as well as the experiments with primary ECs and tumor cells. I analyzed the data, I wrote the first draft and revised the manuscript. I planned the experiments for the revision and discussed the results with my supervisors and with the collaborators of this work.

Contribution to Annex I: "ILC2s: New Actors in Tumor Immunity" (Ercolano *et al*.)
This work was published as a review in the journal *Frontiers in Immunology* in December 2019. Together with MF, I wrote the first draft and revised the manuscript, and I designed the Figure 1.

Contribution to Annex II: "CD56 as a marker of an ILC1-like population with NK cell properties that is functionally impaired in AML" (Salomé *et al.*)
This work is presented as a research article published in October 2019 in the journal *Blood Advances*. For this work, I performed the isolation, expansion and characterization of ILC1-like cells from the peripheral blood of healthy donors and contributed to the revision of the manuscript.

Contribution to Annex III: "Immunosuppressive Mediators Impair Proinflammatory Innate Lymphoid Cell Function in Human Malignant Melanoma" (Ercolano *et al.)*
This work is presented as a research article published in February 2020 in the journal *Cancer Immunology Research*. For this work, I performed the isolation, expansion and characterization of ILC1s from the peripheral blood of healthy donors and contributed to the revision of the manuscript.

During my PhD I also actively contributed to an interdisciplinary project, sponsored by the Faculty of Biology and Medicine, aiming to investigate the impact of virtual reality on the immune system. Due to patent pending though, I cannot disclose any detail about this project.

6. Human primed ILCPs support endothelial activation through NF-κB signaling

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Short running title: Primed hILCPs activate ECs

6.1. Abstract

Innate lymphoid cells (ILCs) represent the most recently identified subset of effector lymphocytes, with key roles in the orchestration of early immune responses. Despite their established involvement in the pathogenesis of many inflammatory disorders, the role of ILCs in cancer remains poorly defined. Here we assessed whether human ILCs can actively interact with the endothelium to promote tumor growth control, favoring immune cell adhesion. We show that, among all ILC subsets, ILCPs elicited the strongest upregulation of adhesion molecules in ECs *in vitro*, mainly in a contact-dependent manner through the TNFR- and RANK-dependent engagement of the NF-κB pathway. Moreover, the ILCP-mediated activation of the ECs resulted to be functional by fostering the adhesion of other innate and adaptive immune cells. Interestingly, pre-exposure of ILCPs to human tumor cell lines strongly impaired this capacity. Hence, the ILCP-EC interaction might represent an attractive target to regulate the immune cell trafficking to tumor sites and, therefore, the establishment of an anti-tumor immune response.

6.2. Introduction

Innate lymphoid cells constitute the latest described family of innate lymphocytes with key functions in the preservation of epithelial integrity and tissue immunity throughout the body (49). Besides conventional NK (cNK) cells, three main distinct subsets of non-NK helper-like ILCs have been described so far, mirroring the transcriptional and functional phenotype of $CD4^+$ Th cell subsets (274): ILC1s, ILC2s and ILC3s, that mainly produce IFN-γ, IL-4/IL-5/IL-13, and IL-17A/IL-22 respectively (49).

In human tissues, the majority of ILCs is mainly terminally differentiated, while a population of circulating Lin^- $CD127^+CD117^+CRTH2^-$ ILCs, able to differentiate into all ILC subsets, has been recently identified in the periphery and named ILC precursors (ILCPs) (86). ILCPs are characterized by the expression of CD62L, that drives their migration to the lymph nodes (88). Enriched at surface barriers, ILCs rely on IL-7 for their development and promptly respond to tissue- and cell-derived signals by producing effector cytokines in an Ag-independent manner (275).

The different ILC subsets have important effector functions during the early stages of the immune response against microbes, in tissue repair and in the anatomical containment of commensals at surface barriers (51). In addition, depending on the ILC subset that is involved and on the tumor type (276)(90)(277)(192), ILCs have been shown to also exert pro- and anti-tumoral activity by interacting with different cell types, including endothelial and stromal cells. In a subcutaneous melanoma mouse model, IL-12-responsive $NKp46^+$ ILCs, recruited to the tumor, supported a massive leukocyte infiltration through the upregulation of adhesion molecules in the tumor vasculature (227). In humans, $NKp44^+$ILC3s were found to be present at early stage in NSCLC patients (226) and to correlate with a more favorable prognosis, possibly by promoting intratumoral TLS formation (262).

However, scant data are available about the interaction between human ILCs and the vascular endothelium, which constitutes the physical barrier to be crossed by peripheral blood immune cells to migrate into tissues where to exert their effector functions (278).

In this study, we show for the first time that human primed ILCPs can interact with ECs, upregulate adhesion molecules and stimulate their pro-inflammatory cytokine secretion. This activation occurs through NF-κB, primarily in a contact-dependent manner that engages surface TNF and RANKL. We report that the ILCP-mediated activation of the ECs is functional, i.e., it allows the adhesion of freshly isolated peripheral blood immune cells. Moreover, we show that the ability of ILCPs to activate ECs is dampened after the co-culture with tumor cells. With this study, we have unraveled a cell intrinsic ability of ILCPs that might be selectively impaired by tumors to favor their immune escape.

Materials and methods

6.2.1. Cell isolation

Venous blood was drawn from healthy donors (HDs) at the local Blood Transfusion Center, Lausanne, Switzerland, under the approval of the Lausanne University Hospital's Institute Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation on 1.077g/mL Ficoll-Hypaque (Lymphoprep). Individual human ILC subsets were isolated from HD PBMCs by fluorescence activated cell sorting (FACS) using the following antibodies (Biolegend): FITC anti-CD3, -CD4, -CD8, -CD14, -CD15, -CD16, -CD19, -CD20, -CD33, -CD34, -CD94, -CD203c and –FcεRI (lineage markers); PE anti-CRTH2; APC anti-c-Kit and BV421 anti-CD127. ILC subsets were sorted within the Lin^- $CD127^+$ fraction, according to the expression of c-Kit and CRTH2: ILC1s as c-Kit^-CRTH2^- cells; ILC2s as c-$Kit^{+/-}CRTH2^+$ cells and ILCPs as c-Kit^+CRTH2^- cells. Naïve total $CD4^+$ T cells were firstly isolated from HD PBMCs by FACS by using FITC anti-CD3, PE anti-CD4 and APC-Fire anti-CD45RA antibodies (Biolegend). Following *in vitro* expansion, individual $CD4^+$ Th cell subsets were isolated by FACS using FITC anti-CXCR3, PerCP-Cy5.5 anti-CCR4, PE anti-CRTH2, PE-Cy7 anti-CCR6, APC anti-CD3, A700 anti-CD4, APC-Cy7 anti-CD45RO and BV421 anti-CXCR5 antibodies (Biolegend). Gating on $CD3^+CD4^+CD45RO^+CXCR5^-$ cells, the Th subsets were sorted as follows: Th1 as $CRTH2^-CXCR3^+CCR6^-$ cells; Th* as $CRTH2^-CXCR3^+CCR6^+$ cells; Th2 as $CRTH2^+$ $CXCR3^-CCR6^-$ cells; Th17 as $CRTH2^-CXCR3^-CCR4^+CCR6^+$ cells. Individual ILC subsets, naïve $CD4^+$ T cells and individual $CD4^+$ Th cell subsets were all isolated by FACS on a FACS Aria II or a FACS Aria III (BD).

6.2.2. Cell culture and blocking experiments

Highly purified ILC subsets (≥90%) were expanded *in vitro* for at least 2 weeks in the presence of 100U/mL of rh-IL-2 (PeproTech), 1µg/mL of phytohaemagglutinin (PHA - PeproTech) and irradiated allogenic feeder cells obtained from three different donors (1:10 ILC/feeder cell ratio) in RPMI-1640 (Gibco) supplemented with 8% human serum (HS), 1% penicillin/streptomycin (10'000U/mL, Gibco), 1% L-Glutamine (Gibco), 1% non essential amino acids (Gibco), 1% Na pyruvate (Gibco), 1% Kanamycin 100x (Gibco) and 0.1% 2β-mercaptoethanol 500mM (Sigma). After expansion, content of ILC subset in the cultures was assessed by flow cytometry and, if necessary, re-sorted to obtain pure (≥ 90%) ILC1s, ILC2s and ILCPs, before being employed in co-culture experiments. Similarly, $CD45RA^+$ naïve $CD4^+$ T cells were firstly *ex vivo* isolated and *in vitro*-expanded for 2 weeks in the presence of 100U/mL of rh-IL-2, 1µg/mL of PHA and irradiated allogenic feeder cells obtained from three different donors (1:10 $CD4^+$ T cell/feeder cell ratio) in RPMI-8% HS. Subsequently, individual $CD4^+$ Th cell subsets (i.e., Th1, Th2, Th17 and Th*) were isolated by FACS and

cultured for additional two weeks in RPMI-8% HS in the presence of 100U/mL of rh-IL-2 for Th1 and Th2, 20U/mL of rh-IL-2 for Th17, 10U/mL of rh-IL-2 with 50ng/mL of rh-IL-12 and rh-IL-21 (Peprotech) for Th*. Primary human umbilical cord vein ECs (HUVECs - Lonza) and primary human dermal blood ECs (HDBECs – Promocell) were cultured in supplemented EC growth medium (EGM Ready To Use, Lonza) and used between passages 4 and 6. Non-muscle invasive bladder carcinoma cells (BU68.08), muscle-invasive bladder carcinoma cells (TCC-Sup) and the colon adenocarcinoma cells (SW1116), were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (10'000U/mL, Gibco), 1,15% AAG (Arg,Asp,Glu), 1% Hepes buffer 1M (Gibco) and 0.2g/L ciproxin (Bayer) (EC number 2019-00564). Prior exposure to ILCPs, EC monolayers were incubated during 1h in the presence of 2.5µM of BAY 11-7082 (Adipogen) in EC growth medium, to specifically prevent NF-κB activation in ECs. EC monolayers where then washed once with PBS, before incubation with ILCPs at 1:1 ratio. Similarly, blocking experiments with the use of soluble Fc fusion proteins were performed: when indicated, ILCPs were pre-incubated for 30min with 2µg/mL of TNFR1:Fc (Adipogen), 5µg/mL of TNFR2:Fc (Adipogen), 5µg/mL of RANK:Fc (Adipogen), either alone or in combination. Then, ILCPs were washed once with PBS, then added to EC monolayers. Finally, when indicated, *in vitro*-expanded ILCPs were incubated during an overnight with 50µM of 2-chloroadenosine (CADO - Sigma) in RPMI-8% HS with 10U/mL of IL-2 prior co-culture with ECs.

6.2.3. Co-culture experiments

Following expansion, individual pure (≥90%) ILC and Th subsets were rested overnight in RPMI-8% HS medium supplemented with 10U/mL of rh-IL-2. Then, confluent EC monolayers were either co-cultured for 3h with individual ILC and Th subsets at 1:1 ratio, treated with 20ng/mL of rh-TNF (Peprotech) or left untreated as positive and negative controls, respectively. Co-cultures of ECs with ILCPs were performed both in the presence or absence of 0.4µm pore polycarbonate filter in 24-well transwell chambers (Corning). ILCPs were also incubated overnight with epithelial bladder and colon carcinoma cells in RPMI-8% HS with 10U/mL of IL-2 at 1:1 ratio, before exposure to EC monolayers. The day of the experiment, ILCs were collected, washed with PBS, and re-suspended in the respective EC growth medium (Lonza). At least 3 independent experiments were performed, using individual ILC and Th subsets isolated from a different donor. At the end of the experiment, supernatants were collected and stored at -20°C, EC were washed twice with PBS and detached with Accutase (Gibco) for 5min at 37°C. Cell suspensions were then washed with PBS and stained for flow cytometry analyses.

6.2.4. Phenotypic characterization

The phenotypic characterization of both *ex vivo* and *in vitro*-expanded ILCPs from HDs, as well as the quantification of *ex vivo* ILCPs in the peripheral blood (PB) of bladder cancer patients, was performed by using the same antibodies as the ones used for isolation by FACS together with the following antibodies: PE anti-BAFF (Biolegend), -NKp46 (Biolegend) , -RANKL (Biolegend) and –RORγt (Biolegend); PE-CF594 anti-T-bet (BD); PE-Dazzle anti-CD39 (Biolegend); PerCP-Cy5.5 anti-CD28 (Biolegend); PE-Cy7 anti-NKp44 (Biolegend); APC anti-CD30L (Biolegend) and anti-GATA3 (Biolegend); A700 anti-CD45RA (Biolegend) and -LT$\alpha_1\beta_2$ (Biolegend); APC-Cy7 anti-CD45RO (Biolegend) and anti-CXCR5 (Biolegend); eFluor450 anti-CD73 (eBioscience); BV421 anti-NRP1 (Biolegend); BV650 anti-CD62L (Biolegend), -CD69 (Biolegend) and anti-CCR6 (Biolegend); BV711 anti-CD40L (Biolegend). The activation state of ECs was assessed by flow cytometry using FITC anti-CD31 (Biolegend), PE anti-RANK (R&D), PE-Cy7 anti-CD62E (or E-Selectin - Biolegend), Pacific Blue anti-CD54 (or ICAM-1 - Biolegend) and PE-Cy5 anti-CD106 (or VCAM-1 - Biolegend). For the static adhesion assay, the assessment of PBMCs adhesion to ECs and the EC activation state was analyzed using the following panel of antibodies: APC anti-CD3 (BC), PE-Cy7 anti-CD4 (BC), PE-CF594 anti-CD14 (BD), A700 anti-CD16 (Biolegend), APC-H7 anti CD19 (BD), FITC anti-CD31 (BD), Pacific Blue anti-CD54 (Biolegend), PE anti-CD62E (Biolegend) and PE-Cy5 anti-CD106 (Biolegend). For the characterization of tumor cells, the following antibodies were used: PE-Dazzle anti-CD39 (Biolegend); eFluor450 anti-CD73 (eBioscience) and PE anti-IDO-1 (Invitrogen). All analyses included size exclusion (forward scatter [FSC] area versus side scatter [SSC] area), doublets exclusion (FSC height/ FSC area), and dead cell exclusion (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, ThermoFisher). A minimum of 10’000 events were acquired on either a Gallios Cytometer (Beckman Coulter) or SORPLSR-II Cytometer (BD) and analyzed with FlowJo software (TreeStar).

6.2.5. Static adhesion assay

ECs were plated at 80% confluency in a 24-well plate in complete EGM. Once adherent, the media was removed, ECs were washed with PBS and 500µL of complete EGM, containing or not ILCPs at 1:1 ratio, were added to the wells during 3h. As positive control, ECs were incubated during 3h with 20ng/mL of rh-TNF. After the co-culture, ECs were detached and stained with FITC anti-CD31 antibody and FACS-sorted to remove adherent ILCPs. Recovered ECs were seeded in a 48-well plate and let to adhere overnight in complete EGM. The morning after, the static adhesion assay was performed (adapted from Safuan *et al.*, 2012). The adhesion of freshly isolated PBMCs was assessed by adding 4:1 cells (PBMC:EC) /well for 30min at 37°C. Non-adherent cells were washed away from the EC monolayer by performing 2x washing steps

with PBS. ECs, together with adherent PBMCs, were detached with Accutase (Gibco), and stained for flow cytometry analyses. The number of CD3, CD4, CD8, CD14, CD16, $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16^{low}$ and CD19 expressing cells, as well as $Lineage^{-}CD127^{+}$ total ILCs were quantified by flow cytometry by adding 10µL of CountBright Absolute Counting Beads (Thermofisher) to the cell suspensions. 2000 beads/sample were acquired and cell counts normalized.

6.2.6. RNA purification and qPCR

Total RNA was isolated from highly pure *ex vivo* and *in vitro*-expanded ILCPs, from primary ECs (HUVECs) and from sorted human ILC and CD4 Th cell subsets using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Final preparation of RNA was considered DNA- and protein-free if the ratio of spectrophotometer (NanoDrop, ThermoFischer, Carls- bad, CA, USA) readings at 260/280 nm was ≥1.7. Isolated mRNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-RadLaboratories, Watford, UK) according to the manufacturer's protocol. The qPCR was carried out in the ECO™ Real-time PCR System (Illumina) with specific primers (hA2A 5'-CTCCGGTACAATGGCTTGGT-3', 5'-TGGTTCTTGCCCTCCTTTGG-3'; hA2B 5'-ATGCCAACAGCTTGAATGGAT-3', 5'-GAGGTCACCTTCCTGGCAAC-3'; hA3 5'-TTGACCAAAAGGAGGAGAAGT-3', 5'-AGTCACATCTGTTCAGTAGGAG-3'; hIL-6 5'-GGATTCAATGAGGAGACTTGC-3' , 5'-GTTGGGTCAGGGGTGGTTAT-3'; hIL-8 5'-AGCTCTGTGTGAAGGTGCAG-3' , 5'-TGGGGTGGAAAGGTTTGGAG-3'; hGM-CSF 5'-GCCTCAGCTACGTTCAAGG-3' , 5'-CATAGGAGTTAGGTCCCCACA-3'; hIFN-γ 5'-TGCCTTCCCTGTTTTAGCTGC-3' , 5'-TCGGTAACTGACTTGAATGTC-3'; hTNF 5'-GAGGCCAAGCCCTGGTATG-3' , 5'-CGGGCCGATTGATCTCAGC-3') using KAPA SYBR FAST qPCR Kits (KAPA Biosystems, Inc., MA). Samples were amplified simultaneously in triplicate in one-assay run with a nontemplate control blank for each primer pair to control for contamination or for primer dimerization, and the Ct value for each experimental group was determined. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the Ct values, using the $2^{-\Delta Ct}$ formula.

6.2.7. Immunohistochemical staining

Immunohistochemical staining was performed on 2µm paraffin sections with an automated IHC staining system (Ventana BenchMark ULTRA, Ventana Medical Systems, Italy). Sequential double IHC was performed on Ventana BenchMark ULTRA, using a ultraView Universal DAB detection Kit as the first stain and ultraView Universal Alkaline phosphatase Red detection kit as the second stain. Heat-induced epitope

retrieval pre-treatment was performed using CC1 buffer (standard CC1, Roche Ventana) by boiling for 36 minutes for both CD31 and CD3 and for 64 minutes for RORγt. Afterwards, slides were incubated with primary antibodies: CD31 antibody (clone JC70, Cell Marque, dilution 1:20) for 16 minutes at 37°C or CD3 (clone 2GV6, Ventana, dilution 1:20) for 44 minutes at 37°C and RORγt (clone 6F3.1, Millipore, dilution 1:20) for 36 minutes at 37°C. CD31 and CD3 were visualised with DAB chromogen, and RORγt was visualised with Fast Red chromogen.

6.2.8. Statistical analyses

GraphPad Prism 7 software was used to perform the statistical analyses. Paired or unpaired t-tests were used when comparing two groups. ANOVAs or the non-parametric Kruskal-Wallis test were used for comparison of multiple groups. Data in graphs represent the mean ± SEM, with a P value <0.05 (two-tailed) being significant and labelled with *. P values <0.01, <0.001 or <0.0001 are indicated as **, *** and ****, respectively. Without mention, differences are not statistically significant.

6.4 Results

6.4.1 ILCPs upregulate adhesion molecules on endothelial cell surface and acquire an activated and ILC3-like phenotype *in vitro*

The first evidence of an ILC-EC interaction was reported by Eisenring *and colleagues* in an *in vivo* melanoma model (227). To investigate whether also human ILCs can interact with ECs, individual circulating ILC subsets, identified based on the expression of c-Kit and CRTH2 within the $Lin^{-}CD127^{+}$ fraction (**Fig. 1a**), were *ex vivo*-sorted from the peripheral blood (PB) of healthy volunteers and short-term *in vitro*-expanded and eventually re-sorted at a purity ≥ 90%, before use in co-culture experiments with primary human ECs (HUVECs) (**Supplementary Fig. 1a**). Upon exposure of ECs to *in vitro*-expanded ILC subsets, ILCPs were the only subset that significantly upregulated the adhesion molecules E-Selectin, ICAM-1 and VCAM-1 on the EC surface, if compared to ILC1s and ILC2s (**Fig. 1b**). These adhesion proteins are involved in the different stages of the multi-step process of the leukocyte TEM process, i.e., the movement of leukocytes out of the blood stream and towards the site of tissue damage and/or infection (236). Interestingly, we confirmed the ability of *in vitro*-expanded ILCPs to activate ECs using other primary human dermal blood ECs, i.e., HDBECs (**Supplementary Fig. 1b**). Following *in vitro* expansion, we observed that ILCPs upregulated NKp44 and CD69 as well as CD45RO and RORγt, if compared to their *ex vivo* counterparts, while maintaining similar levels of expression of NKp46 and CD62L (**Supplementary Fig. 1c-d**), suggesting that the *in vitro* expansion process conferred a more committed phenotype to this ILC subset. Interestingly, no difference in T-bet or GATA3 expression in $ROR\gamma t^{+}$ vs $ROR\gamma t^{-}$ cells was observed (**Supplementary Fig. 1e**), indicating that the expression or not of RORγt is not directly involved in the EC-activating capacity of ILCPs. Since we observed that around 60% of *in vitro*-expanded ILCPs acquired NKp44 expression, we investigated the ability of $NKp44^{+}$ vs $NKp44^{-}$ ILCPs to activate ECs. As shown in **Supplementary Fig. 1f***,* no significant difference was observed in the EC-activating capacity of these 2 subpopulations, suggesting that the EC-activating capacity of ILCPs does not depend on the expression of NKp44. Moreover, we observed that *in vitro*-expanded ILCPs upregulated the expression of CCR6 and CXCR5, i.e., two known LTi-like cells markers, compared to their *ex vivo* counterpart. Consistent with previous reports, Neuropilin1 (NRP1) was not expressed by circulating ILC3s (280) and was not upregulated after *in vitro* expansion. Compared to *ex vivo* ILCPs, *in vitro*-expanded ILCPs downregulated the expression of CD28, although only 20% of circulating ILCPs expressed it (**Supplementary Fig. 1c-d**). Overall, these data suggest that not only *in vitro*-expanded ILCPs acquire an activated phenotype *in vitro*, but are also skewed towards an ILC3-like phenotype and

share some phenotypical markers with LTi-like cells, while maintaining multipotent features as shown by the expression of T-bet an GATA3.

To understand if the ability of ILCPs to interact with ECs is an intrinsic property of these cells or if they need to be primed to acquire it, we decided to expose ECs directly to *ex vivo*-sorted ILC subsets. As shown in **Fig. 1c**, none of the isolated ILC subsets could induce a significant activation of ECs, suggesting that the EC-activating capacity of ILCPs is acquired during the *in vitro* expansion process. Since ILCPs were expanded in the presence of feeder cells, PHA and IL-2, it is conceivable that feeder-derived cytokines such as IL-12 and IL-1β are involved in the priming. As ILCs constitute the innate counterpart of $CD4^+$ T cells, we tested if *in vitro*-expanded individual T-helper (Th) subsets, i.e., Th1, Th2, Th17 and Th* (i.e., Th cells with a Th1/Th17 intermediate phenotype (281)) (**Supplementary Fig. 2a**) could also interact, at steady-state, with ECs. Following the same expansion protocol employed for *ex vivo*-isolated ILC subsets, Th subsets were employed in 3h co-culture experiments with ECs. As reported in the **Supplementary Fig. 2b**, except for a statistically significant Th1-mediated upregulation of VCAM-1, still not to the same extent as the ILCP-mediated induction, all Th subsets failed to upregulate adhesion molecule expression on the EC surface. Overall, these data suggest that *in vitro*-expanded ILCPs not only acquire a more activated/ILC3-like phenotype *in vitro*, but also the ability of interacting with ECs by means of mediating the upregulation of adhesion molecule expression on the EC surface.

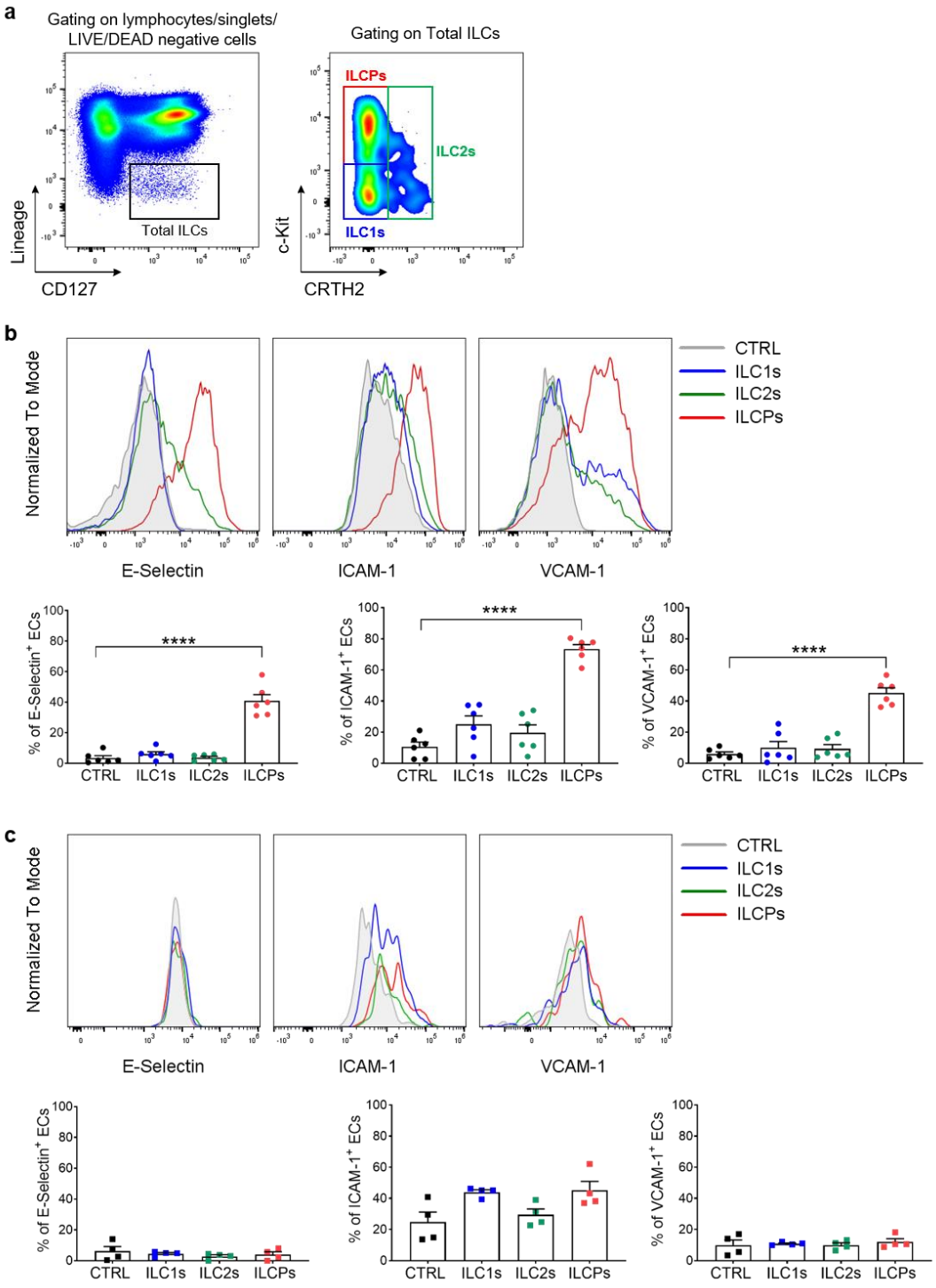
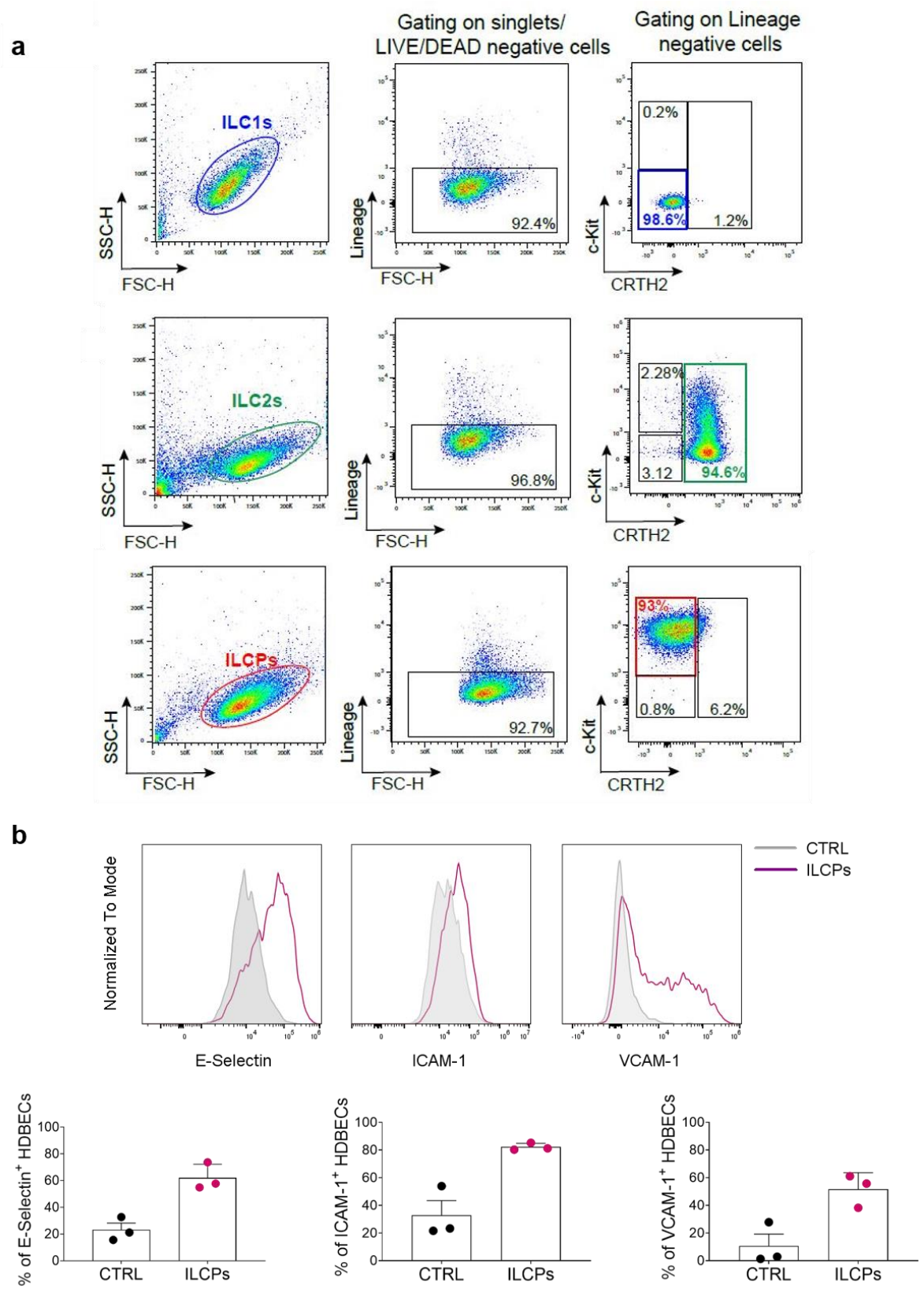


Figure 1. ***In vitro*-expanded ILCPs induce adhesion molecule expression in endothelial cells**. (**a**) Circulating human ILCs are identified as lineage negative CD127$^+$ cells; within this population, we discriminate ILC1s as c-Kit$^-$CRTH2$^-$, ILC2s as CRTH2$^+$c-Kit$^{+/-}$, ILCPs as c-Kit$^+$ CRTH2$^-$ cells. HUVEC cells were co-cultured for 3 h at 1:1 ratio in direct contact with either *in vitro*-expanded (**b**) or directly *ex vivo*-sorted (**c**) ILC1s, ILC2s and ILCPs. Untreated ECs were employed as negative control (CTRL). ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry. Graphs show representative histograms (panels **b** and **c**, top) and the summary (panels **b** and **c**, bottom) of the induction of the indicated adhesion molecules on the EC surface (n=6). Ordinary one-way ANOVA - Tukey's multiple comparison test (panel **b**); Ordinary one-way ANOVA - Friedman test (panel **c**).


a
Gating on singlets/
LIVE/DEAD negative cells
Gating on Lineage
negative cells
ILC1s
SSC-H
FSC-H
Lineage
92.4%
0.2%
98.6%
1.2%
c-Kit
CRTH2
ILC2s
96.8%
2.28%
3.12
94.6%
ILCPs
92.7%
93%
0.8%
6.2%
b
Normalized To Mode
CTRL
ILCPs
E-Selectin
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VCAM-1
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% of ICAM-1+ HDBECs
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CTRL
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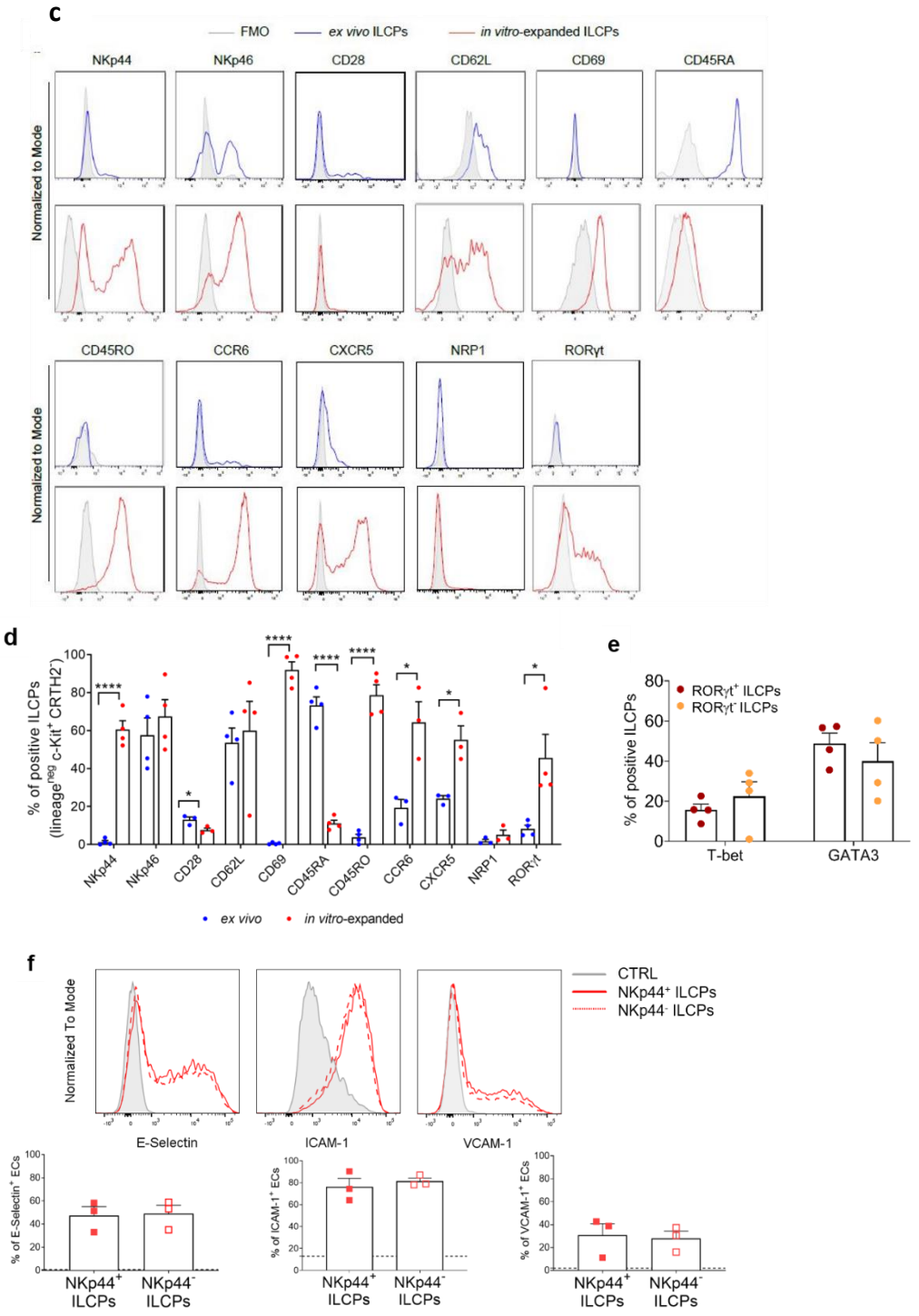
c
FMO
ex vivo ILCPs
in vitro-expanded ILCPs
NKp44
NKp46
CD28
CD62L
CD69
CD45RA
Normalized to Mode
CD45RO
CCR6
CXCR5
NRP1
RORγt
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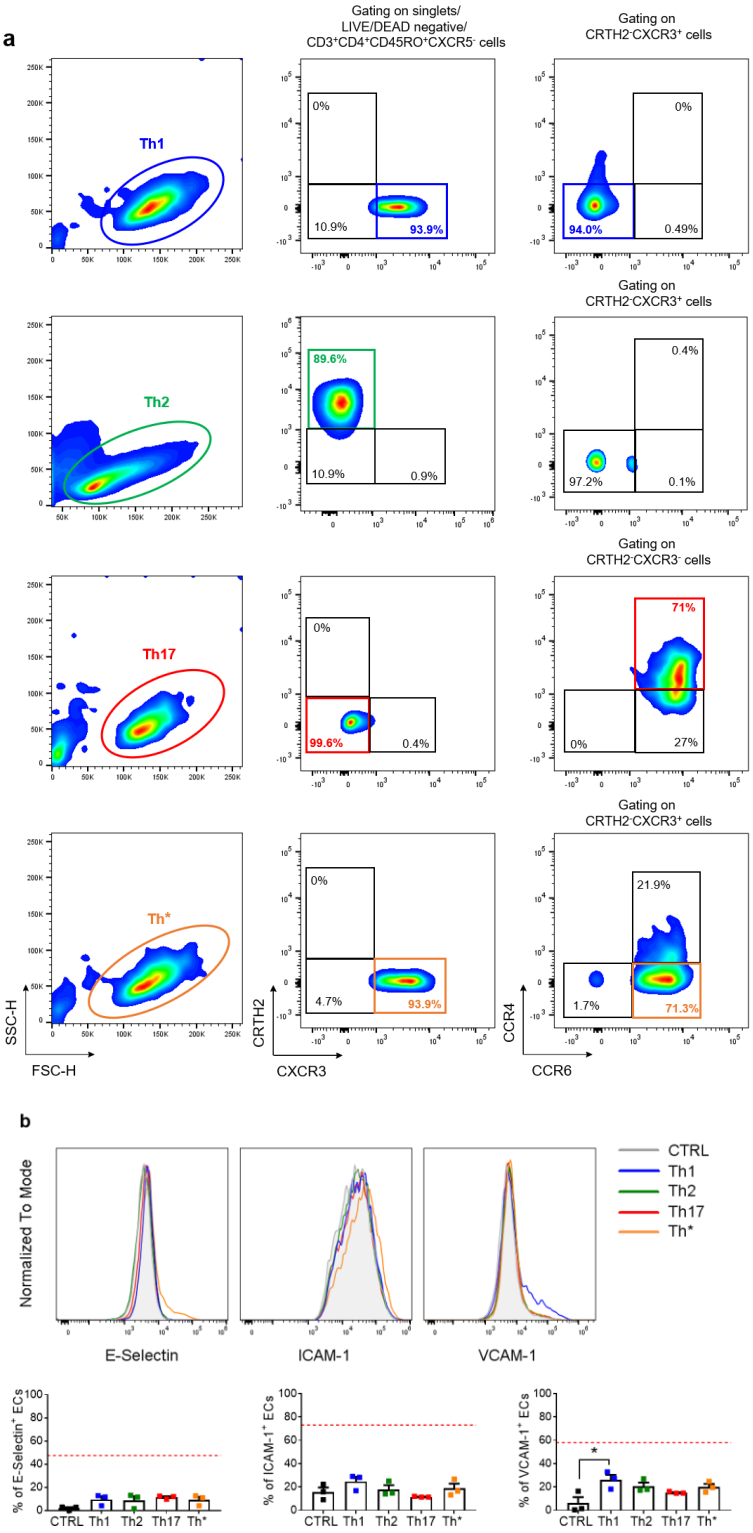
% of positive ILCPs (lineage^neg c-Kit^+ CRTH2^-)
ex vivo
in vitro-expanded
e
RORγt^+ ILCPs
RORγt^- ILCPs
% of positive ILCPs
T-bet
GATA3
f
Normalized To Mode
CTRL
NKp44^+ ILCPs
NKp44^- ILCPs
E-Selectin
ICAM-1
VCAM-1
% of E-Selectin^+ ECs
% of ICAM-1^+ ECs
% of VCAM-1^+ ECs
NKp44^+ ILCPs
NKp44^- ILCPs

Supplementary Figure 1. Human ILCPs acquire an activated phenotype *in vitro*. (**a**) Following *ex vivo* sorting and *in vitro* expansion, the individual ILC subsets were subjected to purity checks before use in functional assays with ECs. ILC1s were re-sorted as $Lin^{-}c\text{-}Kit^{-}CRTH2^{-}$, ILC2s as $Lin^{-}c\text{-}Kit^{-/+}CRTH2^{+}$ and ILCPs as $Lin^{-}c\text{-}Kit^{+}CRTH2^{-}$ cells. (**b**) HDBECs were co-cultured for 3 h at 1:1 ratio in direct contact with *in vitro*-expanded ILCPs. Untreated HDBECs were employed as negative control (CTRL). ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry. Graphs show representative histograms (top) and the summary (bottom) of the induction of the indicated adhesion molecules on the EC surface (n=3). (**c**) Representative histograms showing the expression of NKp44, NKp46, CD28, CD62L, CD69, CD45RA, CD45RO, CCR6, CXCR5, NRP1 and RORγt by *ex vivo* and *in vitro*-expanded ILCPs. (**d**) The graphs show the summary of the results obtained in 4 different donors. (**e**) Expression of T-bet and GATA3 in $ROR\gamma t^{+}$ vs $ROR\gamma t^{-}$ ILCPs. (**f**) HUVEC cells were co-cultured for 3 h at 1:1 ratio in direct contact with *in vitro*-expanded $NKp44^{+}$ ILCPs and $NKp44^{-}$ ILCPs. Untreated ECs were employed as negative control (CTRL). ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry. Graphs show representative histograms (top) and the summary (bottom) of the induction of the indicated adhesion molecules on the EC surface (n=3). The black dotted lines indicate the level of average expression of adhesion molecules by untreated ECs. Statistical tests used: Paired t test (panels **b**,**f**); Multiple t tests (panels **d,e**).



Supplementary Figure 2: *In vitro*-expanded Th subsets fail to activate endothelial cells. (**a**) Circulating human naïve $CD4^+$ T cell subsets were isolated from the peripheral blood of HDs as $CD3^+$ $CD4^+$ $CD45RA^+$ cells and *in vitro*-expanded for 2 weeks in the presence of 100U/mL rhIL-2, 1µg/mL of PHA and irradiated allogenic feeder cells (1:10 T cell-feeder cell ratio). Individual $CD4^+$ T cell subsets were re-sorted by FACS as follows: Th1 as $CRTH2^-CXCR3^+CCR6^-$ cells; Th* as $CRTH2^-CXCR3^+CCR6^+$ cells; Th2 as $CRTH2^+$ cells; Th17 as $CRTH2^-CXCR3^-CCR4^+CCR6^+$ cells. (**b**) HUVEC cells were co-cultured for 3 h at 1:1 ratio in direct contact with *in vitro*-expanded Th1, Th2, Th17 and Th* or left untreated (CTRL). ECs were harvested and analyzed for cell -surface adhesion molecule expression by flow cytometry. Graphs show representative histograms (top) and the summary (bottom) of the induction of the indicated adhesion molecules on the EC surface (n=3). The red dotted lines indicate the level of average expression of adhesion molecules by ILCP-exposed ECs. Statistical test used: Kruskal-Wallis test (panel **b**).

6.4.2. ILCPs activate ECs primarily in a contact-dependent mechanism

Inflammation triggers the upregulation of adhesion molecules in ECs, promoting the accumulation of leukocytes and their adhesion to the blood vessel walls. This phenomenon is mediated by pro-inflammatory mediators, such as TNF and IL-1β (282). As a consequence, to discriminate whether the EC activation by ILCPs was due to contact-dependent or soluble factor(s)-dependent mechanism(s), supernatants from the EC/ILCP co-cultures were analyzed. Significantly higher levels of IL-6, IL-8, GM-CSF and IFN-γ were observed (**Fig. 2a**). To address which cell type was producing the pro-inflammatory cytokines that accumulate in the cell-free supernatants, qPCR analysis of ECs and ILCPs (CD31-based FACS-sorted after 3h co-culture) was performed and compared to untreated ECs and steady-state ILCPs. As reported in **Fig. 2b,** high levels of IL-6 and IL-8 transcripts were found in ECs exposed to ILCPs, whereas TNF transcripts were high only in steady-state ILCPs, indicating that IL-6 and IL-8 measured in the supernatant (**Fig. 2a**) derive from ECs, whereas TNF from ILCPs. GM-CSF and IFN-γ transcripts were observed in both ECs and ILCPs before and after co-culture, indicating that both cell types contribute to the accumulation of these two cytokines in the supernatant. To experimentally verify if the upregulation of adhesion molecules in ECs was dependent on these soluble factors, 0.4μm pore transwell chambers were employed, to allow cytokine exchange between the two compartments yet avoiding the cell contact. In this context, ILCPs failed to induce the expression of adhesion molecules on EC surface (**Fig. 2c**). Of note, the production of the pro-inflammatory cytokines was dramatically reduced in the presence of the transwell insert (**Supplementary Fig. 3a**). To further prove the direct contact-dependency of the EC-ILC interaction, ECs were incubated during 3h in the presence of cell-free supernatant collected from previous EC-ILCP co-culture. As reported in **Fig. 2d**, cell-free supernatant did not lead to the upregulation of the adhesion molecules E-Selectin and VCAM-1 in ECs, although ICAM-1 levels were found to be significantly increased if compared to unstimulated ECs, yet not to the same extent as for ILCP-exposed ECs. Finally, we analyzed the production of IL-6, IL-8, TNF, GM-CSF and IFN-γ by *ex vivo* and *in vitro*-expanded ILCPs. As shown in **Supplementary Fig. 3b**, no difference in terms of secretion of the indicated cytokines was observed. Indeed, incubation of ECs during 3h with cell-free supernatant collected from pure ILCPs at the end of the *in vitro* expansion did not provoke upregulation of adhesion molecules on EC surface (**Supplementary Fig. 3c**) correlating with the very low amount of the pro-inflammatory cytokines as shown in **Supplementary Fig. 3b**. Overall, these data suggest that ILCPs are superior to other ILC subsets in inducing the upregulation of adhesion molecules on ECs, and can also favor the release of pro-inflammatory cytokines, primarily in a contact-dependent manner.

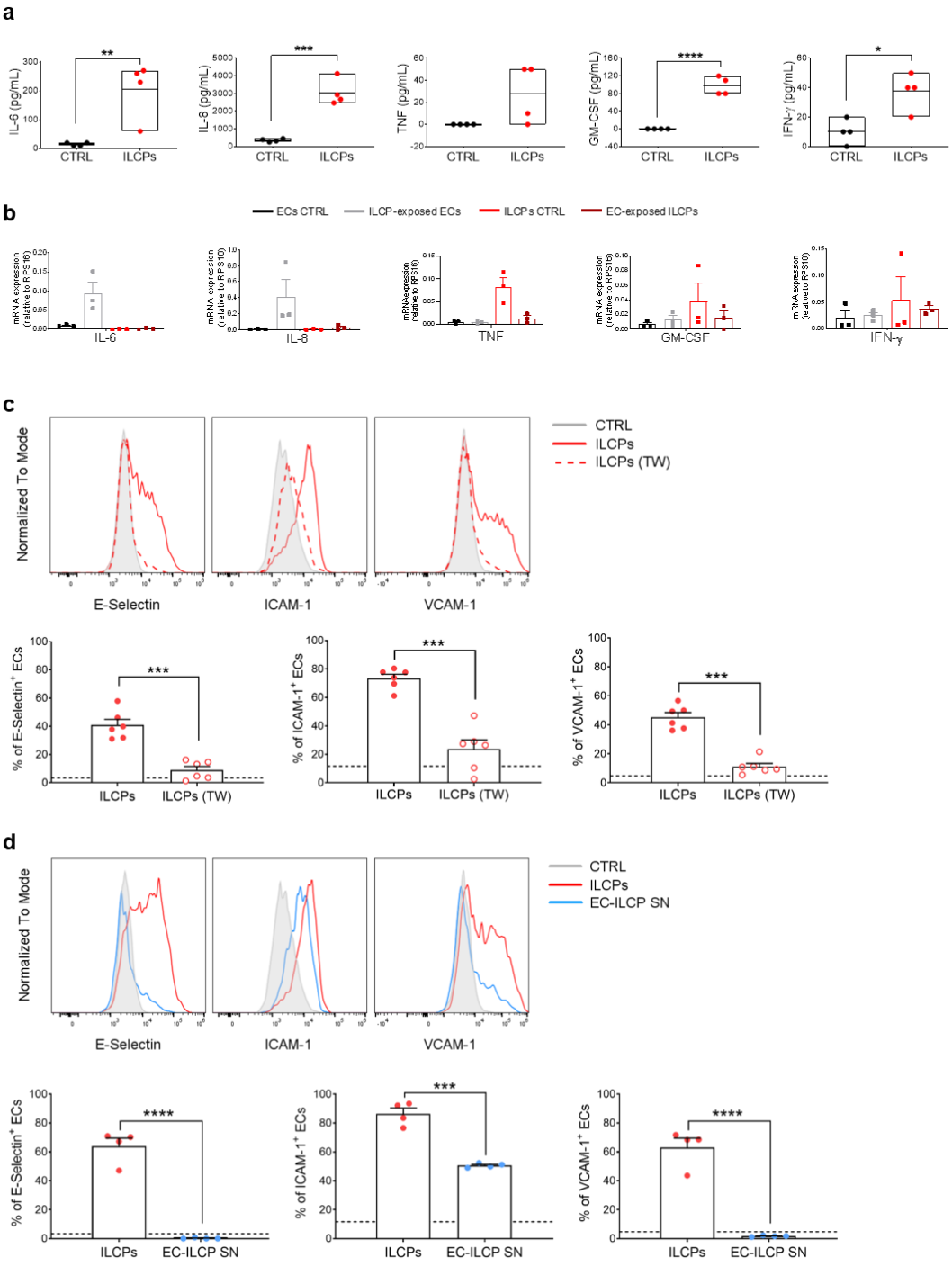
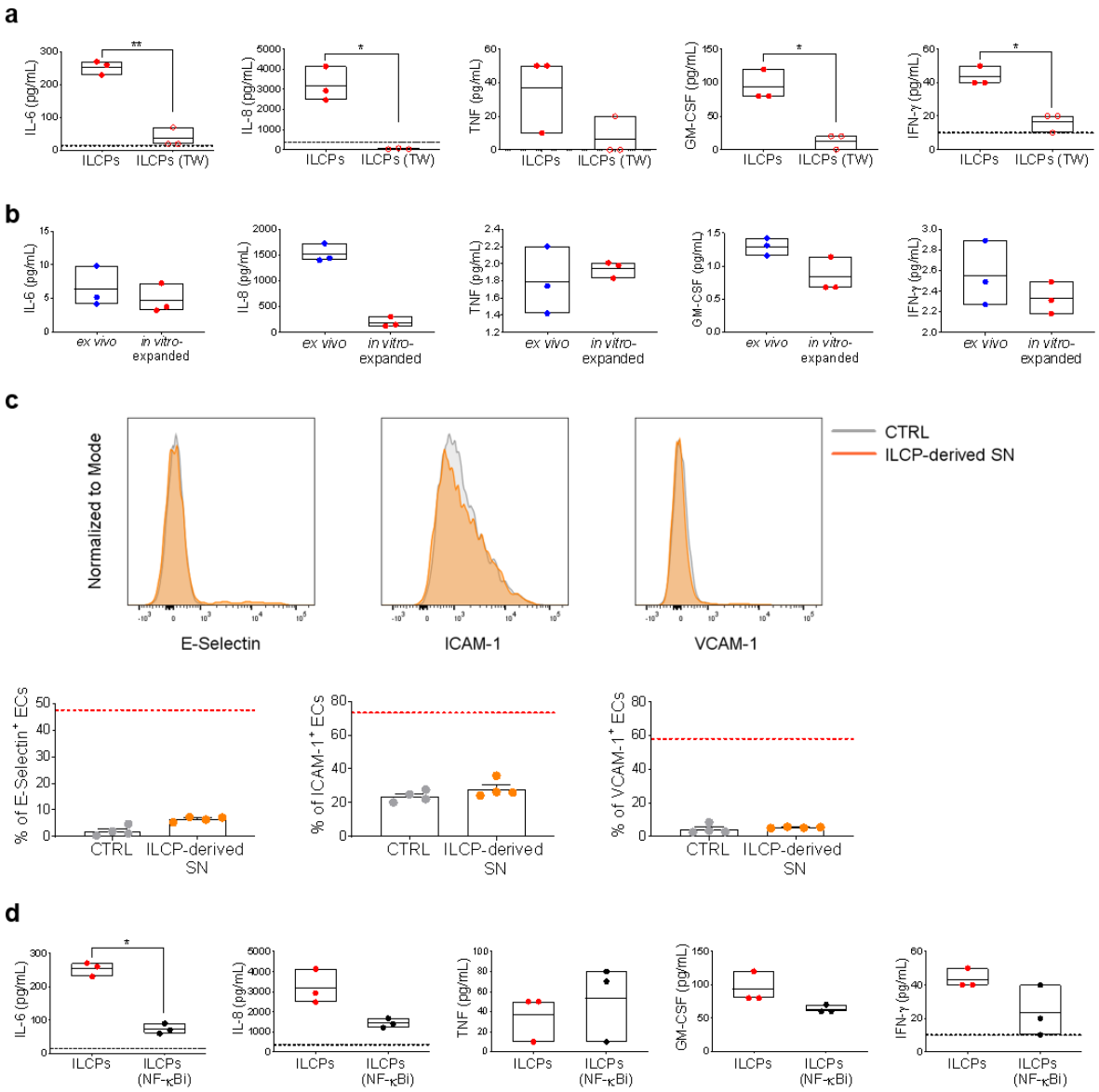


Figure 2. Human ILCPs activate ECs primarily in a contact-dependent mechanism *in vitro*. (**a**) The supernatant of the 3h co-culture experiments between ECs and ILCPs was analyzed for its cytokine contents (n=4). The composition of the supernatant of ECs in EC growth medium was used as negative control (CTRL). (**b**) The expression of IL-6, IL-8, GM-CSF, TNF and IFN-γ was analyzed by qPCR in ECs and ILCPs after being cultured for 3h at 1:1 ratio and FACS-sorted according to CD31 expression. Untreated ECs and ILCPs were employed as controls (CTRL). (**c**) HUVEC cells were co-cultured for 3 h at 1:1 ratio in direct contact with *in vitro*-expanded ILCPs either in the absence (red dots) or presence (red circles) of a transwell (TW) insert (0.4µm pore polycarbonate filter) or (**d**) in the presence of pre-conditioned media coming from previous EC-ILCP 3h co-cultures. ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry (n=6). The dotted lines indicate the level of average expression of adhesion molecules by unstimulated ECs. Statistical tests used: Unpaired t test (panels **a, d**); paired t test (panel **c**).



Supplementary Figure 3. Human ILCPs activate ECs primarily in a contact-dependent manner *in vitro*. The supernatant of the 3h co-culture experiments between ECs and ILCPs, with (ILCPs TW) and without transwell (ILCPs) (**a**), and with (ILCPs NF-κBi) or without (ILCPs) NF-κB pre-inhibition in ECs (**d**) was analyzed for its cytokine content (n=3). EC growth medium was used as negative control (black dotted lines). (**b**) The supernatant of *ex vivo*-sorted ILCPs, cultured for 24h in the presence of 100U/mL of IL-2 in RPMI 8% HS, and of ILCPs at the end of the *in vitro* expansion protocol was analyzed for its cytokine content (n=3). (**c**) HUVEC cells were co-cultured for 3h in the presence of pre-conditioned media coming from previous ILCPs at the end of the expansion protocol. ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry (n=4). The red dotted lines indicate the level of average expression of adhesion molecules by ILCP-exposed ECs. Statistical test used: Paired t test (panels **a**,**c**,**d**): Unpaired t test (panel **b**).

6.4.3. ILCPs engage the NF-κB pathway in ECs

It has been shown that adhesion molecule expression can be induced in ECs during inflammatory responses by the activation of different signaling pathways, among which the NF-κB pathway (283). To test whether the induction of adhesion molecules by ILCPs was dependent on NF-κB, ECs were pre-treated during 1h with a IκB kinase (IKK) complex inhibitor (BAY 11-7082)(284) to specifically prevent NF-κB activation. In this context, ILCPs failed to significantly induce the expression of adhesion molecules on pre-treated ECs (**Fig. 3a**), indicating that ILCPs need to engage the NF-κB pathway to activate ECs *in vitro*. Similar to what we observed in the context of ILCPs cultured with ECs in the presence of a transwell insert, the prevention of NF-κB activation in ECs led to a significant decrease of IL-6, as well as reduction in IL-8, GM-CSF and IFN-γ secretion (**Supplementary Fig. 3d**). Next, to understand which molecular players were involved in the ILC-EC cross-talk, we screened ECs and, both *ex vivo* and *in vitro*-expanded, ILCPs for the presence on their surface of receptors and ligands, respectively, known to be involved in the NF-κB pathway activation. On one side, we observed that untreated ECs constitutively expressed the LT-β receptor (LT-βR), as well as the TNF receptors 1 and 2 (TNFR-1 and TNFR-2, respectively), whereas B-cell activating factor receptor (BAFF-R), CD40 and RANK were expressed only at low levels (**Fig. 3b**). Following stimulation with TNF, CD30 expression became detectable and BAFF-R and RANK expression increased, while CD40 and LT-βR expression remained unchanged (**Fig. 3b**). On the other side, when looking at extracellular NF-κB activating ligands on *ex vivo* ILCPs, we observed that they expressed high levels of the transmembrane form of LT ($LT\alpha_1\beta_2$), a described ligand for LT-βR (285), if compared to *in vitro*-expanded ILCPs (**Fig. 3c-d**). Both BAFF and CD30L were undetectable and low levels of CD40L and RANKL were observed. In contrast, *in vitro*-expanded ILCPs upregulated the expression of RANKL and downregulated that of $LT\alpha_1\beta_2$ (**Fig. 3c-d**). It has been reported that pro-inflammatory cytokines, such as IL-12, can induce RANKL on human periodontal ligament cells *in vitro* (286). Since it is known that feeder cells can produce a wide array of cytokines, among which IL-1β and IL-12, we decided to test whether RANKL expression might be upregulated by one of these factors. Surprisingly, after 24h stimulation of freshly *ex vivo* isolated ILCPs with IL-1β (**Fig. 3e**), but not with IL-12 (data not shown), we observed increased expression of RANKL compared to untreated ILCPs. The transmembrane form of TNF (tm-TNF) constitutes another described NF-κB activating ligand. However, the detection of the membrane-bound form of TNF could not be tested due to the lack of a specific antibody. Moreover, the discrimination between the soluble and the membrane forms of TNF at mRNA levels is not possible, since TNF is transcribed (and also translated) as a full-length membrane-bound precursor (287). However, at the end of the *in vitro* expansion ILCPs showed higher levels of TNF transcripts compared to *ex vivo* ILCPs (data not shown). Overall, these data show that

in vitro-expanded ILCPs express TNF, possibly present on the ILCP surface, to *in vitro* interact with ECs via TNFRs and upregulate RANKL expression, possibly via feeder-cell-derived IL-1β, to engage RANK on ECs.

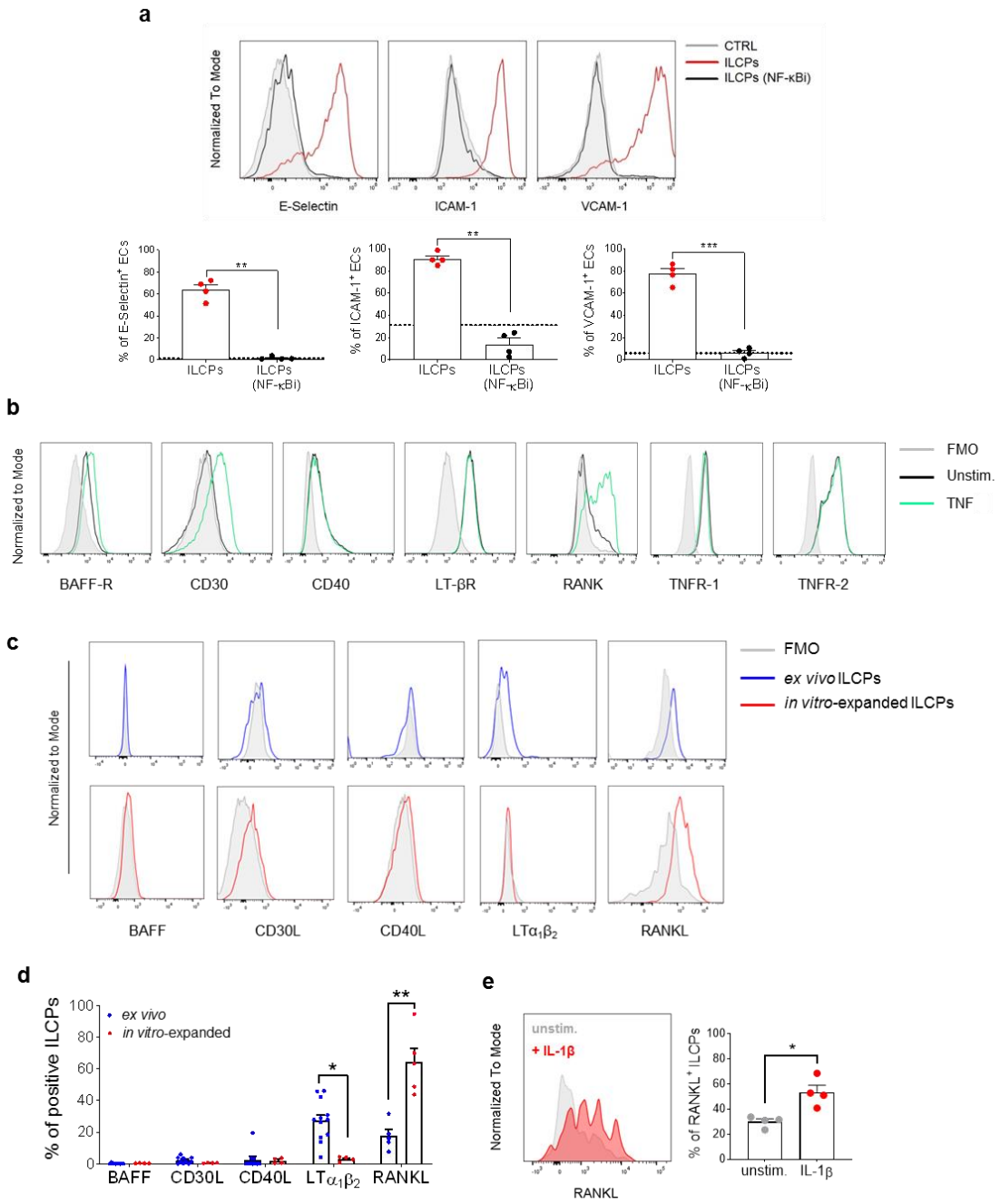


Figure 3. ILCPs induce adhesion molecules expression on the EC surface via NF-κB pathway activation. (**a**) HUVEC cells were treated during 1h with 2.5µM of a specific inhibitor of both canonical and alternative NF-κB pathways (BAY 11-7082, Adipogen) and then exposed to ILCPs at 1:1 ratio for 3h. ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry (n=4). The black dotted line indicates the level of average expression by untreated ECs. (**b**) HUVEC cells were tested for the expression of NF-κB activating receptors, either at steady-state (black line) or following 3h *in vitro* stimulation with 20ng/mL of TNF (green line). (**c-d**) The respective activating ligands were analyzed on both *ex vivo* and *in vitro*-expanded ILCPs. Graphs show representative histograms (panel **c**) and the summary (panel **d**) of the analysis performed on HDs (n=4-11). (**e**) *In vitro*-expanded ILCPs were stimulated during 24h in the presence of 20ng/mL of IL-1β or left untreated and stained for surface RANKL (n=4). Statistical tests used: Paired t test (panels **a**,**e**); Multiple t tests (panel **d**).

6.4.4. ILCPs activate ECs via the engagement of TNFR and RANK

To test which of the NF-κB activating molecules was responsible for the upregulation of adhesion molecules on EC surface, a series of blocking experiments using different soluble Fc fusion proteins were performed, to prevent the binding of defined ligands to their receptors on ILCPs. Since we observed increased levels of RANKL on *in vitro*-expanded ILCPs as compared to their *ex vivo* counterparts (**Fig. 3e**), and higher levels or RANK on ECs following 3h co-culture with ILCPs (**Supplementary Fig. 4a**), we decided to interfere with the RANK/RANKL interaction. As negative control, we performed the blocking experiments with intravenous immune globulins (IVIGs), a pool of human gamma globulins (**Supplementary Fig. 4b**). Although ILCPs were still able to activate ECs in this setting with yet an inhibition of E-Selectin triggering in ECs (**Fig. 4a**), we observed that the levels of IL-6 and GM-CSF were dramatically reduced, if compared to the cytokine composition of ECs cultured with steady-state ILCPs (**Supplementary Fig. 4c**). Therefore, we hypothesized a major involvement of tm-TNF in the induction of adhesion molecules. Thus, we pre-incubated ILCPs in the presence of TNFR1:Fc and/or TNFR2:Fc and we observed that the EC expression of adhesion molecules was significantly reduced (**Fig. 4b**). In all cases, inhibition with TNFR2:Fc was slightly more efficient than with TNFR1:Fc, which could be explained by the greater affinity of TNFR2 for TNF (288). Of note, no difference in the cytokine secreted levels was observed (**Supplementary Fig. 4d**), suggesting that interfering with the TNF-TNFR signaling does not impact cytokine production in both cell types. Addition of RANK:Fc to TNFR1:Fc and TNFR2:Fc further slightly reduced E-Selectin, ICAM-1 and VCAM-1 levels, although the contribution of RANK:Fc was not significant (**Fig. 4c**). However, we could observe a decreased production of the pro-inflammatory cytokine IL-6, IL-8, TNF and GM-CSF (**Supplementary Fig. 4e**) when blocking ligands of TNFR1, TNFR2 and RANK in ILCPs/ECs co-cultures. Taken together, our data suggest that ILCPs activate EC primarily through the engagement of TNFRs to upregulate adhesion molecules expression on EC surface. The engagement of RANK in ECs does not seem to have an additive effect in inducing adhesion molecules expression, but might act in synergy with tm-TNF to control the cytokine secretion and further support the EC activation.

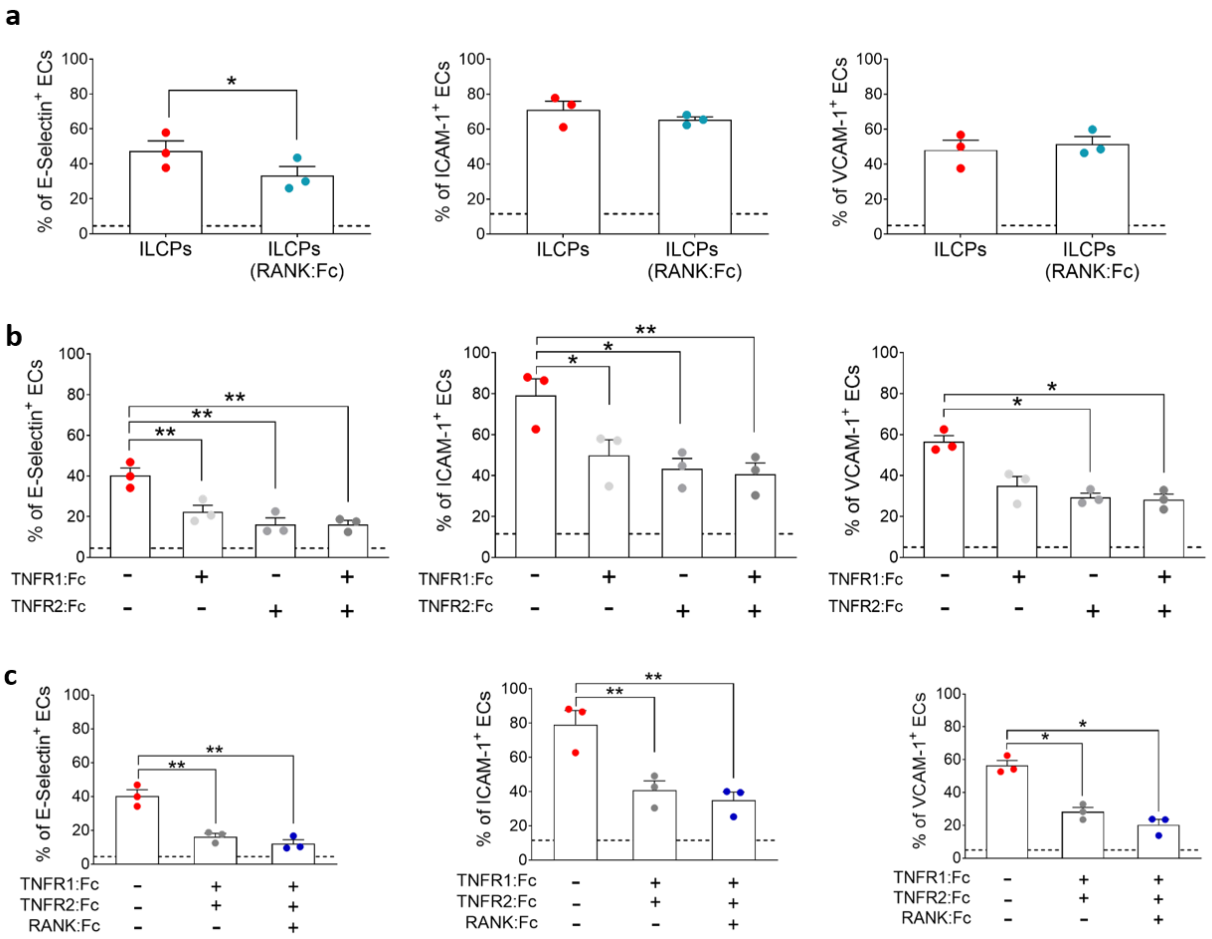
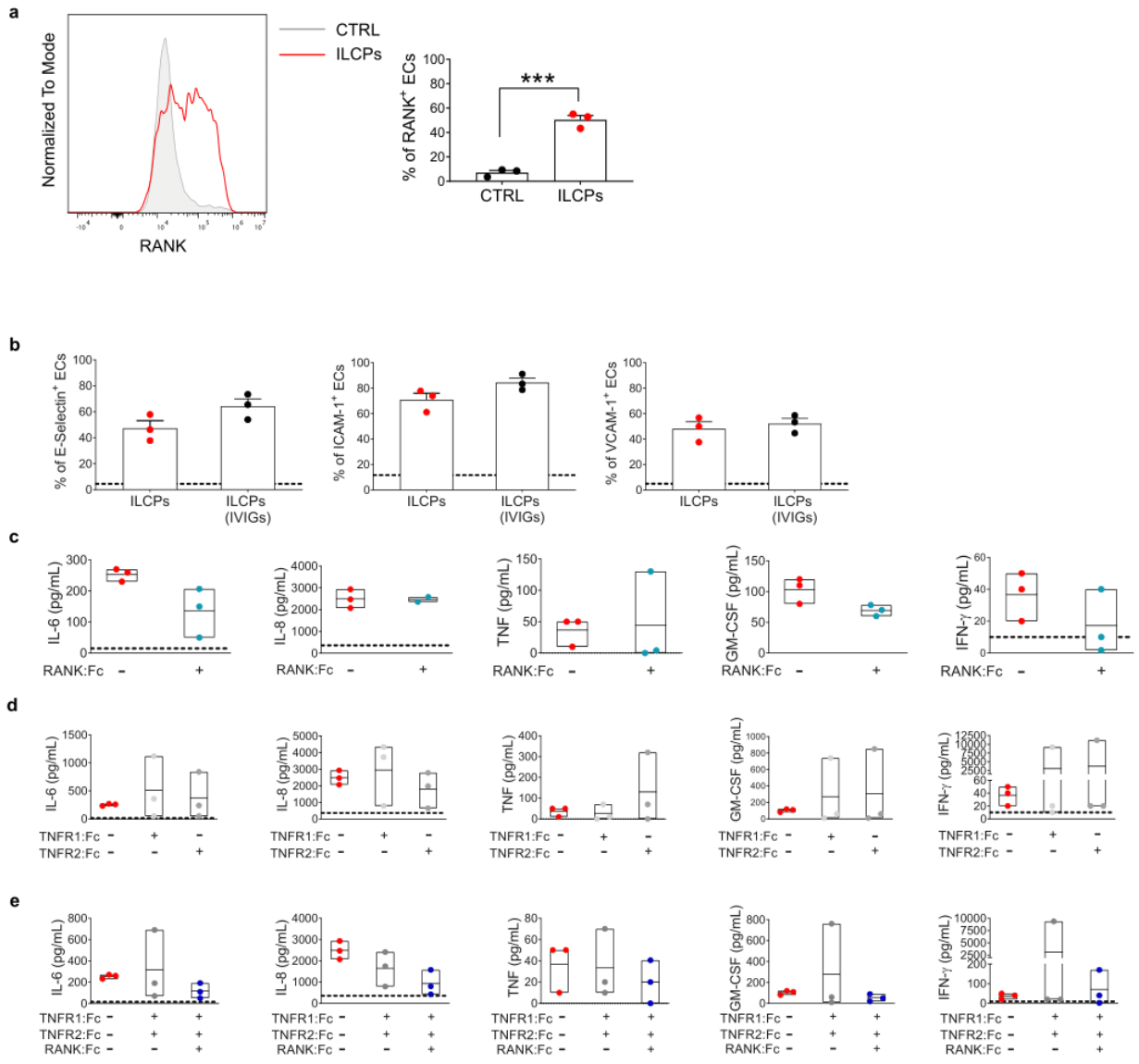


Figure 4: ILCP-mediated upregulation of adhesion molecules on ECs involves the engagement of TNFR1, TNFR2 and RANK. ILCPs were incubated overnight in the presence of 10U/mL of rhIL-2 and an additional pre-incubation of 30min (prior co-culture with ECs) was performed in the presence of 5µg/mL of RANK:Fc (**a**), of 2µg/mL of TNFR1:Fc, 5µg/mL of TNFR2:Fc, and 5µg/mL of RANK:Fc, either alone or in combination (**b,c**). ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry (n=3). The dotted lines indicate the level of average expression of adhesion molecules by unstimulated ECs. Statistical test used: Paired t test.



Supplementary Figure 4: ILCP-mediated modulation of RANK expression on EC surface. (**a**) HUVEC cells were co-cultured for 3 h at 1:1 ratio in direct contact with *in vitro*-expanded ILCPs. Untreated ECs were employed as negative control (CTRL). ECs were harvested and analyzed for cell-surface RANK expression by flow cytometry. Graphs show a representative histogram (left) and the summary (right) of the induction of RANK expression on the EC surface by ILCPs isolated from 3 different donors. (**b**) ILCPs were incubated for an overnight in the presence of 10U/mL of rhIL-2 and an additional pre-incubation of 30min (prior co-culture with ECs) was performed in the presence of 2µg/mL of IVIGs, or left untreated. ECs were harvested and analyzed for cell-surface adhesion molecule expression by flow cytometry (n=3). The dotted lines indicate the level of average expression of adhesion molecules by unstimulated ECs. (**c**, **d, e**) The supernatant of the 3h co-culture experiments between ECs and ILCPs pre-incubated with Fc fusion proteins were analyzed for cytokine content (n=3). The dotted lines indicate the average level of cytokines produced by unstimulated ECs. Paired t tests.

6.4.5. ILCP-mediated EC activation favors the adhesion of freshly isolated PBMCs *in vitro*

To address the functionality of the EC-ILCP interaction, i.e., the adhesion of freshly isolated PBMCs to ILCP-exposed EC, a static adhesion assay was performed. Briefly, following the 3h co-cultures, $CD31^+$ ECs were isolated by FACS, to remove adherent ILCPs, and re-plated. After the sorting, untreated ECs (negative control) did not upregulate adhesion molecule expression on their cell surface, and ILCP-exposed ECs maintained comparable surface levels of adhesion molecule as before the FACS isolation procedure, showing that the sorting procedure did not affect the activation state of ECs in any of the conditions (**Fig. 5a**). The day after, the assay was performed and ECs, together with adherent PBMCs, were detached and stained for flow cytometry analyses. Interestingly, ECs pre-exposed to ILCPs led to the adhesion of a significantly higher number of freshly isolated PBMCs compared to unstimulated ECs. As shown in **Fig. 5b-c**, the ILCP modification of EC allowed a strong adhesion of T, B as well as NK cells and monocytes. To understand if the adhesion of freshly isolated PBMCs is itself dependent on NF-kB, we repeated the experiment by exposing untreated or NF-κB-inhibited ECs to TNF for 3h the day before performing the static adhesion assay. As shown in **Fig. 5d**, the inhibition of NF-κB activation prior stimulation with TNF strongly reduced the numbers of adhered T, B, NK cells and monocytes. In this setting, we could also observe that ILCs themselves could adhere to TNF-treated ECs (**Fig. 5d**). Interestingly, a trend for a reduction in the number of adhered PBMCs to ECs was also observed when NF-κB activation was prevented in ECs 30min before performing the static adhesion assay (**Fig. 5d**) although not significant. Since we showed that NF-κB engagement is crucial for the ILCP-mediated adhesion molecule upregulation in ECs (**Fig. 3a**), it was not surprising to observe the impaired adhesion of PBMCs to ECs *in vitro*. Overall, these data suggest that the adhesion molecule expression induced by the ILCPs is functional, i.e., it supports the adhesion of other immune cell types to ECs *in vitro*, and relies on NF-κB activation.

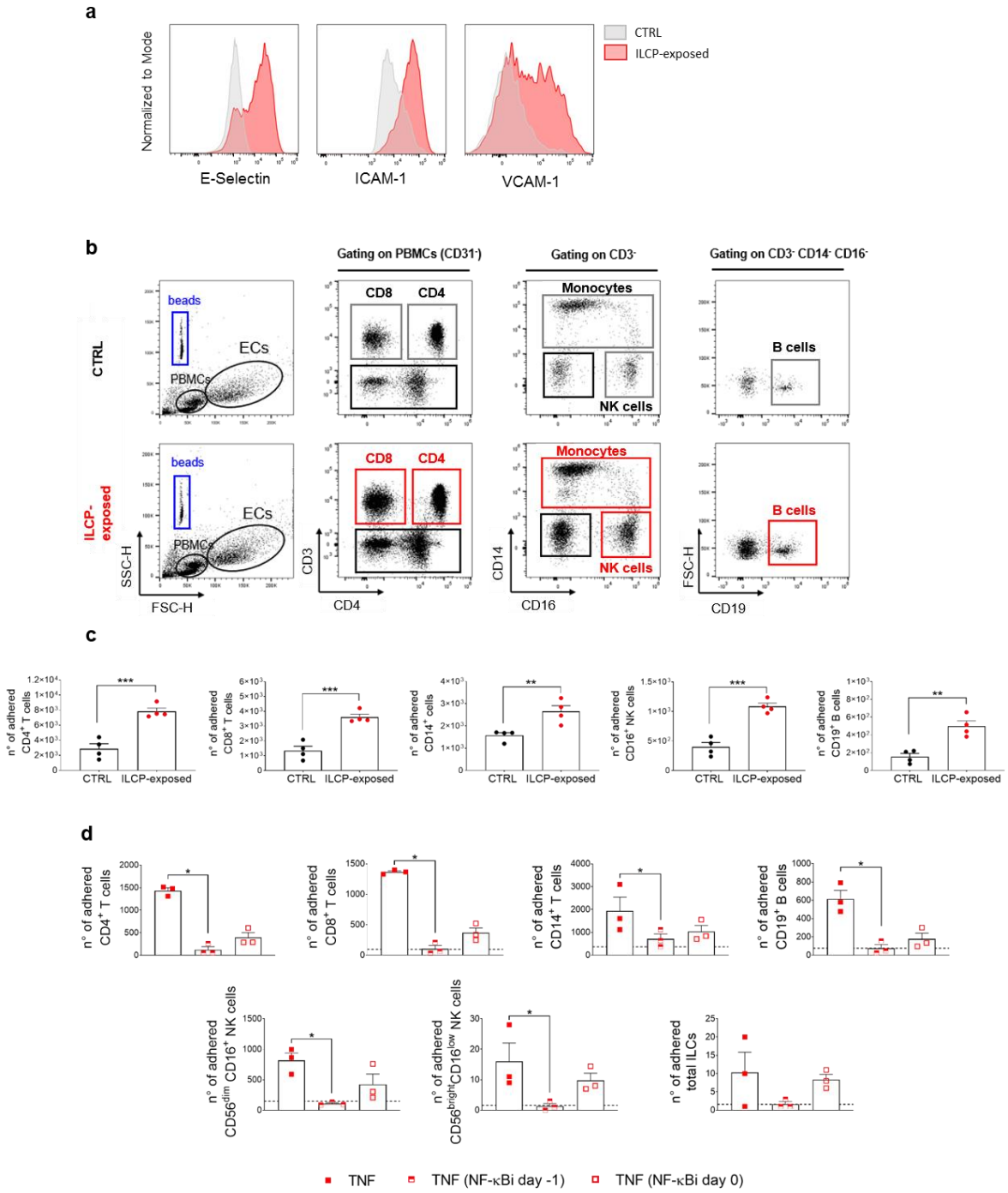


Figure 5. ILCP-exposed ECs favor the adhesion of freshly isolated PBMCs *in vitro*. HUVEC cells were co-cultured for 3 h at 1:1 ratio in direct contact with *in vitro*-expanded ILCPs or left untreated (CTRL). ECs were harvested, FACS isolated to remove adhered ILCPs, and re-seeded. (**a**) The graphs show the level of expression of adhesion molecules by ILCP-exposed ECs after the sorting and before performing the static adhesion assay, compared to untreated ECs (gray). Graphs show representative dot plots (**b**) and the summary (**c**) of the number of adhered CD3, CD4, CD8, CD14, CD16 and CD19 expressing cells assessed by flow cytometry with the use of CountBright Absolute Counting Beads (blue gate in the dot plots). (**d**) The day before the assay, HUVEC cells were cultured for 3h in the presence of 20ng/mL of TNF and treated during 1h with 2.5µM NF-κB inhibitor BAY 11-7082 (Adipogen), either before the TNF treatment (half-full red square dots) or directly on the day of the assay (empty red square dots), before incubation with total PBMCs at 1:4 ratio for 30min. The graphs show the summary of the number of adhered CD3, CD4, CD8, CD14, , $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16^{low}$, CD19 expressing cells and ILCs assessed by flow cytometry with the use of CountBright Absolute Counting Beads. Statistical test used: Unpaired t test (panels **c**,**d**).

6.4.6. Tumor-derived factors impair ILCP ability to activate ECs *in vitro*

The poorly functional and altered structural organization of the vascular bed has an important impact on tumor progression and affects endothelial-leukocyte interactions (240). Hence, we were interested in studying the impact that the tumor and/or the TME could exert on ILCPs and, therefore, on their ability to modulate the EC activation. First, we observed that $CD3^{-}RORγt^{+}$ ILCs are present in low-grade transitional bladder carcinoma in close proximity to $CD31^{+}$ blood vessels (**Fig. 6a, panels 1-4**) but are barely detected in high-grade bladder carcinoma (**Fig. 6a, panels 5-8**). Interestingly, since we also observed that ILCPs are expanded in the PB of NMIBC patients, but reduced in muscle-invasive stage of the disease (MIBC) (**Fig. 6b**), and, following *in vitro* expansion, ILCPs acquire RORγt expression, we hypothesized that the presence of ILCPs in NMIBC patients might underline the attempt of this cell population to support the infiltration of immune cells into the tumor site. To this aim, ILCPs were pre-exposed to human bladder cancer cell lines, originating either from non-muscle invasive (early stage) or muscle invasive (late-stage) epithelial bladder carcinoma, thus allowing us to mimic *in vitro* early and late tumor stage conditions. As shown in **Fig. 6c**, the capacity to upregulate adhesion molecule expression on ECs by ILCPs was significantly reduced after the overnight incubation with bladder carcinoma cell lines, if compared to resting ILCPs. Interestingly, the co-culture with MIBC lines showed the highest capacity to modify ILCP ability to activate the ECs (**Fig. 6c**). Moreover, the analysis of the cytokine composition of the supernatants from 3h EC-ILCP co-culture revealed statistically significant reduced levels of the pro-inflammatory cytokines IL-8, GM-CSF and IFN-γ when ECs where co-cultured with MIBC pre-exposed ILCPs (**Fig. 6d**). Similar observations were obtained using tissue sections of colon adenocarcinoma patients and the SW1116 colon cancer cell line (**Supplementary Fig. 5a-b**). To further understand which could be the mechanisms underlying the tumor cell-mediated impairment of ILCPs, we considered adenosine and/or kynurenine pathways, known for their potent immune-inhibitory effects in the TME (289)(290). As shown in **Fig. 6e**, TCC-Sup did not express IDO-1, suggesting that the tumor-mediated effects on ILCPs might not depend on kynurenines. However, following the overnight incubation with ILCPs, TCC-Sup strongly upregulated CD39 and further increased the CD73 expression (**Fig. 6e**). Interestingly, steady-state ILCPs also expressed CD39, but very low levels of CD73 (**Supplementary Fig. 5c**), suggesting that, in the presence of $CD73^{+}$ cells, ILCPs might process ATP and support adenosine production. Interestingly, as shown in **Supplementary Fig. 5d**, *in vitro*-expanded ILCPs upregulated the expression of A2A, A2B and A3 receptors. Of note, pre-exposure of ILCPs to 2-Chloroadenosine (a stabilized form of adenosine) reduced their EC-activating capacity (**Fig. 6f**). Taken together, these results suggest that tumor cells might impair or deviate, at least in part via adenosine production, the capacity of ILCs to modulate vascular activation through the upregulation of cell surface

adhesion molecules, and affect the production of pro-inflammatory cytokines upon EC-ILCP encounter. Therefore, this could represent a mechanism through which tumors can prevent and block immune cell infiltration into the tumor site.

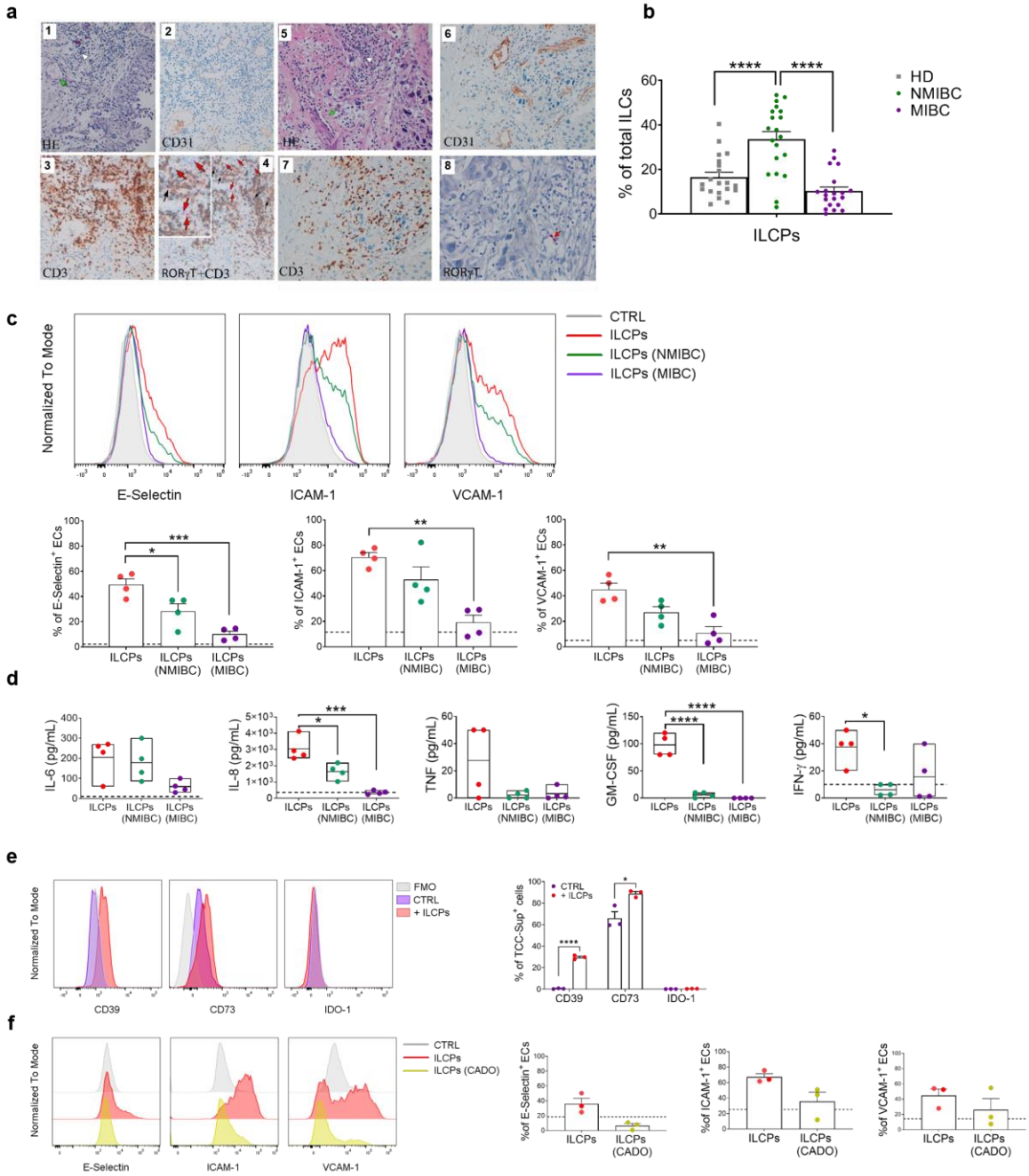
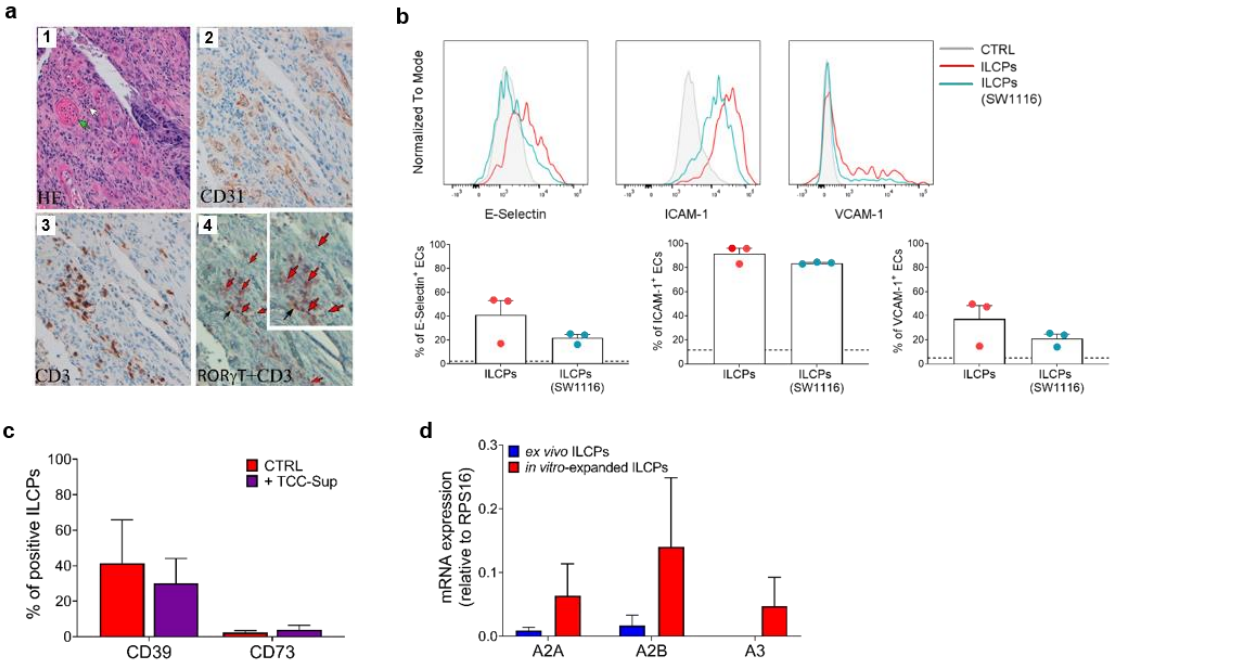


Figure 6. ILCPs are found in proximity of blood vessels in low-grade, but barely detected in high-grade, bladder cancer tumor samples and are functionally impaired by co-cultures with bladder carcinoma cells. (**a**, panels **1**-**4**) Low-grade transitional bladder cell carcinoma. (**a**, panel **1**) In the subepithelial connective, blood vessels (green arrow) and inflammatory lymphocytic infiltrate are observed (white arrow) (hematoxylin-eosin staining, 20x magnification). (**a**, panel **2**) Immunohistochemical CD31 signal showing intense positive ECs of blood vessels (20x magnification). (panel **3**) Immunohistochemical detection of $CD3^+$ cells at level of inflammatory lymphocytic infiltrate (brown signal) (20x magnification). (**a**, panel **4**) Combined staining with antibody to RORγt and CD3. Black arrows indicate $ROR\gamma t^+/CD3^+$ cells; red arrows indicate $ROR\gamma t^+/CD3^-$ cells (red signal) (20x magnification). On the

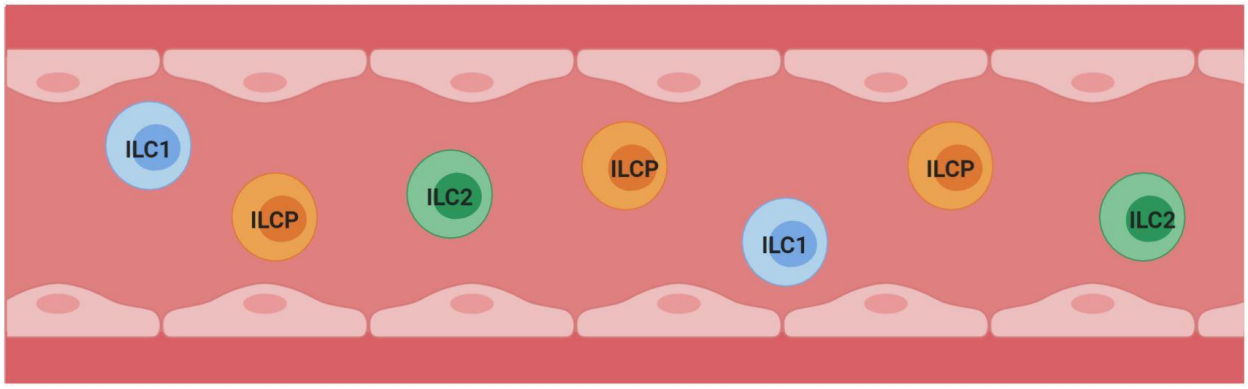
upper left a magnified insert of the main image. Data are representative of five independent experiments. (**a**, panels **5**-**8**) High-grade bladder cell carcinoma. (**a**, panel **5**) In the subepithelial connective, blood vessels (green arrow) and inflammatory lymphocytic infiltrate are observed (white arrow) (hematoxylin-eosin staining, 20x magnification). (**a**, panel **6**) Immunohistochemical CD31 signal showing intense positive ECs of blood vessels (20x magnification). (**a**, panel **7**) Immunohistochemical detection of $CD3^+$ cells at level of inflammatory lymphocytic infiltrate (brown signal) (20x magnification). (**a**, panel **8**) Immunohistochemical detection of $ROR\gamma t^+$ cells at level of inflammatory lymphocytic infiltrate (red signal, red arrow) (20x magnification). Data are representative of three independent experiments. (**b**) Flow cytometry characterization of ILCP distribution in the PB of NMIBC and MIBC patients, compared to HDs, expressed as percentage of total ILCs (n=20). (**c**) Graphs show representative histograms (panel **c**, top) and the summary (panel **c**, bottom) of the induction of adhesion molecules by ILCPs upon different culture conditions, represented as percentage of ECs expressing the indicated adhesion molecules. The dotted lines represent the level of expression of the adhesion molecules in untreated ECs (n=4). (**d**) The supernatants of the 3h co-culture experiments between ECs and ILCPs, pre-incubated or not for an overnight with bladder carcinoma cell lines, were analyzed for cytokine content (n=4). The dotted lines indicate the average level of cytokines produced by unstimulated ECs. (**e**) The expression of CD39, CD73 and IDO-1 in MIBC cells (TCC-Sup) after overnight co-culture with *in vitro*-expanded ILCPs was assessed by flow cytometry. Untreated TCC-Sup cells (purple bar) were used as controls (CTRL) (n=3). (**f**) Graphs show representative histograms (panel **f**, left) and the summary (panel **f**, right) of the induction of adhesion molecules by ILCPs pre-treated with 50μM of 2-Chloroadenosine (a stabilized form of adenosine), represented as percentage of ECs expressing the indicated adhesion molecules. The dotted lines represent the level of expression of the adhesion molecules in untreated ECs (n=3). Statistical tests used: Ordinary one-way ANOVA, Tukey's multiple comparison tests (panels **b**,**c** and **d**); Multiple t tests (panel **f**).



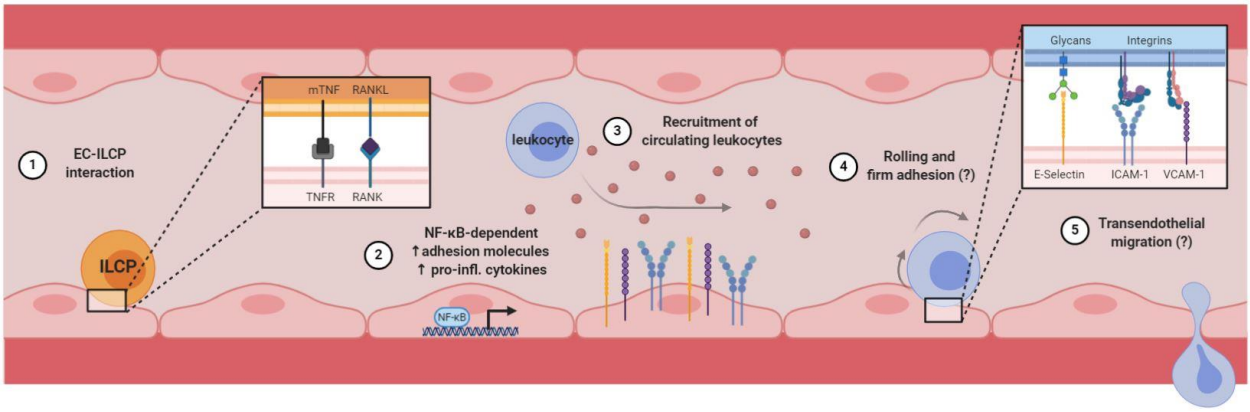
Supplementary Figure 5. ILCPs are found in proximity of blood vessels in low-grade colon adenocarcinoma and are impaired by co-cultures with colon adenocarcinoma cells. (**a**, panels **1-4**) Low-grade invasive colon adenocarcinoma. (**a**, panel **1**) In the stroma, blood vessels (green arrow) and lymphocytic inflammatory infiltrate (white arrow) are observed (hematoxylin-eosin staining, 20x magnification). (**a**, panel **2**) CD31 immunohistochemistry shows an intense signal in the ECs of blood vessels (20x magnification). (**a**, panel **3**) Immunohistochemical detection of $CD3^+$ cells at level of inflammatory lymphocytic infiltrate (brown signal) (20x magnification). (**a**, panel **4**) Combined staining with antibody to RORγt and CD3. Black arrows indicate $ROR\gamma t^+/CD3^+$ cells; red arrows indicate $ROR\gamma t^+/CD3^-$ cells (20x magnification). On the upper right a magnified insert of the main image. Data are representative of five independent experiments. (**b**) Graphs show representative histograms (top) and the summary (bottom) of the induction of adhesion molecules by steady-state ILCPs (red) or by ILCPs pre-exposed to SW1116 colon cancer cells (marine blue), represented as percentage of ECs expressing the indicated adhesion molecules. The dotted lines represent the level of expression of the adhesion molecules in untreated ECs (n=3). (**c**) The expression of CD39 and CD73 in *in vitro*-expanded ILCPs after overnight co-culture with TCC-Sup cells was assessed by flow cytometry. Untreated ILCPs (red bar) were used as controls (CTRL) (n=3). (**d**) The expression of adenosine receptors A2A, A2B, and A3 was analyzed by qPCR in *ex vivo* and in *in vitro*-expanded ILCPs (n=3).

Graphical abstract:

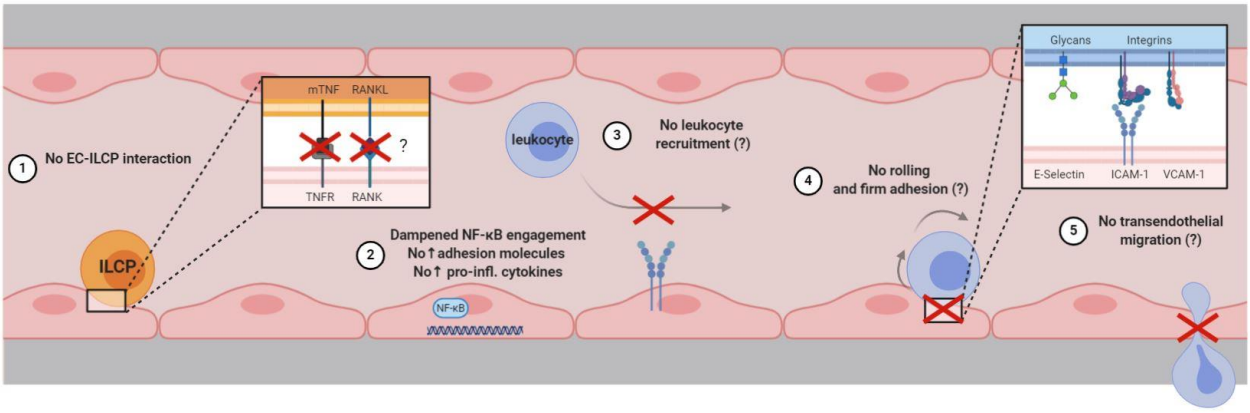
1) Steady-state: no ILC-EC interaction

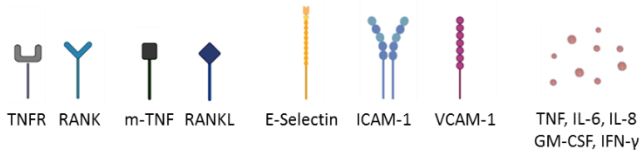


2) Pro-inflammatory setting(s) – functional EC-ILCP interaction



3) Immunosuppressive cancer setting(s) – dysfunctional EC-ILCP interaction





6.5. Discussion

In this study, we characterized for the first time the *in vitro* interaction between circulating human ILCs and vascular ECs. In particular we identify ILCPs as the only competent circulating ILC subset in inducing EC activation through the upregulation of adhesion molecules on the EC surface. Our results are consistent with previously reported data showing that group 3 ILCs (defined as $Lin^{-}CD127^{+}NKp44^{+}$ cells) induce the expression of ICAM-1 and VCAM-1 on MSCs after 4 day co-culture and in the presence of IL-7 (120,291). According to recent findings, circulating ILCPs constitute a distinct subset from ILC3s, although they share the expression of c-Kit on their cell surface and are $CRTH2^{-}$(86). Following *in vitro* priming, the upregulation of RORγt, and the expression of activation markers argue for a conversion of ILCPs into committed ILC3-like cells, possibly supported by IL-1β and/or other factors secreted by feeder cells during the expansion phase. This environment mimics the *in vivo* dynamics observed during inflammatory processes driven by PAMP/DAMP/tumor-dependent DC activation.

However, differently from what was described by Lim *and colleagues* (86), the *in vitro* culture of ILCPs isolated from the PB of HDs did not lead to the expansion of neither ILC1s nor ILC2s, whereas only ILC3-like cells arose. Indeed, the *in vitro* stimulation applied in that context differs from our *in vitro* expansion protocol, with the lack of ILC1-, ILC2- or ILC3-specific cytokines. Overall, our findings support the idea that the *in vitro* expansion of circulating ILCPs in the presence of feeder cells, PHA and IL-2 favors their commitment towards an ILC3-like phenotype.

Interestingly, as far as adaptive immune cells are concerned, a previous publication showed that freshly isolated $CD4^{+}$ $CD45RO^{+}$ lymphocytes are able to induce, to different extents, the expression of VCAM-1 on ECs in a contact-dependent manner (292). We were unable to recapitulate these findings, most probably due to different culture conditions (isolation and *in vitro* expansion of T cells, timing and EC:T-cell ratios). For the innate counterpart, it was shown that the human NK cell line NK92 induces the expression of E-selectin and IL-8 in ECs, which results in EC activation, through the LT-dependent activation of the NF-κB pathway (293). Yet, in our system, we did not observe the upregulation of adhesion molecules when employing *in vitro*-expanded purified primary NK cells (data not shown).

In this work, we define the ILCP-mediated activation of ECs primarily as a contact-dependent mechanism. However, we cannot exclude that pro-inflammatory cytokines (IL-6, IL-8, GM-CSF, IFN-γ and TNF), produced during the EC-ILCP interaction, might contribute to the observed EC activation. It is known that pro-inflammatory cytokines, and especially TNF, constitute potent inducers of adhesion molecule expression in ECs (282). However, in our hands, ECs upregulate adhesion molecules expression only when short-term exposed to TNF, but not to the other cytokines (data not shown). Moreover, the exposure of

ECs to cell-free supernatant recovered from foregoing EC-ILCP co-culture only provoked a significant upregulation of ICAM-1 on ECs, and exposure of ECs to cell-free supernatant collected at the end of the *in vitro* expansion of ILCPs did not upregulate adhesion molecules on ECs, supporting the idea of a primarily contact-mediated interaction between these two cell types. However, it cannot be excluded that the pro-inflammatory cytokines that are produced during the co-culture can support, at the cell-cell contact region, the *in vitro* cross-talk.

Upon interaction, ILCPs possibly engage the NF-κB pathway in ECs via TNFR/tm-TNF and RANK/RANKL interactions, eventually acting in synergy. RANKL has been recently described as a negative regulator of $CCR6^+$ILC3s activation and cytokine production, via the paracrine interaction with its receptor RANK (294). On one side, we observed that the expression of RANK on *ex vivo* ILCPs was not detectable, whereas *in vitro*-expanded ILCPs acquire transient, intermediate levels of RANK after expansion (data not shown). Nevertheless, the contribution of RANKL to EC activation needs further investigation, as well as the formal evaluation of tm-TNF on ILCP surface. Of note, we observed higher levels of transcripts in *in* vitro-expanded ILCPs compared to their *ex vivo* counterparts, but very low levels of soluble TNF at the end of the expansion, suggesting that TNF might be present on the surface of expanded ILCPs. We might speculate that a sequential engagement of these ligand-receptor interactions occurs in the EC-ILCP interface, with initial tm-TNF/TNFR interactions that are needed to induce adhesion molecules expression, together with increased RANK expression in ECs. This could facilitate the sequential RANKL/RANK interactions, possibly required to support the production of pro-inflammatory cytokines, since the prevention of both TNFR/RANK engagement resulted in impaired cytokine production.

By performing a static adhesion assay, we show that the ILCP-mediated EC activation is functional. Therefore, ILCPs might favor the initial tethering of circulating immune cells to vascular ECs via E-Selectin induction, and the subsequent ICAM-1/LFA-1 and VCAM-1/VLA-4-mediated firm adhesion step, and support the EC-dependent recruitment of other immune cell types, thus facilitating their exit from the blood stream through the vessel wall.

As previously reported (227), $NKp46^+$ ILCs were described to be crucial, in a subcutaneous melanoma mouse model, for the establishment of an IL-12-dependent anti-tumor immune response. A similar role was proposed for $NKp44^+$ ILC3s in NSCLC patients (226). Beside their putative role in supporting intratumoral TLS formation, an aspect that has been further recently supported in colorectal cancer patients (Ikeda *et al.*, in press), these cells were suggested to activate tumor-associated ECs and, in turn, favor leukocyte recruitment. Hence, ILCP-EC interactions might represent an early event during a large spectrum of biological reactions, ranging from inflammation, autoimmunity and cancer. In tumors,

leukocytes have to travel across the vessel wall to infiltrate tumor tissue where they contribute to the killing of cancer cells. Further, the vessel wall serves as a barrier for metastatic tumor cells, and the integrity and the activation status of the endothelium serves as an important defense mechanism against metastasis' formation (240).

The infiltration of immune cells in solid tumors often correlates with a better overall survival in cancer patients (296)(297)(298). However, in the TME, ECs are dysfunctional and play a major role in several processes that contribute to cancer-associated mortality. One mechanism by which ECs can actively discourage the tumor homing of immune cells was described by Buckanovich *and colleagues* (299). By transcriptionally profiling the tumor ECs (TECs) isolated from ovarian cancer specimens poorly infiltrated by T cells, the authors describe a mechanism that relies on the interaction between ET_BR, found to be highly expressed by TECs, and its ligand endothelin-1 (ET-1), overexpressed in ovarian cancer cells. ET_BR signaling was shown to be responsible for the impaired ICAM-1-dependent T cell homing to tumors, and in turn, it correlated with shorter patient survival. Another mechanism that prevents T cell infiltration into the tumors relies on the overexpression of FasL on TEC surface (252), that causes the selective killing of tumor-specific $CD8^+$ T cells and to the accumulation of $FoxP3^+$ T regulatory (Tregs) cells within the tumors. Finally, it has been reported that Th1 cells can actively influence vessel normalization processes via the production of IFN-γ, which positively correlated with a more favorable outcome for cancer patients (268). Therefore, committed ILCPs might represent an additional key regulator of efficient immune cell penetration into the tumor.

Tumors can engage multiple mechanisms to discourage the establishment of anti-tumor immune responses (300). The shaping of an immunosuppressive milieu, together with the diversion of the vascular system supports tumor progression and favors metastatic dissemination (301). Here we show that RORγt-expressing ILCs infiltrate both human low-grade bladder and colon cancers and are in proximity to $CD31^+$ vessels, arguing for a potential ILC-EC interaction also *in vivo. In vitro*, we observed that the ability of ILCPs to induce adhesion molecules on ECs was dampened after the co-culture with bladder- and colon-derived tumor cells. ILCs are very plastic cells (77), and it has been reported that, in the cancer setting, tumor-derived TGF-β drives the transition of NK cells to dysfunctional and pro-tumoral ILC1s *in vivo*, a novel mechanism exploited by tumors to prevent the establishment of an innate anti-tumor response (135). One can speculate that a similar conversion also occurs for ILCPs towards a non-EC activating ILC subset. We showed that the mechanism of impairment of ILCPs might rely at least in part on adenosine. *In vitro*-expanded ILCPs express high mRNA levels of the adenosine receptors and CD39, whereas bladder cancer cells express CD73 and potentially also CD39. The presence of these two ectoenzymes, key for adenosine

production, suggest that adenosine might be produced during the co-culture between cancer cells and ILCPs and impact ILCP functions. Indeed, by pre-exposing ILCPs to 2-Chloroadenosine, we could observe reduced EC-activating ability.

In conclusion, our data show that ILCPs, upon proper stimulation, might represent novel players in regulating the trafficking of immune cells to tissues, not only during the early phase of inflammation, but also at early phases of anti-tumor immune responses. Such contact-mediated events may be crucial in supporting further EC activation, to favor tumor-specific T-cell adhesion and, in turn, recruitment to the tumor site.

Author contributions

GV, ST and CJ designed the study and wrote the manuscript. GV, GE and ST performed and analyzed the experiments. GV, ST and CJ performed statistical analyses. PS provided the Fc fusion proteins for the blocking experiments. LD provided the tumor cell lines. EM, SC, MR and ML performed the immunohistochemical stainings and analyses. CJ and ST jointly supervised the study. All authors provided intellectual input, critically revised and edited the manuscript.

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Disclosure of potential conflict of interest

The authors declare no conflict of interest.

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General Discussion and future perspectives

7. *In vivo* relevance

The presented work shows that *in vitro*-expanded ILCPs isolated from the peripheral blood of healthy individuals acquire EC-activating ability that can be altered by the tumor cells or the TME, suggesting the potential role of ILCPs in favoring immune cell infiltration into the tumor site.

7.1. *In vivo* relevance of our observations

Our labelling of tumor tissue sections argues that ILCP-vessels interactions occur *in vivo*. However, due to several experimental hurdles, we were not able to directly address the potential contribution of circulating ILCPs to anti-tumor immune responses *in vivo*. One could envisage to initially use mouse models to test the ability of murine ILCs to activate the endothelium. If our *in vitro* results using human ILCPs would be recapitulated also with murine ILCs, and in particular ILCPs/ILC3-like, the next step would rely on a mouse model lacking the equivalent of the human population. Even though the existence of circulating mouse ILCPs is still under debate, mouse ILC3s share the expression of the transcription factor RORγt with *in vitro*-expanded human ILCPs. Therefore, the targeting of RORγt-expressing cells in tumor-bearing mice might represent a good strategy to address these questions. However, there are several limitations in generating "ILC3-deficient" mouse models. Above all, RORγt-deficient mice show impaired secondary lymphoid tissue and thymocyte maturation, and they fail to generate LTi cells (44). Furthermore, the expression of RORγt by Th17 cells introduces major limitations in exploiting RORγt-deficient animals, since if any effect would be observed, it would involve both Th17 and ILC3 cells (302). Similarly, the pharmacologic or genetic targeting of AHR might be suboptimal, given its expression in ILC3 but also by Th17 cells (303–305). One possible strategy to exclusively deplete ILC3s could be the use of the *RORc(γt)^{Cre} Id2^{flox}* mouse models, a conditional ILC3 KO mice in which the normal fetal development of secondary lymphoid tissues is maintained (302,306). Attempts to generate ILC3 KO models have been made. However, since ILC3s are crucial in the maintenance of intestinal homeostasis, depletion of ILC3s in adult mice results in severe colitis, thus influencing the observations on tumor monitoring. In addition, Diphtheria toxin A (DTA)-mediated depletion models have also been described (302). Once entered in the cytoplasm, DTA leads to cell death by blocking protein translation. DTA expression can be conditionally induced from a floxed-STOP *Dta* gene inserted into the ubiquitously active Rosa26 locus (302). For example, the use of *Ncr1^{Cre} R26R^{Dta}* (94) or *RORc(γt)^{Cre} R26R^{Dta}* (unpublished) models enables the depletion of NKp46^{+} and RORγt^{+} ILC3s,

respectively. However, a common limitation of Cre-recombinase based methods is concealed in the efficacy of the target gene inactivation, that often results to be partial (302).

Considering that the ILCP-mediated activation of ECs *in vitro* primarily relies on the direct contact, and firstly on the engagement of TNFRs on ECs via binding to surface TNF on ILCPs, a strategy to demonstrate this mechanism *in vivo* would be to use knock-in mice with a non-cleavable TNF form (307). The tm-TNF knock-in mice were shown to normally develop and are able to control the acute phase response during *Mycobacterium tuberculosis* infection, thus defining a potential model without phenotypical nor functional defects. Moreover, the genetic deletion of the EC-activating molecule in murine ILC3s would be key in confirming the molecular mechanism(s) underlying the EC-ILC crosstalk *in vivo*. To that aim, one could use ILC3 KO animals reconstituted with genetically modified ILC3s, taking advantage of the ID2-Cas9 model (available in our laboratory), as a source of ILCs to be transduced *in vitro* with any guide RNAs of interest. Further, given the previously published data regarding mouse ILC-EC interaction *in vivo* in a subcutaneous melanoma mouse model (227) and the positive correlation observed between ILC3 NCR^+ presence in early stage NSCLC patients and TLS density (226), it will be interesting to validate our findings in melanoma and lung cancer models, as well as in bladder cancer models, with the use of either wild type and ILC3 KO animals, or animals harboring tm-TNF-deficient or tm-TNF knock-in ILC3s and/or TNFR-deficient ECs, which expression could be knocked-out with the use of short interfering RNA (siRNA)-based gene silencing techniques. As a read out, the activation state of the endothelium and the ILC3-mediated immune cell infiltration in the tumor bed would be monitored.

7.2. EC-ILCP interactions in cancer, and beyond?

Besides the cancer setting, the EC-activating ability of ILCPs might be crucial in other pathological contexts, for example in autoimmune diseases, in which dysregulated responses by the immune system mistakenly attack a functioning body part. In this regard, several groups reported an increase of Th17 cells and higher levels of IL-17, IL-22 (Th17- but also ILC3-related cytokines), IL-12, TNF and IL-1β in multiple sclerosis (MS) patients compared to healthy individuals (308). Although the brain is believed to be immunologically privileged, lymphocytes can infiltrate the brain parenchyma when the blood brain barrier is compromised (309). Interestingly, it was reported that ILC3s are increased in an experimental autoimmune encephalomyelitis (EAE) model (310), where they accumulated in the meninges and supported T cell survival and activation. The disease-induced trafficking of T cells to the meninges was impaired in *Rorc*$^{-/-}$, together with reduced EAE-susceptibility, suggesting that ILC3s might have an active role in MS pathogenesis. Whether and how the MS pathogenesis might be due to blood brain barrier-ILC3

interactions it is not yet known. Furthermore, the high levels of IL-12 in MS patients (308), a cytokine that was shown to recruit anti-tumor NKp46^{+} ILC3 cells to the tumor bed in a melanoma mouse model (227), raise the possibility that, also in MS, ILC3s could be recruited to the brain parenchyma in a IL-12-dependent manner, where the EC-activating ability of ILCPs might be in fact detrimental, thus favoring the infiltration of auto-reactive lymphocytes. Similar observations were also found in psoriasis, where dysregulation of RORγt-dependent ILC3s correlated with dermal microvascular changes, and in increased lymphocytic local infiltration (161).

Of note, our results show that IL-1β increases RANKL expression in ILCPs, and that the binding of RANKL with RANK-expressing ECs is important for inducing the pro-inflammatory cytokine production (i.e., IL-6, IL-8, GM-CSF, IFN-γ and TNF) to further support EC activation and lymphocyte recruitment. Interestingly, we also observed higher levels of RANKL on circulating *ex-vivo* ILCPs isolated from psoriatic patients as compared to healthy individuals (figure 7). However, IL-1β levels were reported as comparable between healthy individuals and psoriatic patients (311), suggesting that RANKL can be upregulated by other factors, for example by IL-8 as it was reported in osteoblastic stromal cells (312). Therefore, depending on the context, RANKL expression might be detrimental, as reported in other types of inflammatory diseases such as rheumatoid arthritis, a known IL-1β rich environment (313).

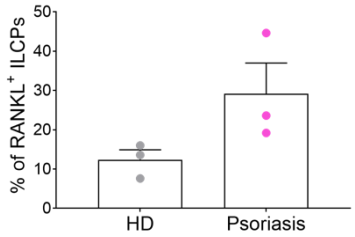

Figure 7: Expression of RANKL in *ex vivo* circulating human ILCPs in psoriatic patients compared to healthy donors (HD).

In cancer, we observed higher numbers of circulating ILCPs in early-stage BC patients, which correlate with the higher presence of RORγt^{+}CD3^{-} tumor-infiltrating lymphocytes (i.e., ILC3s). However, these cells where reduced in late-stage BC patients in favor of an ILC2 expansion and were not anymore detectable in the tumor immune infiltrate of patients in advanced disease stage. Given the increased levels of IL-4 in the serum of the patients compared to healthy individuals, and this particularly in late-stage BC patients, one could hypothesize that ILC3-to-ILC2 plasticity events may take place, also in light of the increased frequencies of ILC2s detected both in the blood and urine of these patients (207). From the RNAseq dataset generated in our lab on circulating ILCs and Th cell subsets, it emerges that ILCPs have high levels of *Il4r* transcripts (68). Since IL-4 is very well known to control the upregulation of GATA3 (314) and,

subsequently, to promote ILC2 identity, in the context of bladder cancer this might represent one mechanism of ILC3-to-ILC2 conversion, although it has not been demonstrated yet. Instead, as for Th cells, a Th1-to-Th2 and Th-17-to-Th2 conversion was observed *in vivo* during infection with *Nippostrongylus brasiliensis*, even though the mechanisms behind this conversion are not clarified (315). Therefore, according to the polarizing condition, Related to our findings, even though we did not observe downregulation of RANKL expression after overnight incubation with bladder cancer cells, we might speculate that the immunosuppressive TME might impair the EC-activating capacity of ILCPs. A thorough characterization of *ex vivo* patient ILCs, both circulating and tumor-infiltrating, at early and late disease stages, would be necessary to monitor the dynamics of RANKL and tm-TNF expression on ILCP surface upon tumor progression.

In terms of chemokines, high levels of serum CCL5 (or RANTES, a potent chemotactic factor for memory T cells and monocytes (316)) and MCP-1 were shown to be upregulated in bladder cancer patients following bacillus Calmette-Guérin (BCG) therapy (317). Interestingly, we also observed higher expression of MCP-1 in ECs exposed to ILCPs after 3h co-culture *in vitro* (figure 8), raising the possibility that ILCPs could also actively favor the immune cell recruitment at inflamed/tumor sites. Finally, the observed increase in GM-CSF production upon EC-ILCP crosstalk might represent a beneficial aspect in the establishment of an anti-tumor immune response, since it was shown that GM-CSF affected tumor progression in a urinary bladder transitional cell carcinoma model through the modulation of anti-tumor immunity in the TME (318).

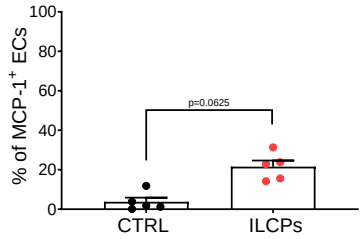

Figure 8: ILCPs induce MCP-1 expression in ECs *in vitro*

8. ILC-lymphatic/tumor cell interactions

8.1. ILC-lymphatic interactions

As of today, there is a lack of knowledge regarding the ability of ILCs to interact with the lymphatic vessels. Besides the ability of ILC3s to promote TLS formation and the potential role of IL-17A (an ILC3 signature-cytokine) in controlling the tumor vasculature (319), nothing has been published on ILC subset-lymphatic cell communication. In preliminary experiments we observed that none of the *in vitro*-expanded ILC subsets, isolated from the PB of healthy donors, could induce adhesion molecule expression in lymphatic ECs (LECs) *in vitro,* in contrast to blood ECs (figure 10).

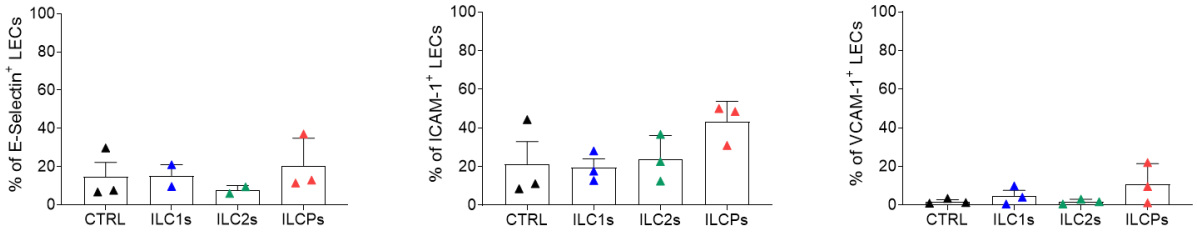

Figure 9: ILCs do not induce adhesion molecule expression in LECs *in vitro*

These preliminary results are not very unexpected. Indeed, blood and lymphatic vessels share a common embryological origin but they significantly differ both in their structure and physiological functions (320). The discontinuous basal lamina and the relative permeable sinusoidal endothelium, in which ECs form overlapping structures, are crucial for the filtering function of the lymphatic system (321). The lymphatic system also plays an important role in immunosurveillance: by transporting the lymph (i.e., the plasma filtered from interstitial fluids, once entered into the lymphatic network) to the lymph nodes, it ensures the optimal encounter between naïve lymphocytes, that enter the lymph nodes via HEVs, and the Ags and DCs contained in the lymph, thus ensuring the onset of immune responses, if needed (322).

In the cancer setting, lymphangiogenesis (i.e., the expansion of the lymphatic network) often correlates with bad prognosis in cancer patients, since it constitutes a better and safer route for cancer cell metastatization at distant sites. Indeed, cancer cells that disseminate via blood vessels are vulnerable to cell death caused by the shear stress and by the activity of NK cells (323,324). In light of our findings with primary ECs, and given the preliminary results shown in Figure 9, rather than monitoring adhesion molecules on LECs, it will be interesting to assess the ability of ILCPs/ILC3s to stimulate lymphangiogenic (but also angiogenic) processes in physiological and pathological settings, and to understand whether and

how the ILC-lymphatic interaction might impact cancer progression. To address these aspects, clonogenic and wound healing assays of tumor cells in the presence of conditioned media derived from ILCPs/ILC3s-LECs co-cultures could be performed, to monitor changes in cancer cell proliferation and motility *in vitro*.

8.2. ILC-tumor cell interactions

The direct ILC-tumor cell crosstalk also represents an interesting research area. We showed that the ability of ILCPs to activate ECs is dampened after the overnight incubation with bladder carcinoma cells. Interestingly, in another set of preliminary experiments (Figure 10), we noticed that ILCPs themselves were able to increase the expression of ICAM-1 and VCAM-1 in TCCSUP bladder cancer cells, suggesting that the tumor cells might hijack the EC-activating ability of ILCPs to upregulate adhesion molecules on their own cell surface, possibly facilitating their direct migration and metastatization at distant sites by exploiting the vascular route.

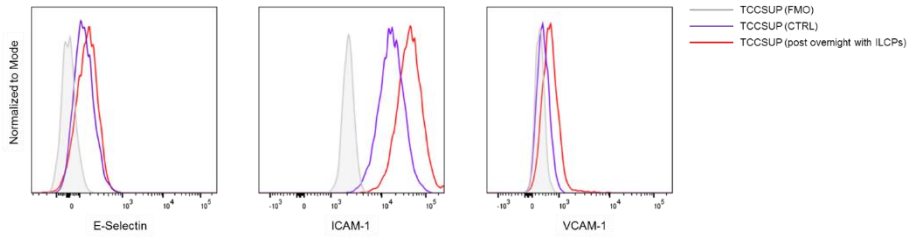

Figure 10: ILCPs increase the expression of ICAM-1, VCAM-1, but not E-Selectin, in TCCSUP cancer cells *in vitro*.

Since the impairment of EC-activating capacity was less evident when ILCPs were incubated with BU68.08 bladder cancer cells (representing an earlier stage of the disease, please refer to figure 6 of the results part of the manuscript), a deeper characterization of the ILCP-tumor cell crosstalk deserves further investigations, to elucidate how the tumors can impact immune cell-EC interactions and, conversely, how ILCs can influence tumor cell behaviors (e.g., proliferation, metastatic potential).

9. Therapeutic applications of our findings

9.1. Current Immunotherapies

Classic ways to treat cancer include surgery, radiation, chemotherapy and anti-angiogenic treatments. However, over the last two decades, immunotherapy, i.e., the use of the body's natural defenses to target neoplastic cells, has deeply changed the way to treat cancer patients. Immunotherapeutic approaches include:

- *immunomodulators*, including cytokines, adjuvants and agonists, aiming to boost the immune system (325–327). However, due to their broad effects on the whole immune system, immunomodulators often associate with high toxicity and low response rates.
- *targeted antibody-based immunotherapy,* including *monoclonal antibodies* (328) that directly target cancer cells, *bispecific antibodies* (329) that, for example, can target cancer cells and immune cells at the same time, or *ADCs*, specific for certain tumor Ags and equipped with anti-cancer drugs (330).
- *oncolytic virus therapy*, i.e., genetically modified viruses that exploit the altered antiviral defense mechanisms in cancer cells to specifically infect and kill them, facilitating the activation of the immune system by inducing immunogenic cell death of cancer cells (331). To date, there is only one FDA-approved oncolytic virus therapy (T-VEC, Lmlygic®), that is a modified herpes simplex virus (HSV) used in a subset of melanoma patients (331).
- *cancer vaccines* (332), composed by Ags and adjuvants that boost the immune system to recognize and to elicit an immune response against cancer cells;
- *adoptive cell transfer (ACT)* (333), that is based on the autologous isolation of tumor-infiltrating lymphocytes (TILs) from the tumor of the patients, their *in vitro* expansion and their re-infusion into the patients that have been pre-conditioned with chemotherapy, to deplete native lymphocytes;
- *immune checkpoint inhibitors (ICIs),* a major breakthrough in cancer treatment (334). *Immune checkpoints*, or ICs, are immunomodulatory molecules that constitute inhibitory factors crucial for the self-tolerance (e.g., CTLA-4) and for modulating and controlling immune responses to avoid tissue damaging (e.g., PD-1). Cancer cells develop immune resistance by inducing the upregulation of ICs on T cells, to negatively regulate T cell activation, differentiation and function, via ligands expressed on their cell surface (e.g., CD80 and PDL-1), limiting the cancer cell recognition and killing by T cells;

Despite the promising expectations, at least under the theoretical point of view, cancer vaccines using self-Ag showed important limitations and unsuccessful results. It has been shown that the activation of the immune system with cancer-specific molecules actually favors the selection of more aggressive and therapy-resistant neoplastic clones, that are so de-differentiated that they do not express the cancer cell specific molecules anymore, ultimately leading to relapses (335). Another promising approach consists in targeting the so-called tumor-specific "neoantigens" to develop more effective cancer vaccines. Neoantigens arise from genomic, transcriptomic or proteomic alterations that exclusively occur in the tumor cells and can be detected via cDNA-, next generation sequencing (NGS)- or immunopeptidomic-based screenings (336). Differently from targeting tumor-associated Ags (TAAs), that are also expressed in normal tissues and often results in off-target and toxic effects (336), the identification of neoantigens represents an attractive alternative strategy. Neoantigens have been targeted in clinical trials and already show promising results in melanoma (337) and glioblastoma patients (338). Still, the majority of neoantigens is mostly private (i.e., not shared among different cancer patients) and requires extremely personalized and expensive approaches for their identification and validation (336).

ACT-based immunotherapy was proposed for the first time in the clinics in 2002 and emerged as the most effective treatment in metastatic melanoma patients, since it provokes an objective tumor regression in 50% of patients, with durable and systemic effects (339). Since the effectiveness of ACT-based approaches depends on the Ag specificity of the transferred T cells, the genetic engineering of patient's own T cells to express modified TCRs (the so called Chimeric Ag Receptors, or CARs) resulted in one of the most remarkable therapeutic advances in the past decade (340). CAR T cell therapies already showed successful results in the treatment of blood malignancies (341), but still account for 10-20% of relapses, due to Ag escape, suboptimal persistence and activation *in vivo* as well as severe systemic toxicity (342,343). Innovative CART T cell design is needed to increase and expand the clinical benefits of CAR T cell-based therapies to patients with different cancer types.

The genetic engineering of different types of lymphocytes opens the possibility to extend ACT approaches to treat patients with other cancer types, especially in the context of radio- and chemo-refractory tumors.

ICIs emerged as a major breakthrough in cancer treatment. The tumor-induced expression of IC receptors on tumor-reactive effector cells and their ligands on tumor cells constitute the primary mechanism of evasion from anti-tumor immune responses. By disrupting the receptor-ligand interaction, ICIs unleash T and/or NK cell functions and restore the anti-tumor immune response. For example, combination

therapies targeting the two checkpoints, i.e., CTLA-4 and PD-1, showed to prolong the progression-free survival in patients with metastatic melanoma (344). Besides T cell and NK cells, several publications reported that ICs can be also expressed by ILCs (345). In humans, for example, ILC3s enriching pleural effusion were reported to express functional PD-1 (although the actual staining for PD-1, including appropriate controls, were not provided in this work) suggesting that they could be impaired in their antitumor function by interacting with PD-L1^{+} tumor cells (225).

Thus, ICIs are potent immunotherapeutic tools that enrich and integrate standard cancer treatment.

9.2. Antiangiogenic therapy

Another standard of care in treating cancer patients has been the use of anti-angiogenic agents. The idea of anti-angiogenic therapy was born in the early 1970s by an intuition of Dr. Judah Folkman (i.e., to interrupt the blood supply to the tumor, hence depriving the tumor cells of nutrients to favor tumor regression) that lead to the development of the first monoclonal antibody against VEGF (Bevacizumab), finally approved in 2004 for treating breast cancer patients (346). Since then, many anti-angiogenic agents have been developed and are often employed in the clinics, in combination or not with other chemotherapeutic/immunotherapeutic agents, to treat different types of cancers (346).

Recently, a subset of pro-angiogenic monocytes, characterized by the expression of the Tek tyrosine kinase ANG2 receptor Tie2, has been identified in the circulation and within the tumor bed both in human and mice (347,348). These Tie2-expressing monocytes were found to be selectively recruited to the tumor, where they promoted tumor neovascularization in a paracrine manner, possibly via basic FGF (bFGF) release (347). Tie2^{+} monocyte recruitment to the tumor site and pro-angiogenic activity are tightly regulated by ANG2 (348). Since the ablation of Tie2-expressing monocytes completely abolished tumor neovascularization in a glioma model (347), and since Tie2^{+} monocytes are enriched in different cancer patients (348) these cells might represent a good target to improve the efficacy of current cancer therapies. Indeed, promising results with Trebananib (a peptide-Fc fusion protein that prevents the binding of ANG2 to Tie2) in combination with chemotherapy have been reported in ovarian cancer patients (349) but also in microsatellite stable (MSS) heavily pre-treated CRC patients, when combined with Pembrolizumab (a FDA-approved anti-PD-1 antibody) (350).

However, the tumor vascular normalization, rather than the blockade of tumor angiogenesis, has emerged as an important aspect to take into account, especially when combining vascular-targeting therapies with ICIs and/or chemotherapy, where a normal vascular network, rather than a disrupted one, could facilitate the delivery of anti-tumor agents and/or the infiltration of anti-tumor specific lymphocytes.

9.3. Innate Immune cell targeted-immunotherapies

Over the past decade, the cells of the innate immune system also emerged as an intriguing target for developing immunotherapeutic strategies. Besides the very well established anti-tumor activities of NK cells that are frequently altered or poorly represented in both solid tumors and hematological disorders (351), other ILCs can be found in the TME and/or directly into the tumors, where they play an active role both in facilitating tumor progression or favoring tumor regression (230,277). Given their distribution throughout the body, ILCs might also be among the first lymphocytes to encounter and respond to tumor cells. Indeed, increasing evidence supports the idea that ILCs might exert either pro- or anti-tumor effects, depending on their phenotype and on the tumor type. ILCs mainly rely on myeloid- and/or epithelial cell-derived signals to adapt their cytokine secreting profile to the surrounding micro-environment (352). Thus, the immunosuppressive microenvironment found in the cancer setting might shape detrimental behaviors in ILCs, and therefore affect their ability to promote or not adaptive anti-tumor reactions.

The ambiguity of ILC functions in cancer is also fostered by their plastic potential. According to the composition of the microenvironment, ILCs can differentiate from one subtype to another one (353). This ability is crucial to fine tune immune responses during pathogenic infections, but could be detrimental in the cancer setting and define a new tumor-escape mechanism, to hijack ILC subset-dependent tumor immune-surveillance.

Currently approved cancer treatments in the clinics, which mainly focus on targeting T cells, might also have an impact on ILCs and NK cells. ILCs respond to several cytokines and, in the cancer setting, express IC molecules (354).

For example, the promising anti-cancer effects of IL-15, able to enhance the cytotoxic activity of NK cells against autologous blasts in AML patients (355), might also promote the conversion of ILC3s into IFN-γ-producing ILC1s (356), but also the stimulation of ie ILC1s, that can produce IFN-γ in response to IL-12 and IL-15 (73). Therefore, ILC plasticity could also be exploited to tip the balance between ILC subsets, depending on their phenotype and the tumor type.

Regarding ICs, activated ILCs showed increased levels of PD-1 in mice (357), whereas ILC2s and ILC3s were reported to express PD-1 in human gastrointestinal tumors and ILC2s were reported to express PD-1 and CTLA-4 in human breast cancer (although the actual staining for PD-1, including appropriate controls, were not provided in this work) (193). Moreover, PD-1$^+$ ILC2s were shown to secrete less IL-5 and IL-13 compared to PD-1$^-$ ILC2s (358). Therefore, anti-PD1 and anti-CTLA-4 checkpoint inhibitor-based therapies, but also cytokine-based strategies, may also influence the ILC compartment. Thus, the monitoring of IC molecule expression by ILCPs might be relevant in the context of combining ILCP-based immunotherapy with immunotherapeutic strategies that are currently used in the clinics (e.g., IC blockade).

In addition to that, we showed that CD56$^+$ ILC1-like cells have cytotoxic potential that is impaired in AML patients, due to the persistent expression of NKG2A (Salomé *et al.,* Annex II)(75). It is known that the binding of NKG2A/CD94 to the non-classical HLA-E molecule results in an inhibitory signal that suppresses the effector cell functions (359). NKG2A/CD94 is often upregulated in tumor-infiltrating CTLs and NK cells, and it has been recently proposed as a novel targetable checkpoint in the TME (360) with promising results in mouse model (in combination with PD-1/PD-L1 blockade) but also in phase II clinical trials, where a humanized NKG2A-blocking antibody (Monalizumab) showed promising results in gynecological malignancies (361) and long-lasting response rates, when combined with VEGF- and PD-L1-blocking agents, in patients with MSS colorectal carcinoma (362).

Furthermore, a TGF-β-dependent detrimental conversion of NK cells into ILC1-like populations with poor tumor immunosurveillance functions was described in different tumor-bearing mouse models (135), thus defining TGF-β as a potential targetable checkpoint on ILCs, e.g., with the use of small molecules inhibiting TGF-β signaling (363).

Therefore, the monitoring of IC expression in innate lymphocytes is important to understand if and how these cells could respond to ICI-based therapies, and therefore provide beneficial or detrimental outcomes in cancer patients.

9.4. Integration of ILCP-targeting in immunotherapy

Despite the increasing evidence that ILCs can have an impact on cancer onset and progression, whether and how their pro- and anti-tumor effects could be exerted, via the interaction with the vascular ECs in humans, remains to be unraveled. Indeed, the potential role of certain ILC subsets to promote leukocyte invasion in mouse models and TLS formation or maintenance could be utilized to enhance the access of activated effector cells to the tumor site (226,227).

Our findings suggest that, under the proper priming conditions, human ILCPs might have an active role in facilitating the infiltration of anti-tumor specific T cells and to support cancer clearance. Therefore, ILCP-EC interaction might constitute a novel target for the development of immunotherapeutic strategies addressed to exploit ILC functions, in combination or not with chemotherapeutic or immunotherapeutic agents, to control tumor growth and progression.

One way to exploit the EC-activating capacity of circulating ILCPs might rely on intratumoral injection of cytokines (e.g., IL-1β and IL-23) to promote polarization of ILCs towards and ILCP/ILC3-like phenotype. Besides being reported as an ILC2-to-ILC3 and ILC1-to-ILC3 polarizing cytokine (77), our *in vitro* observations suggest that IL-1β could also represent one of the priming factors for ILCPs, i.e., responsible for the acquisition of EC-activating potential by ILCPs. However, administration of cytokines can have severe side effects, as shown with IL-2 immunotherapy (364).

Another strategy might consist in the ACT of anti-tumorigenic *in vitro* primed ILCPs, possibly in combination with anti PD-1 and the bispecific A2V antibody (266), shown to promote vessel normalization, to further support the establishment of a potent anti-tumor immune response.

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Annex I

ILC2s: New Actors in Tumor Immunity

frontiers in Immunology

REVIEW
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ILC2s: New Actors in Tumor Immunity

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Innate lymphoid cells (ILCs) represent the most recently identified family of innate lymphocytes that act as first responders, maintaining tissue homeostasis and protecting epithelial barriers. In the last few years, group 2 ILCs (ILC2s) have emerged as key regulators in several immunological processes such as asthma and allergy. Whilst ILC2s are currently being evaluated as novel targets for immunotherapy in these diseases, their involvement in tumor immunity has only recently begun to be deciphered. Here, we provide a comprehensive overview of the pleiotropic roles of ILC2s in different tumor settings. Furthermore, we discuss how different therapeutic approaches targeting ILC2s could improve the efficacy of current tumor immunotherapies.

Keywords: patients, immunotherapy, cancer, ILC2, IL-33, ST2

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INTRODUCTION

ILCs are the most recently described family of innate immune cells that play a key role in the preservation of epithelial integrity and tissue immunity (1). ILCs are rapidly activated by both tissue and immune cell-derived signals providing the first line of defense against bacterial, viral and helminthic infections (2–6). However, ILCs need to be tightly regulated, given that their uncontrolled activation and proliferation has been shown to contribute to severe inflammation and damage in gut, lung, skin, and liver (7). ILCs are classified into three different groups, according to the expression of specific transcription factors and surface markers, and based on their cytokine secretion profile (8).

In humans, ILC1s define the T-bet-dependent ILC subset that mainly produce IFNγ and TNFα (9). ILC3s rely on RORγT for their development and express CD117 (also referred to as c-Kit) on their cell surface (10). ILC2s comprise the GATA-3-dependent ILC subset that is also characterized by the expression of the prostaglandin D2 receptor 2 (CRTH2), the IL-33 receptor (IL1RL1 also referred as ST2) and by variable levels of c-Kit (11). More recently, Nagasawa and colleagues showed that the killer cell lectin-like receptor subfamily G member 1 (KLRG1) is a surface marker that arises during ILC2 development in humans (12). KLRG1 is a co-inhibitory receptor already reported to be expressed also by $CD4^+$ and $CD8^+$ T cells as well as by NK cells, that binds to the members of the cadherin family (13, 14). In mice, the ILC2 phenotype is characterized by the surface expression of both ST2 and KLRG1 (15). Notably, $ST2^+KLRG1^{+/-}$ ILC2s are defined as natural ILC2s (nILC2s) which respond to IL-33 (15), whilst $ST2^-$ $KLRG1^{hi}$ ILC2s represent inflammatory ILC2s (iILC2s) reported to differentiate during infections. iILC2s are highly responsive to IL-25, but not to IL-33, and are able to differentiate into ILC3-like cells under type-17 stimulation, thus defining a distinct subset from nILC2s. ILC2s are able to respond to a wide range of soluble mediators like alarmins [IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)], survival cytokines (such as IL-2, IL-9, and IL-7) and eicosanoids. In addition, ILC2s have been shown also to respond to neuropeptides, including neuromedin U (NMU), vasoactive intestinal peptide (VIP), and calcitonin gene-related peptide (CGRP) (3, 16–20). More precisely, it has been shown that neuropeptides released by

pulmonary endocrine cells (PNECs) can stimulate resident ILC2s to produce cytokines, such as IL-5, which in turn support downstream type-2 immune responses (21). Similarly, VIP can stimulate IL-5 release by ILC2s, regulating eosinophil homeostasis in intestinal tissues (22). On the contrary, an opposite role for the CGRP was described, as it can negatively modulate ILC2 effector functions (i.e., cytokine production) in the context of lung inflammation and also during helminth infections (23, 24). It has also been reported that ILC2s in the small intestine, express high levels of the β_2- adrenergic receptor (β_2-AR), which acts as a negative regulator of the ILC2-mediated anti-inflammatory response (25).

Once activated, ILC2s secrete type 2 cytokines, such as IL-4, IL-5, IL-9, IL-13, and amphiregulin (AREG), that are involved in airway responses, helminth expulsion, and tissue repair (26). More recently, it has been reported that activated ILC2s are able to produce prostaglandin D2 (PGD2) that acts in an autologous manner supporting ILC2 function via the CRTH2 receptor (27). A detrimental role of ILC2s in chronic inflammation is suggested by their increased frequency in the peripheral blood of asthma and chronic rhinosinusitis patients; and additionally, the secretion of AREG by intrahepatic ILC2s is thought to contribute to the process of fibrogenesis in liver diseases (28, 29).

However, in cancer, the role of ILC2s is still controversial. Elevated numbers of ILC2s have been found in many IL-33-enriched tumors, such as breast, gastric and prostate cancer (30–32) as IL-33 is an ILC2 activator that can promote tumor growth, metastatic dissemination and angiogenesis (33). The ILC2 pro-tumorigenic activity is mainly ascribed to the IL-33-triggered IL-4 and IL-13 production. These cytokines have been reported to support tumor development and progression (34), in part by the recruitment and activation of monocytic myeloid-derived suppressor cells (M-MDSCs) that are considered potent inhibitors of the anti-cancer immune response (35). In addition, AREG produced by ILC2s, can further suppress the anti-tumor immune response by boosting the activity of regulatory T cells (Tregs) (36). Conversely, ILC2-produced IL-5 promotes blood and tissue eosinophilia that correlates with reduced tumorigenicity and tumor progression in mice (37). In this review, we summarize the current knowledge concerning the presence and functional characteristics of ILC2 populations in different tumors, using both patient samples and murine tumor models (**Figure 1**). Furthermore, we discuss potential strategies to exploit ILC2 biology to improve the efficacy of current tumor immunotherapies.

ILC2s IN HEMATOLOGICAL MALIGNANCIES

Hematological malignancies represent the fourth most common type of cancer (38). ILCs are a rare cell population, representing ∼0.4% of total circulating peripheral blood lymphocytes in humans (39), however, we have reported that ILC2s are expanded in the peripheral blood of acute promyelocytic leukemia (APL) patients at diagnosis, compared to healthy donors. In particular, we found that ILC2s have a central role in the establishment of an immunosuppressive axis, dictated by the tumor-derived factors PGD2 and B7H6 and their ILC2 receptors CRTH2 and NKp30, respectively. This interaction triggers the production of IL-13 which in turn recruits M-MDSCs supporting the growth of cancer cells [(31); **Figure 1**, left lower corner]. These findings were also confirmed in an APL mouse model raising the possibility of finding the same axis in other tumors, including solid tumors such as prostate cancer (see "*ILC2s in prostate cancer*" section).

In contrast, in treatment-naïve patients with acute myeloid leukemia (AML), we and others have observed an expansion of ILC1s. There was no detection of a change in ILC2 frequency but we observed a lower production of IL-5 and IL-13 following *in vitro* short-term activation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin (40). In this context, the increased ILC1 frequency might be due to the conversion of ILC3s and/or ILC2s into ILC1s driven by tumor-derived factors, among others TGFβ. A putative anti-tumor role of ILC2s has been proposed in a subcutaneous lymphoma mouse model, where sustained production of IL-33 induced the upregulation of CXCR2 on EL4 thymoma cells, the expansion of ILC2s and the concomitant production of CXCR2 ligands (CXCLs). These ligands, mainly CXCL1 and CXCL2, induced apoptosis in a limited proportion of lymphoma cells, thus limiting tumor progression [(41); **Figure 1**, right middle panel].

ILC2s IN UROGENITAL TRACT CANCERS

ILC2s in Prostate Cancer

Prostate cancer is the most common non-cutaneous malignancy in men and responsible for about 20% of male cancer-related deaths (42). Despite the different therapeutic approaches, including the use of immune checkpoint inhibitors, limited clinical benefits have been observed in patients (43). In this context, the tumor microenvironment (TME) seems to play a key role in driving prostate cancer progression and chemoresistance (44, 45). Focusing on ILCs in prostate cancer patients, we have shown that ILC2 levels positively correlate with tumor stage and with M-MDSC frequency (31). Additionally, DU145 and PC3 prostate cancer cells secrete the ILC2 activator PGD2 and express high levels of B7H6, the ligand of NKp30, corresponding with the immunosuppressive axis found in APL patients. Using the spontaneous TRAMP model, in which mice develop orthotopic prostate tumors from puberty (46), we observed an increase of ILC2s both in the blood and the tumor supporting our findings in prostate cancer patients (31). Conversely, Saranchova et al. have showed that ILC2s can acquire anti-tumor activities by influencing the effector functions of cytotoxic lymphocytes, through the release of IL-5 and IL-13 acting on DCs. They used the pTAP-1-EGFP-stably-transfected LMD cell line, derived from a metastatic prostate cancer mouse model, in which TAP-1 activation in tumor cells indirectly correlates with MHC-I and EGFP expression. In order to mimic metastatic prostate cancer conditions *in vivo*, the authors isolated ILC2s from tumors of donor mice and cultured them with the LMD cell lines, $CD8^+$ dendritic cells (DCs), ovalbumin (OVA) peptide as well as $CD8^+$ OT-1 T cells. They observed an increased

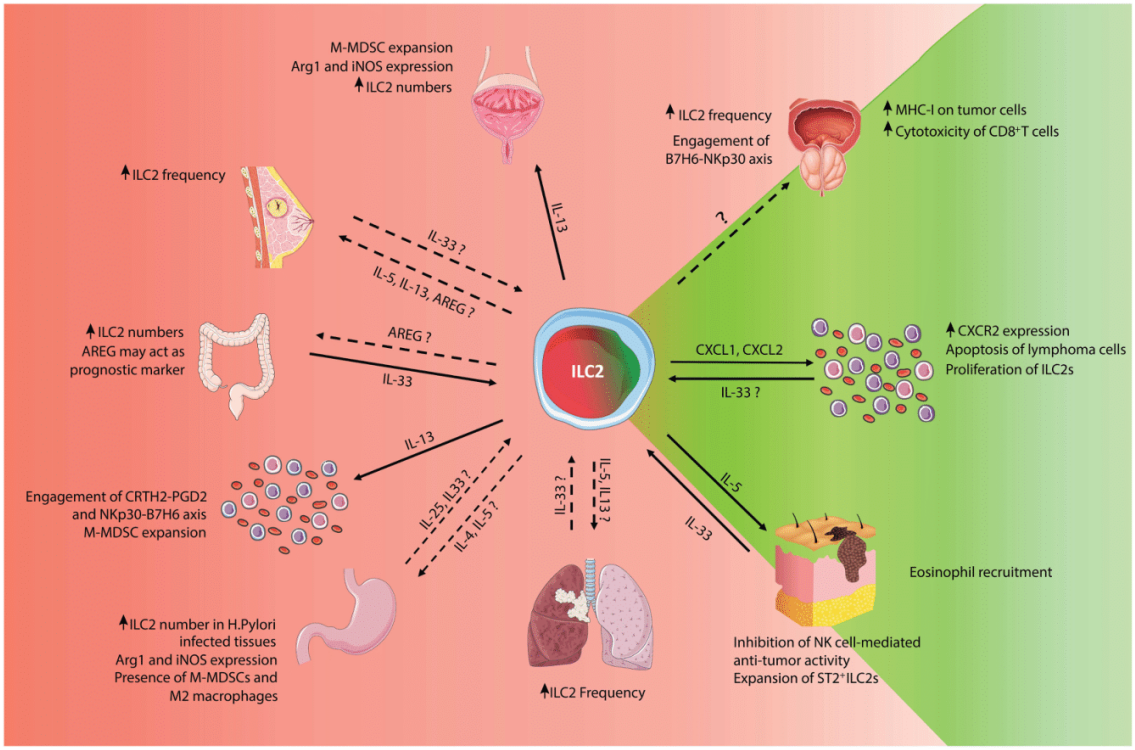

FIGURE 1 | Schematic representation of pro- and anti-tumor roles of ILC2s. Summary of the known pro- and anti-tumor roles of ILC2s, classified by tumor types. For bibliographic details refer to the work cited in the main manuscript.

expression of EGFP, indicating that the LMD cells had been stimulated to express MHC-I on their surface by ILC2s. This suggests that ILC2s via direct interaction or cytokine secretion, facilitate antigen presentation and the recognition of tumor cells by T cells, thus improving the anti-tumor adaptive response [(47); **Figure 1**, right upper panel]. The use of tumor-bearing ILC2 KO mice transferred with ILC2s isolated from wild-type donor mice might represent a good strategy to dissect the contribution of ILC2s in the T-cell mediated anti-tumor responses.

ILC2s in Bladder Cancer

Bladder cancer (BC) is broadly divided into two major stages: non-muscle-invasive (NMIBC) and muscle-invasive bladder cancer (MIBC) and is the ninth most common cancer worldwide (48). NMIBC standard treatment involves intravesical instillation of the Bacillus Calmette-Guérin antigen (BCG) (49), whereas MIBC treatment involves neoadjuvant cisplatin-based chemotherapy (NAC) followed by radical cystectomy (50). Despite these approaches, the rate of recurrence of BC remains high (51). To better understand the reasons behind BC treatment failure, immune cell distribution was analyzed in the urine of NMIBC patients during BCG treatment and ILC2s were found to be the most abundant innate lymphoid cell subpopulation present (52). ILC2 frequency positively correlates with M-MDSC frequency but not with T cell numbers suggesting that ILC2s may promote the expansion of M-MDSCs. This correlation has also been confirmed in the blood of patients with MIBC. In addition, ILC2-associated cytokines measured in blood and urine samples of NMIBC and MBIC patients showed a significantly elevated level of IL-13 compared to healthy donors. IL-13 secretion could explain the ILC2-dependent recruitment of M-MDCS which were shown to express the IL-13 receptor α1 (IL-13Rα1). At mRNA level, the immunosuppressive properties of IL-13 were demonstrated with upregulation of monocytic suppressive markers such as arginase 1 (Arg1), inducible nitric oxide synthase (iNOS) and C/EBPβ [(52); **Figure 1**, upper central panel]. These results highlight the concept that the BC immunosuppressive environment is, at least in part, driven by ILC2-derived IL-13 that may be contributing to the failure of current BC therapies. Furthermore, the ratio between T cells and M-MDSCs may also have an impact on the response to treatment, since patients with a high T cell/MDSC ratio show improved survival with reduced risk of recurrence. However, more research is needed to better understand the role of ILC2s in this type of cancer.

ILC2s IN CANCERS OF THE GASTROINTESTINAL SYSTEM

ILC2s in Colorectal Cancer

Colorectal cancer (CRC) is the third and second most common cancer diagnosed in men and women, respectively (53). CRC mortality rate has decreased over recent years due to improved cancer screening methods (54). A variety of genetic, environmental and nutritional factors play a key role in the pathogenesis and progression of CRC (55). Several immune cell populations infiltrate the CRC TME by modulating the tumor response (56). Among them, ILC2s, that are abundant in the intestinal mucosa (57), have been reported in CRC patients, to be recruited to the tumor site suggesting their potential role in CRC development and progression (58). However, there is still no robust data in human or mouse models, clarifying the role of ILC2s in colorectal tumorigenesis. Nevertheless, analysis of human resected CRC specimens has shown that SW480 and SW620 cells at different stages of the disease are positive for IL-33 and its receptor ST2 (59–61). IL-33 has been shown to promote the *in vitro* proliferation of freshly isolated primary CRC cells (the HT-29 CRC cell line and the murine MC38 cell line), through the activation of the ST2 receptor. The IL-33/ST2 axis activates NF-kB signaling which in turn induces cyclooxygenase-2 (COX2) expression and prostaglandin E2 (PGE_2) synthesis, triggering CRC cell proliferation (62). Further evidence for involvement of the IL-33/ST2 axis in CRC pathogenesis comes from an inflammation-driven model in which ST2 deficiency in mice conferred protection against tumor development (61) and secondly from a polyposis mouse model ($Apc^{Min/+}$), where abrogation of IL-33 signaling reduced the tumor burden, Th2-associated cytokine production and mast cell activation (59). Conversely, Akimoto et al. have reported that sST2, a soluble form of the IL-33 receptor, is down-regulated in patient serum and correlates inversely with disease progression. This data has also been confirmed in nude mice, in which injection of short hairpin RNA (shRNA) targeting sST2, triggered tumor development, and progression (60). These findings underline the potential dual role of the IL-33/ST2 axis in colon cancer (63) and the need for further analysis of this pathway in different CRC models. AREG is another important molecule that regulates cancer cell proliferation, invasion and angiogenesis (64) and has been proposed as a prognostic marker in CRC (65). AREG upregulation is associated with increased migration and invasion of CRC cells which is essential for metastasis [(66, 67); **Figure 1**, left middle panel]. AREG can be produced by different immune cell types under pro-inflammatory conditions, such as mast cells, basophils, tissue resident CD4 T cells (68). However, no data is available to date on ILC2-derived AREG in CRC development and progression.

ILC2s in Gastric Cancer

With a 65% overall survival rate, gastric cancer is one of the most common malignancies affecting the digestive system, with more than one million people newly diagnosed each year worldwide (69). However, due to poor population strategies for primary prevention and lack of early symptoms, most patients are diagnosed at an advanced stage with limited benefit from existing therapies (70). The use of immunotherapy for the treatment of metastatic gastric cancer such as pembrolizumab has showed promising effects in Phase I clinical trials (71), but other strategies are still needed to improve patient survival. Gastric tumors are multifactorial in etiology and one of the main risk factors for disease is chronic infection with Helicobacter Pylori *(H. Pylori*) (72). *H. Pylori* infection causes chronic inflammation of gastric tissue, favoring the development of gastric carcinoma (73). Higher numbers of ILC2s have been observed in the tumors of gastric cancer patients infected with *H. Pylori*, suggesting a role for ILC2s in this immunosuppressive type 2 environment [(74); **Figure 1**, left lower panel]. Moreover, the frequency of ILC2s in the peripheral blood mononuclear cell (PBMC) compartment is higher in gastric cancer patients than in healthy volunteers and ILC2-associated cytokines, such as IL-4, IL-5, and IL-13, are increased in gastric cancer patients, both at mRNA and protein level in PBMCs and plasma, respectively. In addition, Arg1 and iNOS, expressed in M-MDSCs and M2 macrophages as well as in group 2 ILCs (75, 76) were found to be highly expressed at mRNA level in PBMCs of gastric cancer patients (77). Moreover, type 2 cytokines derived from ILC2s have been reported to mediate Arg1 and iNOS secretion by MDSCs and M2 macrophages suggesting a role for ILC2s in promoting M-MDSCs and M2 macrophage phenotype and favoring their immunosuppressive function (78, 79). However, using the $gp130^{FF}$ mouse model, validated as a model of spontaneous gastric cancer, Eissmann et al. (80), demonstrate that mast cells, rather than ILC2s, promote tumor growth upon IL-33 stimulation. The authors show that mast cells are more abundant than ILC2s in gastric tumors and secrete macrophage-chemoattractant colony-stimulating factor 2 (CSF2), CCL3, and IL-6 in response to activation by tumor-derived IL-33. In ST2 deficient animals ($gp130^{FF}$ $ST2^{-/-}$ mice), the authors observed lower tumor burden, which was increased upon adoptive transfer of $ST2^{+}$ wild type bone marrow-derived mast cells (BMMC). Therefore, additional studies with adoptive transfer of $ST2^{+}$ wild type ILC2s could help to determine the individual contribution of mast cells and ILC2s in this cancer setting.

ILC2s IN BREAST CANCER

Breast cancer is the most common cancer affecting women and its incidence rate in younger women is expected to increase (81). Despite the progress in breast cancer detection and treatment (82), aggressive tumors, such as triple negative breast cancer (TNBC), still lack targeted therapies (83). Immunotherapeutic strategies provide hope of finding new treatment approaches (84), but due to the high heterogeneity of breast cancer (85), much more needs to be done to fully understand the interactions between immune and breast cancer cells (86). ILC2 frequency has been shown to be higher in malignant compared to benign breast tissue in humans (32). Using the 4T1 mammary carcinoma model, Jovanovic et al. have reported an increase in endogenous levels of IL-33, that correlated with cancer progression and metastasis. Using the parental 4T1 cell line overexpressing IL-33, they showed elevated frequencies of IL-5 and IL-13-expressing ILCs in tumor-bearing mice [(33); **Figure 1**, left upper panel].

More precisely, in this model they found that ILC2s trigger tumor progression and metastasis development in response to IL-33, sustaining the immunosuppressive milieu that characterizes breast cancer patients. This data suggests that ILC2s could be activated by IL-33 to secrete IL-5 and IL-13 in the 4T1 model of breast cancer, but further investigation is required to confirm this finding also in patients. Moreover, it has been shown that AREG regulates the proliferation and the migration of different mouse and human estrogen-receptor positive ($ER2^+$) breast cancer cell lines (87). However, it is still unknown whether ILC2s and ILC2-derived AREG are involved in this pro-tumoral axis. The use of ILC2 KO mice could represent a strategy to address the role of AREG-producing ILC2s in the context of breast cancer.

ILC2s IN MELANOMA

Melanoma is the most aggressive form of skin cancer with a high mortality rate (88). Whilst early stage melanoma is usually curable with surgery, metastatic melanoma is difficult to treat and often fatal. Nevertheless, in the last few years, treatment for metastatic melanoma has advanced due to the introduction of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and the programmed cell-death protein 1 (PD-1) checkpoint inhibitors (89). However, despite these promising discoveries, a high percentage of patients still experience treatment resistance (90) emphasizing the need to find new therapeutic approaches. The TME has been identified recently as a potential target for metastatic melanoma immunotherapy (91). Among the different TME mediators, IL-33 has been reported to inhibit tumor growth in a melanoma mouse model, by stimulating the anti-tumor activity of $CD8^+$ T cells and natural killer (NK) cells (92). However, this cytokine has also been shown to bind to and expand $ST2^+$ tumor-infiltrating ILC2s, characterized by the expression of the immunosuppressive ectoenzyme CD73. In this setting, ILC2s partially antagonized the IL-33 dependent, NK cell-mediated anti-tumor response, as evidenced by cell depleting experiments in which the lack of ILC2 $CD73^+$ cells led to enhanced NK cell activity and better tumor control (92). This data shows that IL-33 has both a beneficial anti-tumoral role via adaptive immune cells but also a pro-tumoral role via ILC2s. IL-33 is also able to stimulate ILC2s to produce IL-5, a potent eosinophil chemoattractant. Ikutani et al. showed that, in a murine model of metastatic melanoma, the main source of IL-5 was a $CD3^{neg}$ population, characterized by the expression of CD90, CD127, CD25, and ST2 (*bona fide* ILC2s). IL-5 was crucial to induce tumor rejection via eosinophil recruitment, also resulting in reduced lung metastases [(93); **Figure 1**, right lower panel]. The use of neutralizing antibodies directed against IL-5 may be useful to confirm the involvement of ILC2s in metastatic melanoma.

ILC2s IN LUNG CANCER

Lung cancer is generally divided into two types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (94). It is strongly correlated with cigarette smoking (95, 96) and is the most common cause of cancer-related deaths. Different targeted immunotherapies are now being used in lung cancer patients including anti-PD-1 antibodies that have been recently approved for the treatment of SCLC (97–99). Nonetheless, a significant percentage of patients do not respond or develop resistance to treatment, leading to consequent cancer progression (96, 100, 101). ILC2s constitute the most prominent ILC subset in the respiratory tract under physiologic conditions, although their overall numbers are low (26). They respond rapidly to tissue-derived alarmins (102), therefore, unsurprisingly, circulating ILC2s and M-MDSCs were found to be increased in a cohort of 36 lung cancer patients at diagnosis and correlated with a strong type 2 phenotype (103). The expansion of ILC2s in the periphery was accompanied by higher levels of IL-5, IL-13, IL-33, and Arg1 in the plasma of lung cancer patients compared to healthy donors. Simoni et al. have also detected ILC2s within lung tumor tissues. However, no functional assays were performed in these studies to define the pro- or anti-tumor roles of ILC2s in lung cancer [(58); **Figure 1**, central lower panel]. It can be speculated that the observed strong type 2 phenotype may represent a targetable axis for the development of new immunotherapeutic strategies, fostering the anti-tumor immune response. In contrast to these observations, Carrega et al., reported a reduced frequency of ILC2s in tumors compared to normal lung tissue (104). However, in the absence of sufficient data on the function of ILC2s in lung cancer, it is too early to define their role in this setting.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Tumorigenesis is the result of multiple cell intrinsic (e.g., uncontrolled proliferation, cell migration) and cell extrinsic (e.g., pro-inflammatory or immunosuppressive microenvironment, growth factors, angiogenesis) factors (105). Among the latter, the contribution of the immune system to tumor development and/or tumor cell clearance has become more and more accepted/relevant (106). Even though the impact of ILC2s in malignancy is not currently well defined, the number of studies focusing on the role of ILC2s in tumor immunity has multiplied (107), highlighting the importance of this cell type during cancer development and progression. However, many aspects still need to be elucidated to achieve a better understanding of the mechanisms behind ILC2 pro- and anti-tumoral functions. Moreover, it is known that ILC2s are highly plastic cells that can easily adapt to the environment to which they are exposed (12). Hence, the cytokines present in the TME may stimulate the conversion of ILC2s into other ILC subsets within the tumor tissues, suggesting that the environment that they are exposed to can dictate their pro- and/or anti-tumoral roles. In the nasal polyps of cystic fibrosis patients, ILC2s are reported to be capable of differentiation into IL-17 producing cells when stimulated with IL-1β, IL-23, and TGF-β, the concomitant downregulation of GATA-3 and increased expression of RORγt were also observed (108). Therefore, ILC2s may be detrimental in the pathogenesis of IL-17-associated diseases, including some

types of cancer. Efforts to understand the role of *bona fide* and/or plastic ILC2s in tumors represents the next challenging step. In this endeavor, the use of mouse models will be crucial, for example, the use of genetically engineered ILC2-depleted mice would allow dissection of the real contribution of this cell type to tumor development and/or progression (109). However, ILC2 characterization at any given time point in tumor-bearing mice will always be difficult due to their inherent plasticity. Moreover, the contribution of nILC2s and iILC2s remains to be elucidated in the tumor setting. Given their different abilities to respond to cytokines and, therefore, their potential distinct pro- and/or anti-tumor roles, further investigation should consider both subsets separately. The use of reporter mice, such as the $Il13^{GFP}$ or other type-2 cytokine reporter animals, represent helpful tools to track ILC2s, independently of their transcriptional profiles that can be shaped by the interaction with tumor cells and/or by the TME. The use of humanized mice (BRGST HIS mice) to establish patient-derived xenograft (PDX) models would provide unique environments for interrogation of the function of the innate immune system, in particular, the contribution of ILC2s to cancer development and progression (110). Collectively, these strategies are expected to accelerate our knowledge of ILC2 biology, and provide new insight into potential therapeutic targets. One approach may be to target Th2-associated cytokines and ILC2-secreted molecules using neutralizing antibodies. This is the same technique employed by some NK cell-based immunotherapies, for example the use of a transforming growth factor beta (TGF-β) antibody to block TGF-β signaling, restores NK anti-tumor activity and synergy with α-PD-1 can be achieved (111). These anti-cytokine-based immunotherapies may also be effective for altering ILC2 function. However, they are a challenging and non-specific target due to the diversity of cell types producing them and their multiple roles in different physiological and pathophysiological processes. Another attractive strategy for targeting ILC2s may involve the disruption of transcription factor signatures, that are emerging as indispensable in ILC2 biology, or the manipulation of their metabolic programs. Lastly, remarkable success has been recently achieved in the clinics by immunotherapy based on immune checkpoint blockade, including agents targeting CTLA4, PD-1, or PD-L1. While the pattern of CTLA4 and PD-1 expression in various subsets of $CD4^+$ T and $CD8^+$ T cells are well-understood, little is known on the expression of immune checkpoints in ILCs. Of note, PD-1 has been reported as an intrinsic negative regulator of the functions of the ILC2 subset in mice, raising the possibility that current treatments targeting PD-1 might significantly impact on ILC functions (112). Ultimately with constant new discoveries in the ILC2 field in health and disease, immunotherapies focusing on the functional targeting of ILC2 are fast approaching clinical realization.

AUTHOR CONTRIBUTIONS

GE, MF, and GV wrote the manuscript. ST and CJ wrote and critically revised the manuscript. All authors provided approval for publication of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annex II

CD56 as a marker of an ILC1-like population with NK cell properties that is functionally impaired in AML

REGULAR ARTICLE

blood advances

CD56 as a marker of an ILC1-like population with NK cell properties that is functionally impaired in AML

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Key Points

- Human ILC1-like cells kill tumors in a KIR-independent manner.
- The cytotoxicity of human ILC1-like cells is impaired in AML at diagnosis but is restored in remission.

An understanding of natural killer (NK) cell physiology in acute myeloid leukemia (AML) has led to the use of NK cell transfer in patients, demonstrating promising clinical results. However, AML is still characterized by a high relapse rate and poor overall survival. In addition to conventional NKs that can be considered the innate counterparts of CD8 T cells, another family of innate lymphocytes has been recently described with phenotypes and functions mirroring those of helper CD4 T cells. Here, in blood and tissues, we identified a $CD56^+$ innate cell population harboring mixed transcriptional and phenotypic attributes of conventional helper innate lymphoid cells (ILCs) and lytic NK cells. These $CD56^+$ ILC1-like cells possess strong cytotoxic capacities that are impaired in AML patients at diagnosis but are restored upon remission. Their cytotoxicity is KIR independent and relies on the expression of TRAIL, NKp30, NKp80, and NKG2A. However, the presence of leukemic blasts, HLA-E–positive cells, and/or transforming growth factor-β1 (TGF-β1) strongly affect their cytotoxic potential, at least partially by reducing the expression of cytotoxic-related molecules. Notably, $CD56^+$ ILC1-like cells are also present in the NK cell preparations used in NK transfer–based clinical trials. Overall, we identified an NK cell–related $CD56^+$ ILC population involved in tumor immunosurveillance in humans, and we propose that restoring their functions with anti-NKG2A antibodies and/or small molecules inhibiting TGF-β1 might represent a novel strategy for improving current immunotherapies.

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with a 3.7/100 000 incidence per year. AML has a high relapse rate, which decreases patients' 5-year overall survival to

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†S.T. and C.J. contributed equally to this study as joint last authors.

The data for this study have been deposited in the European Nucleotide Archive at EMBL-EBI under accession number PRJEB34980 (https://www.ebi.ac.uk/ena/data/view/PRJEB34980).

The full-text version of this article contains a data supplement.

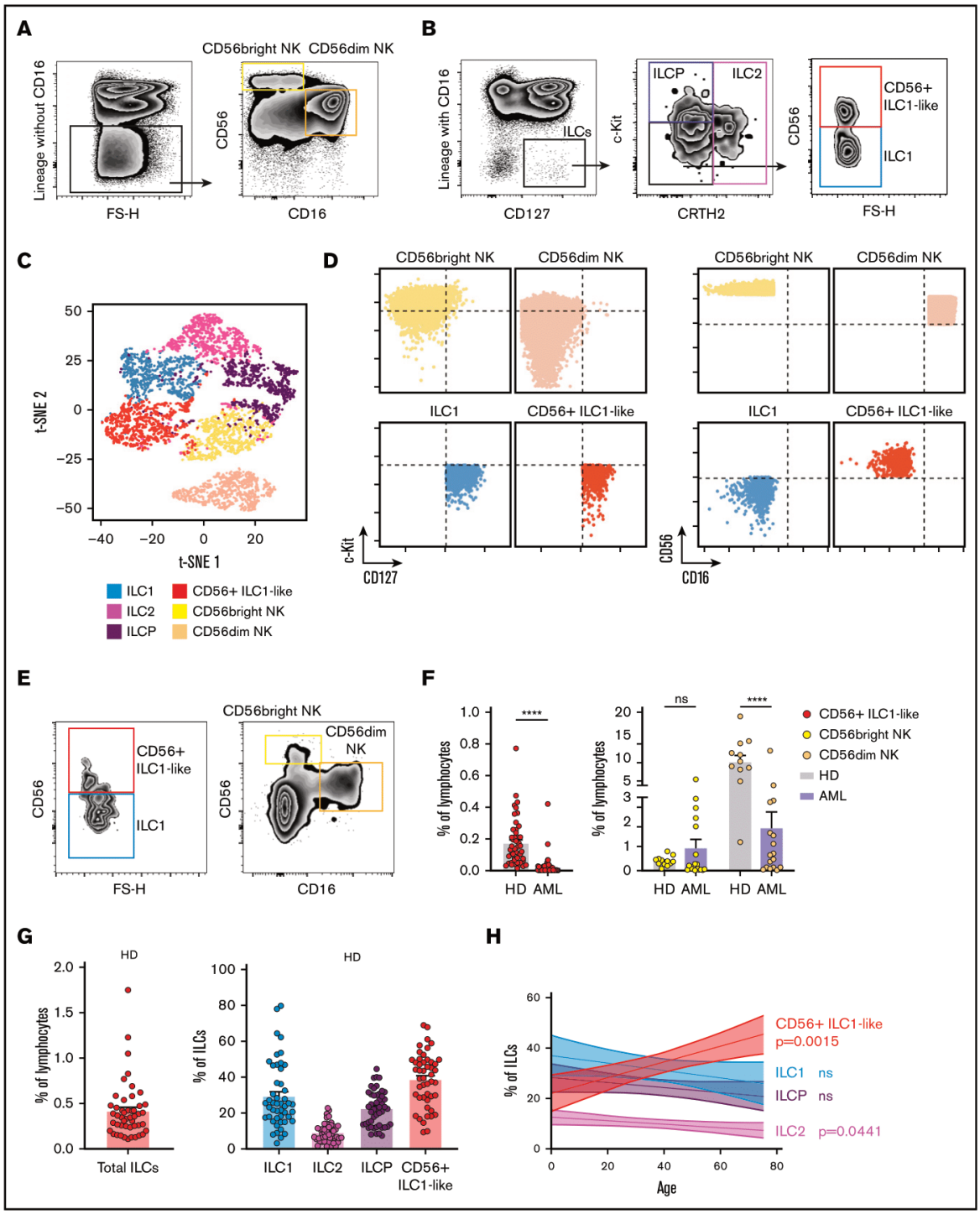
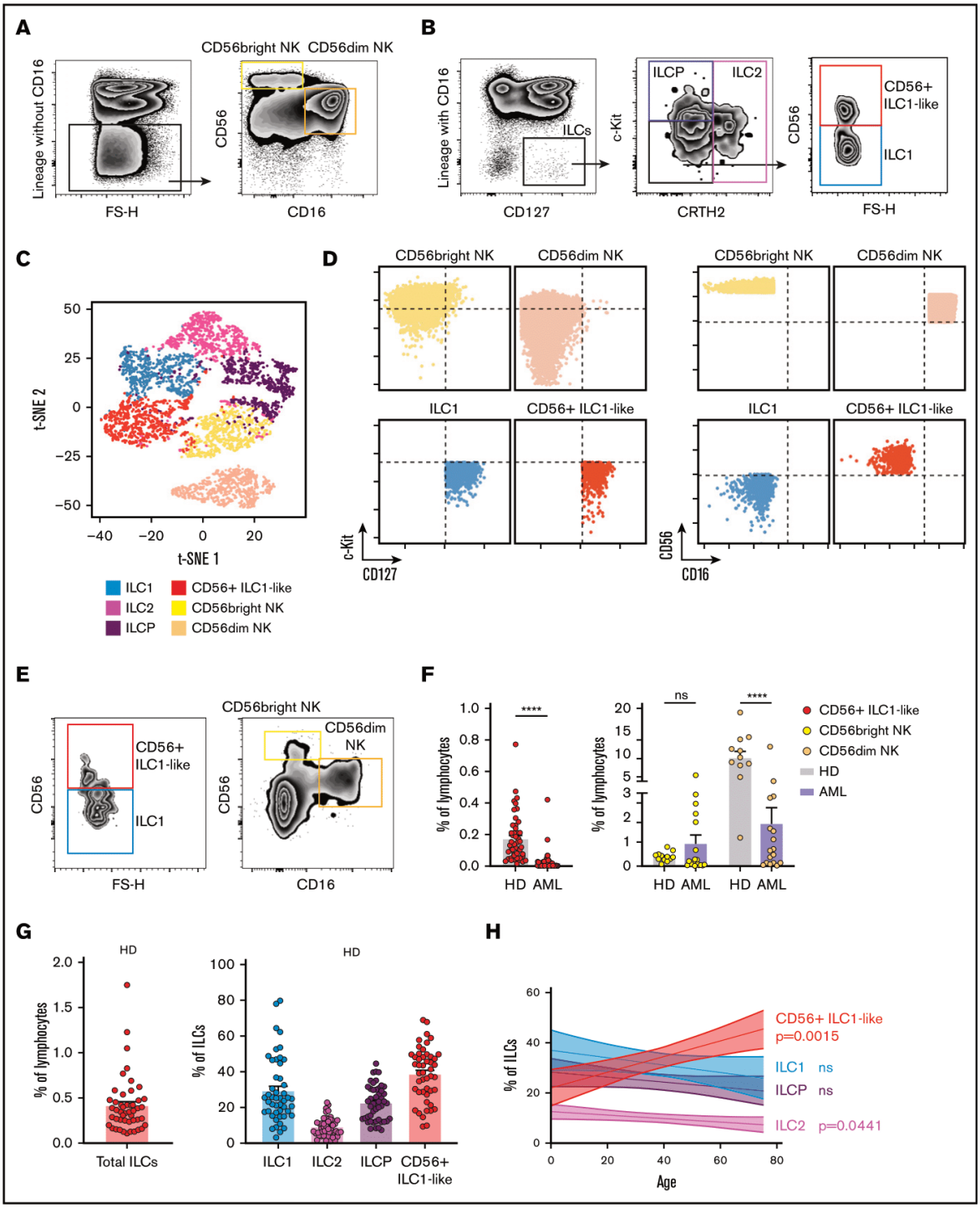


Figure 1. Identification of a CD56$^+$ ILC1-like population with NK properties that is impaired in AML patients at diagnosis. (A-B) Representative density plots of the extracellular flow cytometry panel used to identify the cNK cell subsets (A) and the ILC subsets (B) in peripheral blood (PB) mononuclear cells (PBMCs; ILC1 as CRTH2$^-$ c-Kit$^-$ CD56$^-$, ILC2 as CRTH2$^+$ c-Kit$^{+/-}$ CD56$^{+/-}$, ILCP as CRTH2$^-$ c-Kit$^+$ CD56$^{+/-}$, and cNKs as CD16$^-$ CD56^{bright} and CD16$^+$ CD56^{dim}).[21,22] Lineage markers used



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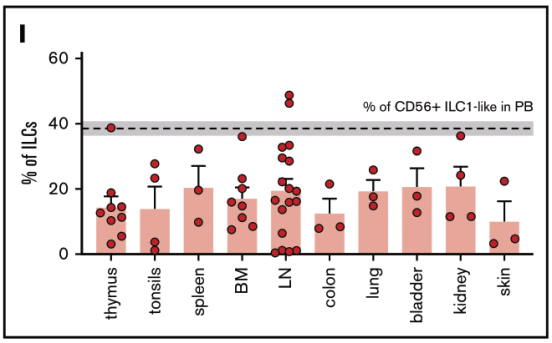

Figure 1. (Continued). for the "helper" ILC staining include CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c, and FcεRIα; same lineage markers, except for CD16, were used for the cNK staining. (C) CD127, CD56, CD16, CRTH2, c-Kit fluorescence intensity on ILC and NK subsets were concatenated from 5 HDs and analyzed with *t*-distributed stochastic neighbor embedding (*t*-SNE). c-Kit, CD127, CD56, CD16 expression levels on ILC1, $CD56^+$ ILC1-like cells and NKs are represented in panel D. Representative gating (E) and quantification of $CD56^+$ ILC1-like cell and cNK proportions among lymphocytes in blood from HDs and AML patients at diagnosis (F) ($CD56^+$ ILC1-like cells: HD, n = 47; AML patients: n = 60; cNKs: HD: n = 12, AML: n = 18). (G) Summary of the results of the total ILC proportions and ILC1, ILC2, ILCP, and $CD56^+$ ILC1-like cell subset frequencies among the total ILCs in HD peripheral blood (N = 47, age median 48, interquartile range 31 to 64). (H) Correlation between ILC subsets' relative frequencies in blood and age (cord blood: n = 9, children: n = 6, [3 to 12] years old, adults: n = 47, age mean 48). (I) $CD56^+$ ILC1-like cell relative frequencies among the total ILCs in tissues from healthy adults (n = 3-18). Spearman correlations were used in panel H. One dot = 1 donor. Mann-Whitney unpaired *U* tests were used in panel F. $^{****}P < .0001$. BM, bone marrow; LN, lymph node. ns, not significant.).

pathway GO0046649), immune effector process (GO0002252), leukocyte migration (GO0050900), and metabolism (GO0008152).

To confirm that $CD56^+$ ILC1-like cells display a specific metabolism compared with the conventional ILC and NK subsets, we evaluated their nutrient uptake and mitochondrial activity (supplemental Figure 4). The $CD56^+$ ILC1-like cells display a lower glucose analog (2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) uptake than the ILC2 and cNKs and a higher fatty acid uptake than the $CD56^{dim}$ NKs. To assess their specific mitochondrial activity, we evaluated the ratio of the uptake of MitoTracker deep red to that of MitoTracker green.[26] The ratio in $CD56^+$ ILC1-like cells is higher than in the ILC1 and tend to be higher compared with cNKs. Hence, the distinct metabolic transcriptional pattern of $CD56^+$ ILC1-like cells is reflected by their specific nutrient uptake and mitochondrial activity.

$CD56^+$ ILC1-like cells are cytotoxic effectors regulated by the NKp30, NKp80, TRAIL, and HLA-E pathways

Based on the $CD56^+$ ILC1-like cell-specific transcriptomic signature involving genes participating in immune effector processes and lymphocyte activation, we directly evaluated the $CD56^+$ ILC1-like cell cytotoxic activity (Figure 3). $CD56^+$ ILC1-like cells express NKp30, NKp80, CD94/NKG2A, and DNAM-1 at a high level, whereas low levels of NKp44, NKp46, and NKG2C were observed (Figure 3A). TRAIL expression is comparable between the $CD56^+$ ILC1-like cells and $CD56^{bright}$ NKs but varies depending on the donor's age (data not shown). Subsequently, we aimed to ascertain whether $CD56^+$ ILC1-like cells contain death-inducing mediators. We observed a significant $CD56^+$ ILC1-like cell production of granzymes A, B, K, and M, perforin and granulysin, with the highest levels measured for granzyme A and M (Figure 3B; supplemental Figure 5A). Next, we cocultured $CD56^+$ ILC1-like cells with the standard NK-sensitive $MHCI^-$ K562 cell line (Figure 3C). After 4 hours, $CD56^+$ ILC1-like cells upregulate CD107a, demonstrating their ability to degranulate. To confirm their cytotoxicity, we analyzed $CD56^+$ ILC1-like cell-mediated target lysis using ^{51}Cr-release assays. $CD56^+$ ILC1-like cells lyse K562 to an extent comparable to that of cNKs, whereas the helper ILCs fail to induce any target cell lysis (Figure 3D). In accordance with the absence of KIR receptors, $CD56^+$ ILC1-like cells are also able to lyse the $MHCI^+$ BJAB and U937 cell lines (Figure 3E; supplemental Figure 5B). To investigate whether DNAM-1, NKp30, NKp80, and TRAIL, which are expressed at the highest levels by $CD56^+$ ILC1-like cells (Figure 3A), are involved in cytotoxicity, we cocultured $CD56^+$ ILC1-like cells with targets in the presence of specific blocking reagents (Figure 3F). The addition of anti–DNAM-1 blocking antibodies does not affect the $CD56^+$ ILC1-like cell killing potential. However, the presence of anti-NKp30 blocking antibodies strongly reduces their killing activity. We also confirmed that TRAIL is involved in their cytotoxicity as the $CD56^+$ ILC1-like cell-mediated killing of the TRAIL-sensitive BJAB line[27-29] was decreased in the presence of a TRAIL decoy receptor. Subsequently, we cocultured the $CD56^+$ ILC1-like cells with the U937 line, which expresses the ligand for NKp80 (ie, Activation-Induced C-type Lectin (supplemental Figure 5C). The addition of NKp80 blocking antibodies decreases the $CD56^+$ ILC1-like cell-killing capacity. Finally, based on the high expression of CD94/NKG2A by $CD56^+$ ILC1-like cells (Figure 3A), we compared the $CD56^+$ ILC1-like cell-mediated lysis of wild-type (HLA-E negative) and HLA-E–transfected ($HLA\text{-}E^+$) 721.221 tumor cells (Figure 3G; supplemental Figure 4D). $CD56^+$ ILC1-like cell cytotoxicity is impaired when the targets express HLA-E. Overall, we show that $CD56^+$ ILC1-like cells display high cytotoxicity triggered by the NKp30, NKp80, and TRAIL pathways and inhibited upon HLA-E binding.

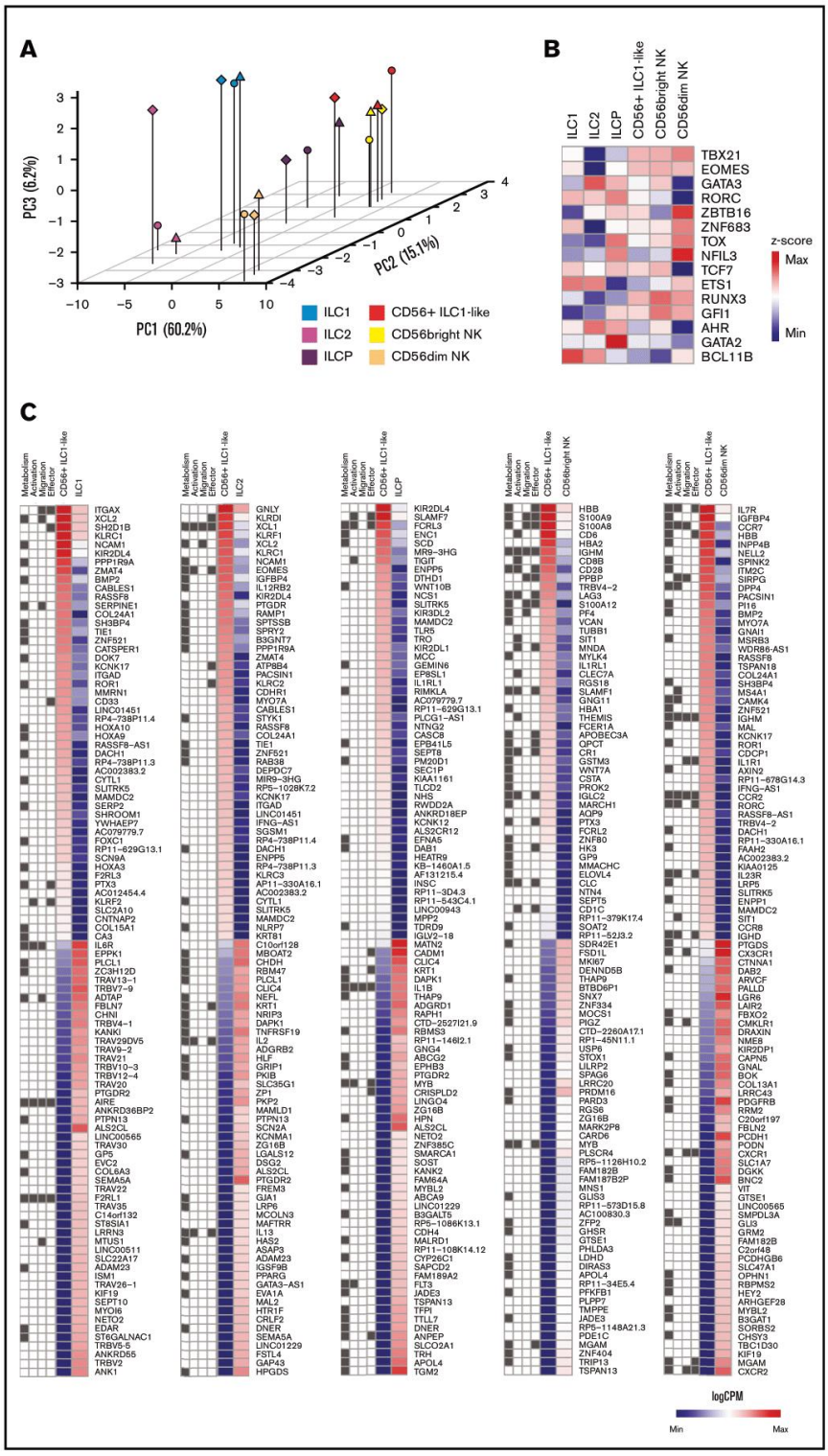

Figure 2. Transcriptomic signature of ex vivo CD56^{+} ILC1-like cells in peripheral blood from HDs. (A) Principal component analysis (PCA) of ex vivo fluorescence-activated cell–sorted ILC and NK subsets from HDs peripheral blood (n = 3). (B) Heat map of *z* scores of the expression levels of genes encoding ILC/NK transcription factors (n = 3). (C) Heat map of log counts per million (CPM) of the 100 most differentially expressed genes between CD56^{+} ILC1-like cells and ILC1, ILC2, ILCP, CD56^{bright} NKs or CD56^{dim} NKs. The GO pathway to which each gene belongs is represented at the left of each heat map: Metabolic process GO0008152 ("Metabolism"); Lymphocyte activation GO0046649 ("Activation"); Leukocyte migration GO0050900 ("Migration"); Immune effector process GO0002252 ("Effector"). Max, maximum; Min, minimum.

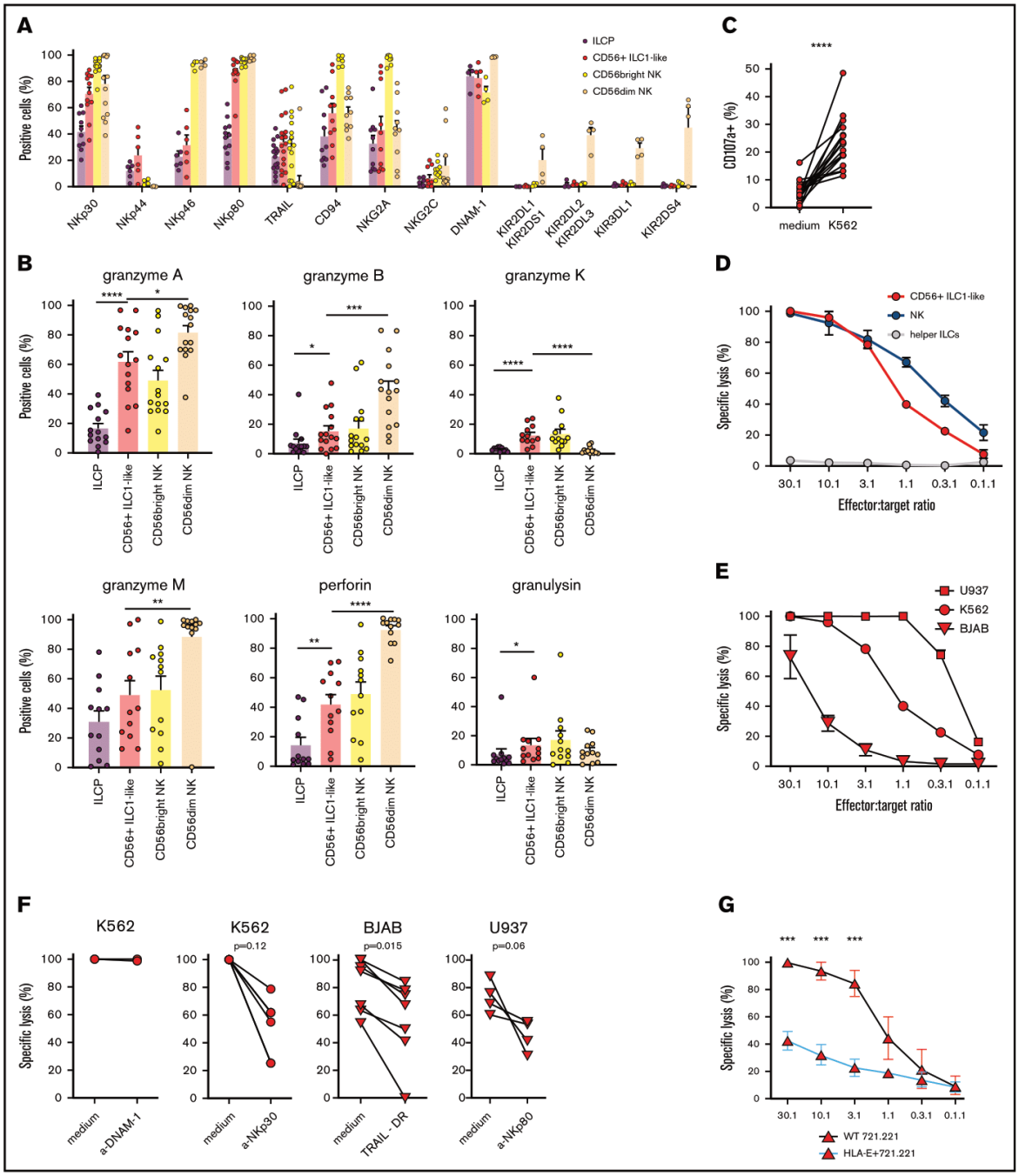

Figure 3. CD56$^+$ ILC1-like cells are cytotoxic effectors regulated by the NKp30, NKp80, TRAIL, and HLA-E pathways. (A) Extracellular flow cytometry analysis of NK receptor expression in ILCP, CD56$^+$ ILC1-like cells, and cNKs (n = 4-18). (B) Intracellular flow cytometry was performed using HD PBMCs to assess CD56$^+$ ILC1-like cell production of granzyme A (n = 15), granzyme B (n = 15), granzyme K (n = 12), granzyme M (n = 12), perforin (n = 12), and granulysin (n = 12). (C) Extracellular flow cytometry was performed after a 4-hour coculture of ILC/NK-enriched PBMCs with K562 (ratio E:T 1:1), anti-CD107a, and Golgistop to assess CD56$^+$ ILC1-like cell degranulation (n = 16). (D) Specific lysis of the K562 tumor cell line by CD56$^+$ ILC1-like cells, cNKs, and helper ILCs (results in duplicate). (E) Specific lysis of the U937, K562, and BJAB tumor cell lines by CD56$^+$ ILC1-like cells (results in duplicate). (F) Specific lysis by CD56$^+$ ILC1-like cells of K562 (ratio E:T 20:1), BJAB (ratio E:T 20:1), and U937

CD56$^+$ ILC1-like cells possess common features with NK cell developmental intermediates

Freud and colleagues recently showed that ILC3 and NKs share a common progenitor that is distinct from ILC2 progenitors.[30] They also demonstrated that S4a NK developmental intermediates possess both NK- and ILC3-related characteristics.[31] We thus compared the CD56$^+$ ILC1-like cells with the NK developmental intermediates in terms of phenotype, based on their previously published data[31] (Table 1). NKp80 is acquired in NKs upon maturation from the S4b stage, alongside the production of cytotoxic mediators. Based on their expression of NKp80 and cytolytic activity, CD56$^+$ ILC1-like cells are more mature than the S3 and S4a NK cells. However, being CD16-negative, these cells are clearly distinct from S5 NKs. CD56$^+$ ILC1-like cells share similar features with S4b NKs, because they express CD94/NKG2A heterodimers, NKG2D, CXCR3, and CD122, while being devoid of KIR receptors in blood. However, S4b NKs are reportedly CD127$^{low/-}$ and c-Kit$^{+/-}$. S4b mainly comprises CD56^{bright} NKs, which have a distinct transcriptomic profile compared with CD56$^+$ ILC1-like cells (Figure 2C). In order to formally evaluate the capacity of CD56$^+$ ILC1-like cells to differentiate into other ILC subsets or NK developmental stages, we sorted these cells and cultured them on OP9 stromal cells in the presence of IL-7 with or without the addition of IL-15 (supplemental Figure 6A-B). Their phenotype was analyzed after 10 days. The CD16$^-$ CD94^{high} c-Kit$^-$ cell population remains the major one upon culture, in particular, in the presence of IL-15. Two minor cell populations emerge by the upregulation of CD16 or c-Kit. Next, to determine whether CD56$^+$ ILC1-like cells represent an effector population generated in the periphery, we monitored their presence in human fetal tissues and during immune reconstitution in humanized mice. CD56$^+$ ILC1-like cells were detectable in all human fetal tissues analyzed (supplemental Figure 6C) and could be identified as early as 8 weeks postreconstitution in humanized mice (supplemental Figure 6D-E). To further dissect their developmental requirement, we monitored the CD56$^+$ ILC1-like cell frequencies in patients with severe combined immunodeficiency (supplemental Figure 6F-G). As conventional ILCs and NKs, CD56$^+$ ILC1-like cells require JAK3 and ADA genes for their development, whereas they are able to develop in the absence of ARTEMIS and CD3D genes. We identified CD56$^+$ ILC1-like cells and cNKs, yet at reduced levels, in an IL2RG-deficient patient, suggesting a hypomorphic mutation with reduced penetrance.[32,33] These cells, as cNKs and helper ILCs, are also present in a RAG1-deficient patient, whereas they are decreased in a RAG2-deficient patient, in contrast to cNK and helper ILCs. RAG1 is expressed in common lymphoid progenitors that are committed to the NK lineage[34] and might thus be required for the differentiation of CD56$^+$ ILC1-like cells. The absence of CD56$^+$ ILC1-like cells in the RAG1-deficient patient might also be due to the ILC and NK genomic instability observed in mice upon RAG deficiency.[35] Overall, these results suggest that CD56$^+$ ILC1-like cells are related to S4b NK cells and therefore might share similarities with cNKs in terms of development.

Table 1. Extracellular phenotype of CD56$^+$ ILC1-like cells compared to previously published NK development stage phenotypes

Marker	S3	S4a	S4b	S5	CD56$^+$ ILC1-like
CD16	−	−	−	+	−
CD56	+/−	+	+	+	+
CD127	+	+/−	Low/−	−	+
c-KIT	+	+/−	+/−	+/−	−
CD94	−	+	+	+/−	+
NKG2A	−	+	+	+	+
NKG2C	−	−	+/−	+/−	+/−
NKG2D	−	+	+	+	+
KIR2D	−	−	−	+	−
NKp80	−	−	+	+	+
CXCR3	−	+/−	+	+	+
CD122	−	+	+	+	+
Perforin	−	−	+	+	+
Granzyme A	−	−	+/−	+	+
Granzyme B	−	−	+/−	+/−	+
Granzyme K	−	−	+	+	+

+, expression of the marker; −, no expression of the marker; Low, low expression of the marker.

CD56$^+$ ILC1-like cytotoxicity is impaired in AML patients at diagnosis

In order to evaluate the role of CD56$^+$ ILC1-like cells in AML disease, we investigated their cytotoxic profile at diagnosis. The CD56$^+$ ILC1-like cell expression of TRAIL, NKp30, and NKp80 is strongly reduced in the patients compared with HDs (Figure 4A). Notably, TRAIL and NKp80 are specifically decreased in the CD56$^+$ ILC1-like cells but not in the cNKs. Then, we analyzed the CD56$^+$ ILC1-like cell release of cytotoxic mediators. The patients' CD56$^+$ ILC1-like cells produce amounts of granzymes A, B, and K and perforin that are similar to those in the HDs, but no granulysin is produced. In contrast, the granulysin levels in the cNKs are not impaired in the patients, and the granzyme A level is even increased in CD56^{bright} NKs in AML (Figure 4B). The degranulation of CD56$^+$ ILC1-like cells in AML is significantly impaired as assessed in cocultures with the K562 line or autologous blasts (Figure 4C-D). This impairment could be explained by the regulation of CD56$^+$ ILC1-like cell cytotoxicity through NKp30, TRAIL, NKp80, or HLA-E in the case of primary AML blasts. Indeed, we observed HLA-E expression in primary AML blasts and NKG2A expression in patients' CD56$^+$ ILC1-like cells, suggesting that the HLA-E/CD94-NKG2A pathway negatively regulates CD56$^+$ ILC1-like cells (Figure 4E-F). In line with this, in the patients, the CD94/NKG2A expression is preserved on CD56$^+$ ILC1-like cells. Overall, we show that the CD56$^+$ ILC1-like cell cytotoxic machinery is impaired in AML patients at diagnosis, suggesting that these cells are unable to limit AML oncogenesis.

Figure 3. (continued) (ratio E:T 10:1) tumor cell lines in the presence of anti-DNAM-1, anti-NKp30, and anti-NKp80 blocking antibodies or TRAIL decoy receptor. (G) Specific lysis of wild-type (WT) or HLA-E–transfected 721.221 tumor cell lines by CD56$^+$ ILC1-like cells (results in triplicate). One dot = 1 donor. Statistical tests used for analyses: panel B: Mann-Whitney unpaired *U* test; panel C: Wilcoxon paired *t* test, panel G: multiple Holm-Sidak *t* tests. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

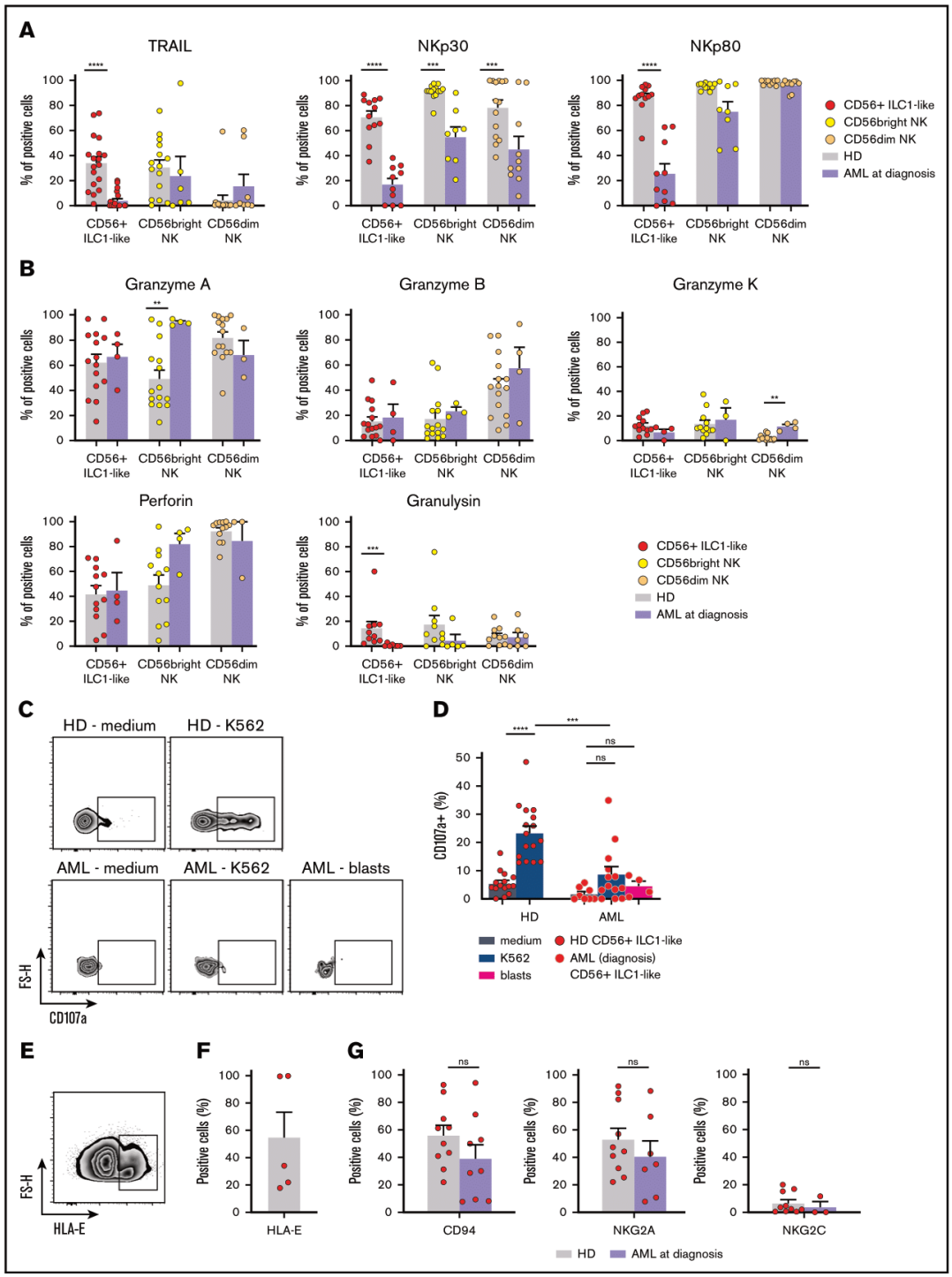

Figure 4. CD56$^+$ ILC1-like cell cytotoxicity is impaired in AML patients at diagnosis. (A) TRAIL, NKp30, and NKp80 expression in CD56$^+$ ILC1-like cells and cNKs in HDs (CD56$^+$ ILC1-like: n = 12-18, cNKs: n = 11-15) and AML patients at diagnosis (CD56$^+$ ILC1-like: n = 11-20, cNKs: n = 8-10) by extracellular flow cytometry. (B) Intracellular flow cytometry was performed using PBMCs from AML patients at diagnosis to assess granzyme A (n = 4), granzyme B (n = 4), granzyme K (n = 4), perforin

CD56$^+$ ILC1-like cell cytotoxicity is restored in AML patients during remission and might be modulated by leukemic blasts and TGF-β1

The CD56$^+$ ILC1-like cell proportions in the AML patients at diagnosis and during remission are comparable (n = 14) (Figure 5A). However, we observed a trend toward a CD56$^+$ ILC1-like cell increase upon remission in 5 of 7 patients with paired diagnosis/remission peripheral blood samples (Figure 5B). Then, we evaluated the CD56$^+$ ILC1-like cell degranulation potential in the AML patients during remission. Notably, the CD56$^+$ ILC1-like cell degranulation capacity toward the K562 cells is restored in remission (Figure 5C). As we previously showed that TRAIL, NKp30, and NKp80 were involved in CD56$^+$ ILC1-like cell cytotoxicity (Figure 3F) and that their expression was decreased in CD56$^+$ ILC1-like cells from AML patients at diagnosis (Figure 4A), we investigated their expression in CD56$^+$ ILC1-like cells in remission (n = 6) (Figure 5D). Interestingly, the expression of these molecules is restored in the patients during remission.

We aimed to explore the putative mechanism(s) inhibiting the cytotoxicity of CD56$^+$ ILC1-like cells in patients. To determine whether AML blasts directly impair CD56$^+$ ILC1-like cell functions, we removed the leukemic blasts from PBMCs from AML patients at diagnosis and cultured the blast-depleted cells for 24 hours. Although NKp30 and NKp80 expression is not modulated by the absence of blasts, TRAIL expression is upregulated in the CD56$^+$ ILC1-like cells following blast removal (Figure 5E). A positive correlation was observed between the circulating AML blast frequencies and the extent of TRAIL restoration following blast removal (Figure 5F). To identify potential candidates leading to the CD56$^+$ ILC1-like cell impaired function in AML, we cultured CD56$^+$ ILC1-like cells from HDs with different inhibitory molecules known to be involved in the AML immunosuppressive microenvironment. Interestingly, we observed that the TRAIL and NKp30, but not NKp80, expression levels are decreased following a 24-hour culture with human recombinant TGF-β1 (Figure 5G). TGF-β1 possibly binds TGF-βR, which we found to be expressed on CD56$^+$ ILC1-like cells (Figure 5H). We also found circulating TGF-β1 in the patient serum (Figure 5I). An analysis of data from TCGA shows that overall survival is lower in the patients with a high TGF-β1 expression level (Figure 4J), in line with its immunosuppressive effect.[37] We thus hypothesize that TGF-β1 might impair CD56$^+$ ILC1-like cell cytotoxicity in AML patients. AML blasts have been shown to produce AhR ligands.[38] We thus incubated NK/ILC-enriched PBMCs from HDs with the AhR ligand FICZ overnight in the presence of IL-2 and evaluated its impact on cNKs and CD56$^+$ ILC1-like cell degranulation toward K562 (supplemental Figure 7A). AhR ligand slightly decreases the degranulation capacity of CD56$^+$ ILC1-like cells and CD56dim NKs in 5 out of 8 donors. Preincubating NK/ILC-enriched PBMCs with the AhR agonist before IL-12, IL-15, and IL-18 stimulation also impairs CD56$^+$ ILC1-like cell function, in terms of their interferon-γ production (supplemental Figure 7B). Overall, CD56$^+$ ILC1-like cell cytotoxicity in AML might be impaired by TGF-β1 and AhR ligands and is restored upon remission.

CD56$^+$ ILC1-like cells are present in NK-cell preparations used for NK-cell transfer therapy

Finally, because CD56$^+$ ILC1-like cells constitutively express the CD56 marker (Figure 1B), we hypothesized that these cells might be present in NK preparations used for NK cell transfer to AML patients. Indeed, haploidentical NK cell transfer consists of injecting enriched CD3$^-$CD56$^+$ haploidentical cells.[3-6] To verify this hypothesis, we applied NK cell purification protocols used for transfer therapy to PBMCs from HDs (n = 10). After the 2-step purification process used in the clinical setting, we evaluated the presence of ILCs in the preparation by flow cytometry. Interestingly, the fraction of purified CD3$^-$CD56$^+$ cells contains CD56$^+$ ILC1-like cells in addition to CD16$^-$CD56^{bright} and CD16$^+$CD56^{dim} cNKs (Figure 6A-B). Of note, the CD56$^+$ ILC1-like cell phenotype is conserved after the enrichment (Figure 6C). Therefore, cytotoxic CD56$^+$ ILC1-like cells are present in cell preparations used for the haploidentical transfer of NK cells in AML patients.

Discussion

We report the identification of an unconventional CD127$^+$ CD56$^+$ c-Kit$^-$ ILC population sharing features with both ILCs and S4b NKs. This innate effector cell type might represent an attractive target for expanding the current options of immunotherapy for AML patients, whose prognosis remains overall unsatisfactory.[36]

Despite their relatedness with helper ILCs and cNKs in terms of nomenclature, we observed that CD127, c-Kit, CD56, CRTH2, and CD16 expression at protein level are sufficient to discriminate these CD56$^+$ ILC1-like cells. RNA sequencing of these cells shows distinct transcriptomic signature compared with helper ILCs and cNKs.

CD56$^+$ ILC1-like cells share similar features with S4b NK cells in terms of phenotype and function, based on published data.[30,31] However, the S4b NK cells are reported to be CD127$^{low/-}$. Upon culture on OP9 stromal cells, CD56$^+$ ILC1-like cells are able to partially differentiate into both S5 and S4a NK cells. This finding is consistent with their closeness to S4b NKs known to differentiate into S5 NKs. Future studies should further compare the differentiation potential of CD56$^+$ ILC1-like cells and S4b NK cells using OP9 stromal cell culture and humanized mice. The dynamics of transcription factor expression by CD56$^+$ ILC1-like cells throughout the differentiation process and in disease should be further assessed in humanized mice and in AML patients. Based on current data, we hypothesize that CD56$^+$ ILC1-like cells might represent a developmental intermediate subpopulation between ILCs and NK cells. CD56$^+$ ILC1-like cells are present at fetal stage, yet their proportion increases with the age, suggesting an age-driven maturation of these cells. ILCs and NK cells lack the requirement for prior sensitization or exposure to elicit a response to a pathogen or tumor. We offer a hypothesis that CD56$^+$ ILC1-like

Figure 4. (continued) (n = 4), and granulysin (n = 7) expression in the CD56$^+$ ILC1-like cells and cNKs. The results are compared with the values obtained in the HDs. (C-D) CD107a expression in CD56$^+$ ILC1-like cells is assessed by flow cytometry after a 4-hour incubation of ILC/NK-enriched PBMCs from HDs (n = 16) or AML patients at diagnosis (n = 4-13) with medium, the K562 tumor cell line, or blasts at a ratio of 1:1. Representative density plot of CD107a expression is shown in panel C, and the summary results are shown in panel D (1 dot = 1 donor). (E) Representative density plot of HLA-E expression in primary leukemic AML blasts. (F) Summary of HLA-E expression in primary leukemic AML blasts (n = 5). (G) Flow cytometry analysis of CD94, NKG2A, and NKG2C in CD56$^+$ ILC1-like cells from AML patients at diagnosis (n = 3-10) and HDs (n = 5-17). One dot = 1 donor. Statistical tests used: panels A-B,D,G: Mann-Whitney *U* test. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

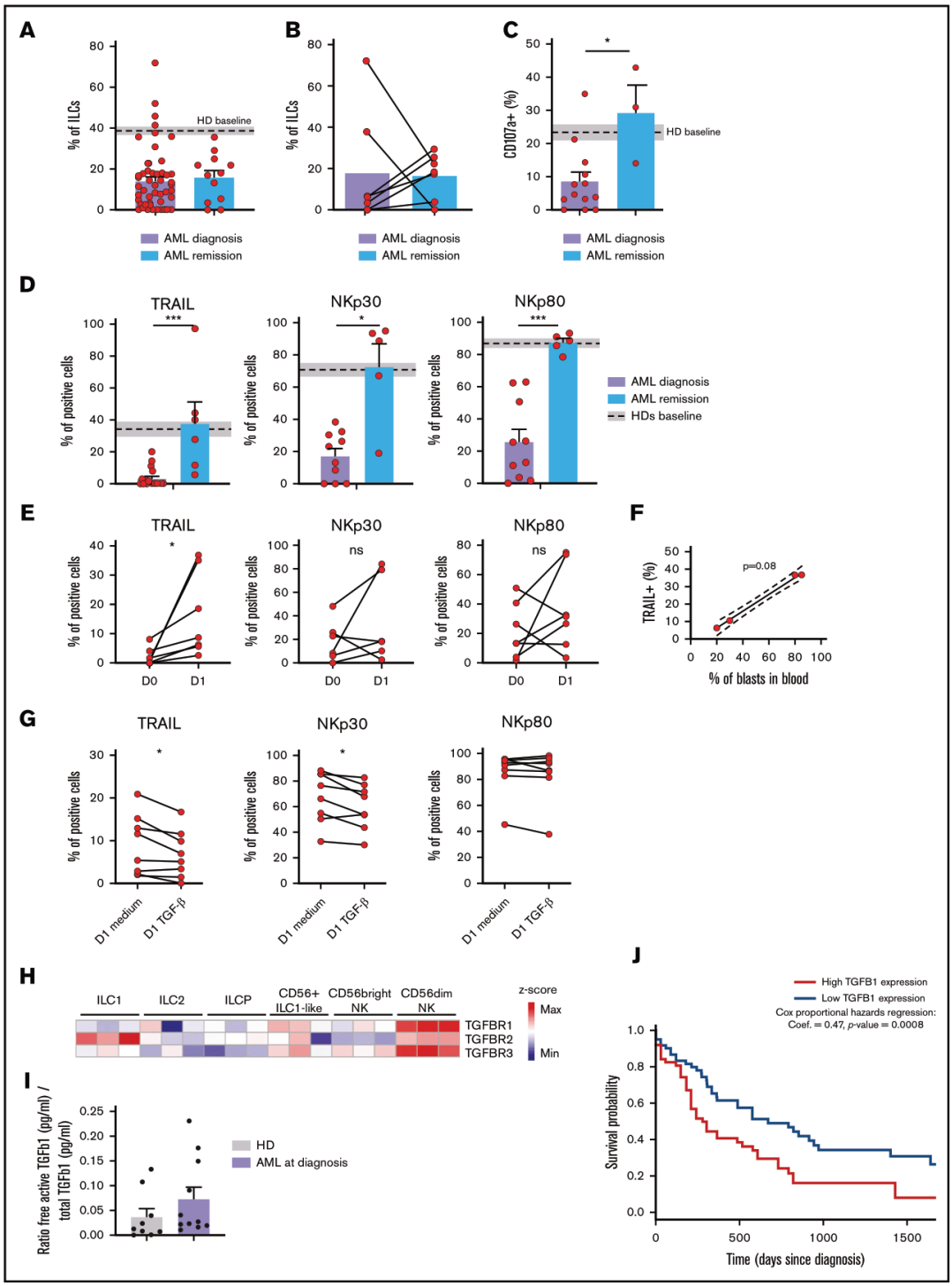

Figure 5. CD56$^+$ ILC1-like cell cytotoxicity is restored in AML patients during remission and is modulated by blasts and TGF-β1. (A) Comparison of peripheral blood (PB) CD56$^+$ ILC1-like cell relative frequencies in AML patients at diagnosis and during remission (n = 12). (B) Comparison of PB CD56$^+$ ILC1-like cell frequencies in

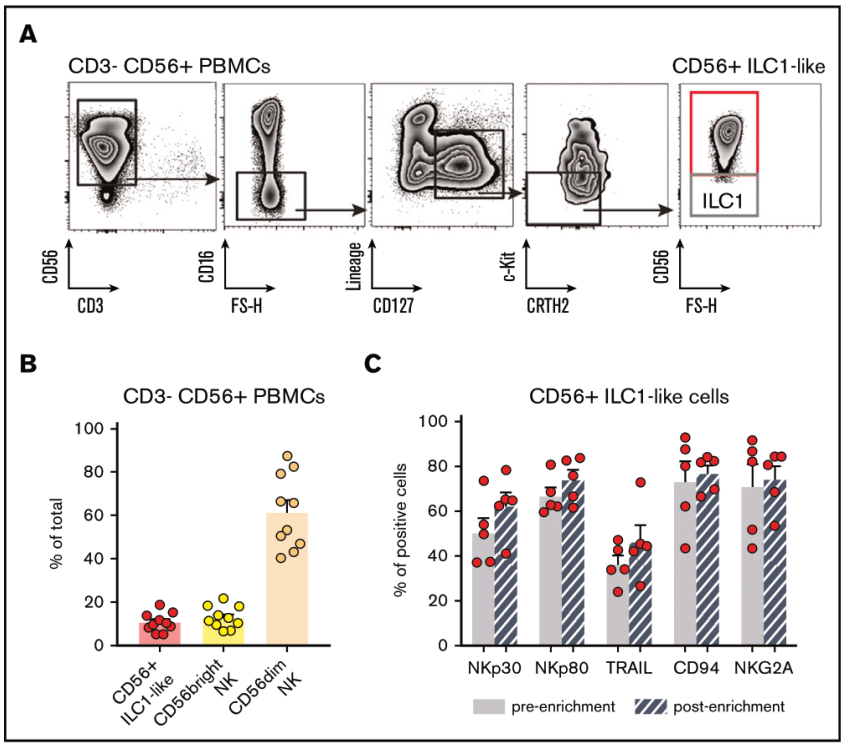

Figure 6. CD56$^+$ ILC1-like cells are present in NK-cell preparations used for NK-cell transfer therapy. (A) Representative density plots of the extracellular flow cytometry panel used to identify the ILC subsets in CD3$^-$ CD56$^+$-enriched fractions of HD PBMCs. (B) Proportions of total ILCs (Lineage$^-$CD127$^+$), CD56^{bright} CD16$^-$, and CD56^{dim} CD16$^+$ NKs in NK-cell transfer therapy products from HD PBMCs (n = 10). (C) NK marker expression on PB CD56$^+$ ILC1-like cells before and after the CD3$^-$ CD56$^+$ enrichment (n = 5).

cells might continue to promote surveillance of tumorigenesis or microbial infections in settings of waning antitumor or antimicrobial cell-mediated immunity. These observations further support the hypothesis that triggering these cells in patients with cancer might help to restrict tumor burden.

Although CD56$^+$ ILC1-like cells can secrete cytokines, their main function resides in their KIR-independent cytotoxic activity. Uncontrolled CD56$^+$ ILC1-like cell activities might be limited by NKG2A/CD94, whereas NKp30, NKp80, and TRAIL are the main receptors conferring cytotoxic potential. We previously described that NKp30 overexpression in ILC2 in patients with acute promyelocytic leukemia[39] is linked to their hyperactivity. NKp80 has been studied in the context of NK cell maturation[31] and has been described as an NK cell coactivating receptor,[40] but no reports regarding its expression and role in human ILCs are available. Our results suggest that its presence might be relevant in the context of Activation-Induced C-type Lectin$^+$ targets. Finally, unconventional Eomes$^-$ group 1 ILCs have been reported to express TRAIL in the liver and exert TRAIL-dependent cytotoxicity.[41-43] Here, we show that the CD56$^+$ ILC1-like cell cytotoxic machinery is impaired in AML patients. Therefore, by therapeutically activating the NKp30, NKp80, and TRAIL pathways and inhibiting NKG2A/HLA-E interactions, the cytotoxicity of CD56$^+$ ILC1-like cell might be restored, and subsequently AML could be controlled. Hence, the administration of the checkpoint inhibitor anti-NKG2A antibody[44] could unleash hypofunctional CD56$^+$ ILC1-like cells in AML patients for whom alloreactive NK cell donors cannot be found. Future studies comparing the activity of anti-NKG2A antibodies on CD56$^+$ ILC1-like cells and NKs in AML patients with HLA-E$^+$ and HLA-E$^-$ blasts will provide valuable insights on this regard.

Figure 5. (continued) paired AML patient samples at diagnosis and during remission (n = 7). (C) Comparison of CD56$^+$ ILC1-like cell AML patients at diagnosis or during remission to determine the degranulation capacity after a 4-hour coculture of ILC/NK-enriched PBMCs with K562 (ratio E:T 1:1), anti-CD107a, and Golgistop (remission: n = 3). (D) Comparison of TRAIL, NKp30, and NKp80 expression in PB CD56$^+$ ILC1-like cells from AML patients at diagnosis (n = 10) and during remission (n = 5). (E-F) PBMCs from AML patients at diagnosis were depleted of CD33$^+$ blasts and cultured for 24 hours in complete medium. (E) Extracellular flow cytometry was performed to assess TRAIL, NKp30, and NKp80 expression in CD56$^+$ ILC1-like cells (n = 6-7). Correlation between TRAIL expression after the 24 h culture and blast frequencies in PB (n = 4, panel F). (G) PBMCs from HDs were enriched in ILC/NK cells and cultured for 24 hours with medium only or supplemented with rhTGF-β1 at 5 ng/mL (n = 8). TRAIL, NKp30, and NKp80 expression was assessed by flow cytometry after the culture. (H) Heat map of *z* scores of expression levels of genes encoding TGF-β receptors in ILCs and cNKs (n = 3). (I) Free/total TGF-β1 ratio in sera from HDs and AML patients. Sera with concentrations above the limit of detection of the assay are shown (HDs: n = 9, AML patients: n = 11). (J) Kaplan-Meier overall survival analysis based on TGF-β1 expression in AML patients from The Cancer Genome Atlas program (TCGA) (n = 125). We excluded patients presenting a t(15;17) translocation (ie, patients with acute promyelocytic leukemia as classified according to the 2017 European LeukemiaNet recommendations[36]) from our analysis since this condition represents a distinct AML pathophysiological entity. Statistical tests used: panels C-D: Mann-Whitney unpaired *U* test; panels E,G: Wilcoxon paired *t* test; panel F: Spearman correlation; panel J: difference in overall survival (OS) between AML patients with high (n = 64) or low (n = 61) TGF-β1 expression based on TCGA data. *$P < .05$, ***$P < .001$.

Furthermore, our data show that in vitro removal of leukemic blasts contributes to the recovery of TRAIL expression in CD56$^+$ ILC1-like cells. This observation might partially explain the in vivo restored expression of TRAIL in CD56$^+$ ILC1-like cells in AML patients during remission. It has been recently reported that TGF-β controls TRAIL expression in salivary gland ILCs,[45] whereas it has been previously reported that leukemic-blast derived microvesicles containing TGF-β1 inhibit TGF-β–receptor expressing immune cells in AML.[46] Our findings that TGF-β1 in vitro induces the downregulation of TRAIL and NKp30 in CD56$^+$ ILC1-like cells and that elevated TGF-β1 concentrations are associated with patients' poor survival suggest that blocking TGF-β1 using small molecule inhibitors, such as galunisertib, might restore the CD56$^+$ ILC1-like cell activity. However, future studies directly assessing the impact of TGF-β1 inhibitors on HDs and AML patients' CD56$^+$ ILC1-like cell cytotoxicity should be performed to confirm these correlative observations. Then, coadministration with anti-NKG2A antibodies might heighten the therapeutic benefits with rather favorable toxicity profiles as suggested by preclinical studies using combinations of TGF-βRI inhibition and checkpoint blockade.[47] Furthermore, AML blasts have been shown to impair NK cell functions in AML patients through the secretion of AhR ligands.[38] Our results argue that these ligands also partially impair CD56$^+$ ILC1-like cell functions and might thus be involved in their decreased cytotoxicity in AML patients.

It has been reported that NKp46 triggers TRAIL expression in ILC1 in mice.[48] However, in AML patients, we found no correlation between NKp46 and TRAIL expression in CD56$^+$ ILC1-like cells ($n = 8$, $P = .76$), suggesting that distinct mechanisms underlie the regulation of receptor expression across species. The observed downregulation of activating receptors might be due to the long-lasting contact with their ligands,[49] ultimately leading to CD56$^+$ ILC1-like cell dysfunction. Alternatively, the overexpression of inhibitory receptors might contribute to CD56$^+$ ILC1-like cell impaired function in AML patients. Although the expression of "common" immune checkpoints (eg, PD-1, CTLA-4, and BTLA) was absent in CD56$^+$ ILC1-like cells from both the HDs and the patients, we observed significant levels of IL-1R8 in the HDs' CD56$^+$ ILC1-like cells (data not shown). IL-1R8 represents a novel immune checkpoint that was shown to negatively regulate NK cell maturation and antiviral and antitumor functions in mice, but it has not been reported in human ILCs to date.[50] Future studies are needed to assess the impact of this receptor on human CD56$^+$ ILC1-like cells.

Finally, given that the CD56 marker is a major discriminator of the CD56$^+$ ILC1-like cell phenotype from that of helper ILCs, we hypothesized and confirmed that CD56$^+$ ILC1-like cells are present in the CD3$^-$CD56$^+$-enriched NK cell fractions infused in AML patients. Since several studies have demonstrated that patients' responses can be influenced by the composition of the NK cell graft,[51] the impact of the CD56$^+$ ILC1-like cell presence on parameters, such as relapse and survival, urgently needs to be investigated. In that regard, we envisage to characterize and quantify CD56$^+$ ILC1-like cells in NK preparations that will be transferred to patients to determine the correlation with the clinical outcome (Clinical trial Bologna NKAML Trial 035/2017/0/Sper). Triggering the CD56$^+$ ILC1-like cell compartment in these enriched fractions by anti-NKG2A, monoclonal antibodies could potentiate their KIR-independent antileukemic action. Alternatively, enriching NK fractions with the CD56$^+$ ILC1-like cell compartment or ex vivo expanding CD56$^+$ ILC1-like cells similarly to NK cell expansion, which has been proven to be safe and feasible,[52] might help obtain better clinical results without the need of KIR-mismatch.

Overall, this work highlights the importance of improving our knowledge of this CD127$^+$ c-Kit$^-$ CD56$^+$ population to help select the best donors for patients and maximize the effects of immunotherapy in AML.

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Authorship

Contribution: B.S., A.G.-C., R.L., G.V., D.F.R., S.T., and C.J. performed the experiments; V.S., M.R., C.R., P.T., E.B., E.-M.J., C.C., M.H., A. Schulz, K.M., A.T., S.S., A.O., M.G.D.P., D.M., A.C., P.-C.H., and A. Steinle provided reagents and patient samples; B.S., T.W., M.S., and D.G. conducted the analysis of the RNA sequencing data; A.H., P.J., and E.M. interpreted and discussed the results; and B.S., A.G.-C., P.R., S.T., and C.J. designed the research, analyzed the experiments, discussed the results, and wrote the manuscript.

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Annex III

Immunosuppressive Mediators Impair Proinflammatory Innate Lymphoid Cell Function in Human Malignant Melanoma

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Immunosuppressive Mediators Impair Proinflammatory Innate Lymphoid Cell Function in Human Malignant Melanoma

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ABSTRACT

Innate lymphoid cells (ILC) are a family of immune cells that are emerging as potent orchestrators of immune responses. In cancer, ILCs display both pro- and antitumorigenic functions depending on the nature of the tumor and the involved ILC subset. Little is known about the ILC–tumor cross-talk in human melanoma. Here, we showed that ILC1s were enriched but functionally impaired in cytokine secretion in both peripheral blood mononuclear cells and tumor-infiltrated lymph nodes of melanoma patients. These findings were confirmed *in vivo* in murine cutaneous melanoma. Multiple immunosuppressive mechanisms are described in the melanoma microenvironment. Among others, adenosine and kynurenines were shown to suppress antitumor immune responses. By exposing ILCs to adenosine and kynurenines, we observed a similar shift toward the ILC1 subset distribution and impairment in proinflammatory cytokine production to that of patient samples studied *ex vivo*. Thus, we hypothesized that the immunosuppressive microenvironment of malignant melanoma might shape ILC subpopulations. Hence, we provide a rationale for the use of drugs targeting adenosine and kynurenine pathways in melanoma patients.

Introduction

Immunotherapy holds substantial promise for metastatic melanoma treatment, as demonstrated by the U.S. Food and Drug Administration (FDA) approval of oncolytic viruses that directly kill tumor cells and antibodies that target cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1). Although these treatments significantly increase overall survival, many patients do not respond or develop resistance (1). Experimental and clinical evidence has highlighted the importance of the tumor microenvironment (TME) in promoting cancer development and progression by fostering a local state of immunosuppression (reviewed in ref. 2). The TME is a heterogeneous ecosystem composed of a variety of cancer cells, stroma, and immune cells that engage in an intricate cross-talk, thus shaping antitumor immunity. In particular, there is increasing interest in the immunosuppressive circuits established within the TME, including the upregulation of indoleamine-2,3-dioxygenase 1 (IDO or IDO1) and the accumulation of adenosine (ADO ref. 3, 4). IDO1 is the key rate-limiting enzyme involved in the regulation of tryptophan catabolism, resulting in the production of L-kynurenine (reviewed in ref. 5). IDO1 activity inhibits the proliferation and activation of immune cells in the TME; in fact, tryptophan starvation and high kynurenines synergistically drive $CD8^+$ T cells, $CD4^+$ T cells, and natural killer (NK) cells to undergo cell-cycle arrest and functional anergy, and reduce the immunogenicity of dendritic cells (DC), leading to a tolerant state that further suppresses antitumor immune responses (6–9). The IDO1 inhibitor, indoximod, in combination with the anti–PD-1, pembrolizumab, increases the overall response rate (ORR) of advanced-melanoma patients in a phase II clinical trial (10); however, no survival benefit was shown in the phase III ECHO-301 trial that combined the IDO inhibitor epacadostat and pembrolizumab, suggesting the need for better designed IDO1-targeting trials (11).

ADO is another potent immunosuppressive metabolite that is mainly produced by the hydrolysis of ATP. This is mediated by the ectoenzyme CD39, which converts ATP to ADP and AMP, and by the rate-limiting ectonucleotidase CD73, which transforms ADP/AMP into adenosine (12). ADO's immunosuppressive effects are mainly exerted via activation of G protein–coupled adenosine receptors (ADORA), such as A2A and A2B, which are expressed in several immune cell populations, including $CD4^+$ T cells, $CD8^+$ T cells, and NK cells (13–15). Monteiro and colleagues characterized CD73 expression in human metastatic melanoma and in tumor-infiltrating mononuclear cells, supporting the idea that the use of anti-CD73 antibodies or A2AR inhibitors can enhance the antitumor activity of both PD-1 and CTLA-4 blocking antibodies (16). This idea is further demonstrated by various clinical studies focusing on targeting adenosine in solid tumors (4, 16, 17).

Innate lymphoid cells (ILC) contribute to the complex cell network within the TME by directly impacting the balance of antitumor immune responses (reviewed in refs. 18–20). ILCs are lineage-negative cells (as described in the Material and Methods section) constitutively expressing CD127 (IL7Rα chain). They are divided into

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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ILC1s, ILC2s, and ILC3s that are defined as the Th1, Th2, and Th17 counterparts, respectively, because they share the same transcription factor expression (T-bet, GATA3, and RORC, respectively) and similar cytokine profiles as adaptive helper T cells (21). In addition to the three mature ILC subsets, ILC progenitors (ILCP) can also circulate in the blood and share the $cKit^{+}CRTH2^{-}$ phenotype with ILC3, but lack other conventional ILC3 attributes (RORγt and IL17A secretion; ref. 22). Although the development, classification, plasticity, and functional diversity of ILCs has been widely studied, there are still little data regarding the role of ILCs in cancer development and progression. In particular, little is known about ILCs and their interaction with cellular and soluble components of the TME in malignant melanoma. In this study, we showed that ILC1s were enriched, but functionally impaired in both peripheral blood mononuclear cells (PBMC) and tumor-infiltrated lymph nodes (TILN) in melanoma patients. These findings were confirmed by using an *in vivo* model of cutaneous melanoma. In addition, we demonstrated that the immunosuppressive metabolites ADO and kynurenines directly modulated the ILC subset distribution and impaired their proinflammatory cytokine secretion, which might have direct implications for the optimization of current immunotherapy strategies.

Materials and Methods

Human cell collection

Venous blood was drawn from 48 unselected healthy, routine donors at the blood transfusion center of Lausanne, approved by the Human Research Ethics Committee of the Canton of Vaud, Switzerland. All subjects gave their written consent. PBMCs were isolated by Lymphoprep centrifugation (1,800 rpm, 20 minutes centrifugation without break, room temperature), washed twice and immediately cryopreserved in 90% fetal calf serum (FCS) and 10% DMSO. Peripheral blood (PB) from 41 stage III/IV malignant melanoma patients (median age 47; range, 24–80) was obtained from the Department of Oncology, University Hospital (CHUV), Lausanne, Switzerland (NCT00112242), under approval of the Lausanne University Hospital's Institute Review Board. PBMCs were isolated as for healthy blood donors (HD). Lymph node (LN) samples were collected during surgical intervention and immediately processed by mechanical disruption with a metallic strainer and a syringe piston, followed by filtering with a 100-μm filter. Mononuclear cells were subsequently isolated using the same protocol as for human PBMCs and immediately cryopreserved. Written informed consent was obtained from all HDs and patients in accordance with the Declaration of Helsinki.

ILC evaluation by flow cytometry

Human ILCs were identified as lineage (Lin)-negative and CD127-positive cells. Lineage markers, all FITC conjugated, include anti-human CD3 [UCHT1, Beckman Coulter (BC)], anti-human CD4 (SFCI12T4D11, BC), anti-human CD8 (MEM- 31, Immunotools), anti-human CD14 (RMO52, BC), anti-human CD15 (80H5, BC), anti-human CD16 (3G8, BC), anti-human CD19 (J3-119, BC), anti-human CD20 (2H7, BioLegend), anti-human CD33 (HIM3-4, BioLegend), anti-human CD34 (561, BioLegend), anti-human CD203c (E-NPP3; NP4D6, BioLegend), and anti-human FcεRIα (AER-37, BioLegend). Additional markers used include Brilliant Violet 421 anti-human CD127 (IL-7Rα; A019D5, BioLegend) or PE Dazzle anti-human CD127 (IL-7Rα; A019D5, BioLegend), PE anti-human CD117 (cKit; 104D2, BioLegend) or APC anti-human CD117 (cKit; YB5.B8, BD Biosciences), PE anti-human CRTH2 (CD294; BM16, BioLegend) or Brilliant Violet 421 anti-human CRTH2 (CD294; BM16, BD Biosciences) or PCF594 anti-human CRTH2 (CD294; BM16, BD Biosciences), PerCPCy5.5 anti-human CD335 (NKp46; 9E2, BD Biosciences), or PECy7 anti-human CD335 (NKp46; 9E2, BD Biosciences). Cells were stained for 20 minutes at room temperature in 50 μL of FACS buffer. Dead cells were excluded using the viability dye Live/Dead Aqua or Vivid IR (Invitrogen) diluted in 200 μL of PBS for 30 minutes at 4°C. Where indicated, additional markers were evaluated using PerCPCy5.5 anti-human CD14 (HCD14, BioLegend), PECy7 anti-human CD39 (A1, BioLegend), and PCF594 anti-human CD73 (AD2, BD Biosciences). Intracellular staining was performed after fixation and permeabilization with 0.1% saponin (Sigma), using APC anti-human IL13 (JES-10-5A2, BD Biosciences), PerCPCy5.5 anti-human IL13 (JES10-5A2, BioLegend), PeCy7 anti-human IFNγ (4S. B3, BD Biosciences), Alexa Fluor 700 anti-human IL17A (BL168, BioLegend), PerCPCy5.5 anti-human TNFα (MAb11, BioLegend), APC anti-human TNFα (MAb11, BD Biosciences), PE anti-human IgG1κ isotype (MOPC-21, BioLegend), PE anti-human IDO (eyedio, eBioscience), Alexa Fluor 647 anti-human IDO (2E2/IDO1, BioLegend), and Brilliant Violet 421 anti-human IL5 (TRFK5, BioLegend). Samples were acquired on a Gallios flow cytometer (Beckman Coulter) or LSRFortessa (BD), and data were analyzed using FlowJo software (TreeStar V.10). For ILC isolation, we used the same panel described above for ILCs identification, aliquots of cells were sorted to 98% purity using a FACSAria (Becton Dickinson), based on the gating strategy illustrated in **Fig. 1**.

Murine ILCs were identified as $CD45^{+}$, Lin^{-}, $CD90.2^{+}$ among live cells. Lineage markers, all FITC conjugated, include anti-mouse CD3e (17A2, in house), CD5 (53.7, in house), CD19 (ID3, in house), CD11b (M1/70, in house), CD11c (N418, in house), B220 (RA3-6B2, in house), CD49b (DX5, Miltenyi Biotec), FCεRIα (MAP-1, Miltenyi Biotec), Ter119 (Ter119, in house), TCRγδ (2M31/11, in house), and TCRαβ (H57, in house). Additional markers used to identify the ILC subpopulations include Alexa Fluor 700 anti-mouse CD45.2 (AL1–4A2, in house), PECy5 anti-mouse CD127 (A7R34, eBioscience), APCCy7 anti-mouse CD117 (cKit; 2B8, eBioscience), and PE anti-mouse ST2 (RMST2-2, Invitrogen).

Cell culture

The murine melanoma cell line B16-F10 was obtained from the laboratory of Dr. Lars French, University Hospital of Geneva, in 2000 and maintained by serial *in vitro* passages from cryopreserved stocks in DMEM supplemented with 1% HEPES, 1% streptopenicillin, and 10% FCS. The human Me290 and Me275 melanoma cell lines were established at the Ludwig Institute for Cancer Research, Lausanne branch in 1996 to 1997 from metastases excised from patients LAU50 and LAU203. They have been maintained since then by serial *in vitro* culture passages, from a master cell bank stock preserved at −110°C (23). Cells were not authenticated in the past year. Cells were cultured for approximately 3 to 4 weeks in RPMI-1640 (Eurobio) supplemented with 10% heat-inactivated fetal bovine serum (Eurobio), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.01 M HEPES buffer and 1 mmol/L sodium pyruvate (Eurobio) and were regularly tested for *Mycoplasma* contamination by PCR. Cells were grown until 75% confluency and passaged using trypsin/1× EDTA (Eurobio), washed with PBS and resuspended in supplemented RPMI-1640. Human PBMCs were cultured in RPMI, supplemented with 8% heat-inactivated pooled human serum, in the presence of 20 U/mL rhIL2 (Proleukin, Roche). Cells were grown at 37°C in a humidified incubator under 5% CO_2. When indicated, D-kynurenine (diluted

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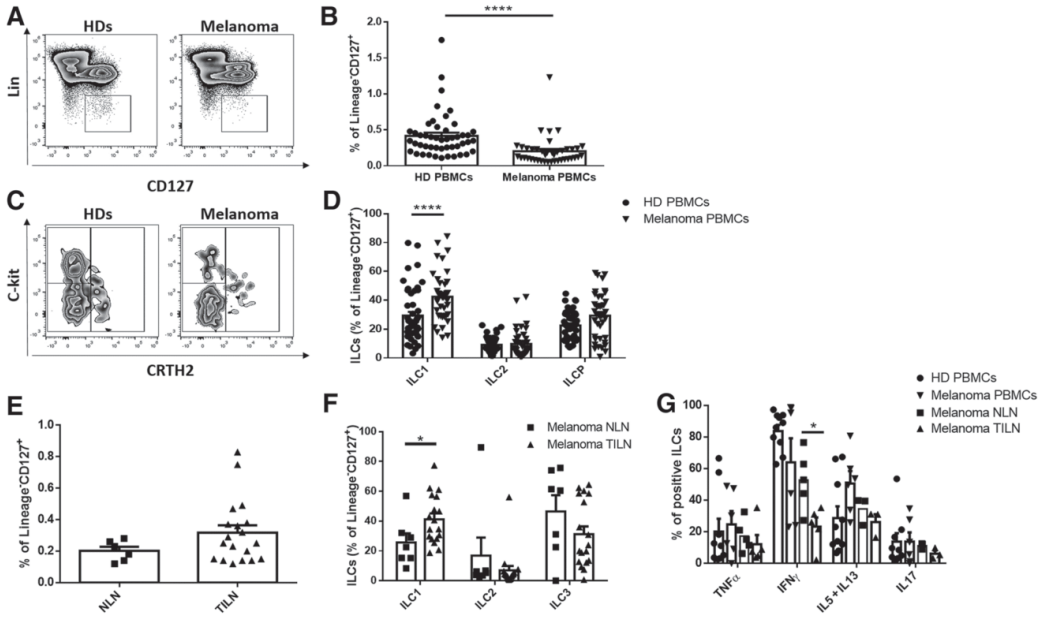

Figure 1.

ILC1s were significantly increased in human melanoma. **A,** Representative examples of flow cytometry analysis of ILCs in HD and melanoma patients' PBMCs. **B,** Frequencies (HD $n = 48$ and melanoma $n = 41$) of total ILCs identified as $Lin^{-}CD127^{+}$ cells in lymphocytes. **C,** Representative examples of flow cytometry analysis of ILC subsets in HD and melanoma patients' PB. **D,** Frequencies of ILC subsets among total ILCs. **E,** Frequencies of total ILCs in melanoma patients' NLN and TILN. **F,** Frequencies (NLN $n = 6$ and TILN $n = 19$) of ILC subsets among total ILCs. **G,** Frequencies of cytokine production by ILCs from *ex vivo* HD PBMCs, melanoma patients' PBMCs, and NLN or TILN (HD PBMCs $n = 9$, melanoma PBMCs $n = 6$, NLN $n = 5$, and TILN $n = 5$). In all box charts, data, mean ± SEM (*, $P < 0.05$; ****, $P < 0.0001$). Wilcoxon and/or two-way ANOVA tests.

in HCl), L-tryptophan, C-adenosine (Cado), 1-methyl-L-Tryptophan, and ZM241385 (all diluted in DMSO and from Sigma-Aldrich) were added at the indicated concentrations. For the evaluation of cytokine production, cells were stimulated with 1 μg/mL PMA plus 0.5 μg/mL ionomycin (P1585 and I9657, respectively from Sigma-Aldrich), in the presence of brefeldinA (B6542, Sigma-Aldrich) for 3 hours prior to intracellular staining.

Generation of an IDO-expressing melanoma cell line

Human IDO-1 (UniProt P14902) was cloned in a pMSGV retroviral vector downstream of the blasticidin resistance gene followed by a P2A element. Viral particles were produced by mixing in 250 μL of Optimem medium (Life Technologies) pMSGV (1.25 μg) and packaging plasmids pMD.gagpol (1.25 μg) and pMD.G (1.25 μg, VSV-G envelope protein) with 7.5 μL of MIRUS reagent (Mirus Bio LLC). After 20 minutes at room temperature, the mix was added slowly to 1 million of 293T cells. After 48 hours, 5 mL of virus-containing supernatant was collected and added to Me275 melanoma cells at 50% of confluency in 25 mm^2 flasks. The following day, the virus was removed, and blasticidin (15205, Sigma-Aldrich) was added to the culture medium at a concentration of 500 μg/mL until selection of a pure population of cells expressing IDO was established. IDO expression and function were determined, respectively, by flow cytometry staining using an anti-IDO antibody (clone 2E2, BioLegend) and by Erhlich test (Sigma-Aldrich) following the manufacturer's instruction.

Cutaneous melanoma model in C57BL/6 mice

Female C57BL/6J mice, 6 weeks old and weighing 18 to 20 g, were purchased from Envigo. B16-F10 murine melanoma cells (1×10^5) in 100 μL saline were injected subcutaneously (s.c.) into the right flank of C57BL/6 mice. Mice were observed daily and humanely euthanized by CO_2 inhalation 21 days after injection; tumor and draining LNs were then collected. Tumors were minced into small pieces in C-tubes followed by mechanical dissociation using the tumor02 program in the gentleMACS dissociator (Miltenyi Biotec). Samples were digested using the Mouse Tumor Dissociating kit (130-096-730, from Miltenyi Biotec) according to the manufacturer's recommendations. After the last mechanical disruption step, the digested tumors were harvested, filtered using a 70-μm strainer, and the lymphocyte fraction enriched using a 40% to 70% Percoll gradient (1,800 rpm, 20-minute centrifugation without break, room temperature). Draining LNs were harvested, dissociated mechanically with the piston of a syringe directly into the 40-μm strainer. Experiments were performed in accordance with Swiss ethical guidelines and with the protocols described in the animal license VD-3255.a.

In vitro coculture experiments

PBMCs from HDs were cultured with the Me290 melanoma cell line in a ratio of 10:1 (1 melanoma cell: 10 PBMCs) in RPMI complete medium. After 48 hours, PBMCs were harvested and analyzed to detect ILC frequencies and cytokine production by flow cytometry analysis.

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IDO activity assay

The amount of L-kynurenine in culture supernatants was measured by spectrophotometric analysis, as previously described (24, 25). Cells (either the Me290 line or magnetically purified human CD14$^+$ monocytes; Miltenyi Biotec) were pre-stimulated with 1,000 U/mL human IFNγ (PeproTech) or 50 ng/mL human TNFα (PeproTech) for 24 hours. Next, cells were washed and resuspended in Hanks buffered saline solution containing 500 μmol/L L-tryptophan (Sigma-Aldrich) and incubated at 37°C for 4 hours. Supernatants were then harvested and mixed with 30% trichloroacetic acid. After centrifugation at 8,000 × *g* for 5 minutes, the solution was added in a ratio of 1:1 with 4-(dimethylamino) benzaldehyde (Ehrlich reagent, Sigma-Aldrich) in a 96-flat bottom well plate. Optical density was measured at 490 nm, using a BioTek Epoch Microplate Spectrophotometer. Triplicate samples were run against a standard curve of defined kynurenine concentrations (0–1000 μmol/L; Sigma-Aldrich).

RNA purification and quantitative real-time PCR

Total RNA was isolated from highly pure, sorted human ILC subsets using the TRIzol reagent according to the manufacturer's instructions (15596026, Invitrogen). Final preparation of RNA was considered DNA- and protein-free if the ratio of readings at 260/280 nm was ≥ 1.7 after quantification with nanodrop. Total RNA (1 μg) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) according to the manufacturer's protocol. The quantitative real-time PCR (qPCR) was carried out in the Applied Biosystems 7900HT Fast Real-Time PCR Sequence Detection System (Applied Biosystems) with specific primers (hA1 5′-CCTCCATCTCAGCTTTCCAG-3′, 5′-AGTAGGTCTGTGGCCCAATG-3′; hA2A 5′-CTCCGGTACAATGGCTTGGT-3′, 5′-TGGTTCTTGCCCTCCTTTGG-3′; hA2B 5′-ATGCCAACAGCTTGAATGGAT-3′, 5′-GAGGTCACCTTCCTGGCAAC-3′; hA3 5′-TTGACCAAAAGGAGGAGAAGT-3′, 5′-AGTCACATCTGTTCAGTAGGAG-3′) using KAPA SYBR FAST qPCR Kits (KK4601, Roche). Samples were amplified simultaneously in triplicate in one-assay run with a non-template control blank for each primer pair to control for contamination or for primer dimerization, and the Ct value for each experimental group was determined. The housekeeping gene (ribosomal protein S16) was used as an internal control to normalize the Ct values, using the $2^{-\Delta Ct}$ formula.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 6. For comparison of two groups, the *t* test was used, for comparison of multiple groups, ANOVAs or the equivalent nonparametric (Mann–Whitney or Kruskal–Wallis tests) tests were used. The data are shown by plotting individual data points and the mean ± SEM. A $P < 0.05$ (two-tailed) was considered statistically significant and labeled: *, $P < 0.01$; **, $P < 0.001$; or ***, $P < 0.0001$.

Results

ILC function was dysregulated in melanoma patients

We compared the frequency of total ILCs in PB samples from 48 HDs and 41 age- and sex-matched melanoma patients (median age 47; range, 24–80) by flow cytometry analysis. Total ILCs were gated as lineage$^-$CD127$^+$ cells within the live lymphocyte population (**Fig. 1A**). A significant reduction in the frequency of circulating ILCs was found in melanoma patients compared with HDs (**Fig. 1B**). Subsequently, the distinct ILC subsets were identified based on the expression of CD117 (cKit) and CRTH2 (CD294) surface markers (**Fig. 1C**). More specifically, ILC1s were identified as CRTH2$^-$cKit$^-$, ILC2s as CRTH2$^+$cKit$^{+/-}$, and ILCPs as the cKit$^+$CRTH2$^-$ population. We cannot exclude that ILCPs might contain CD127$^+$CD56^{bright} NK cells, as we did not include CD56 in the lineage markers. As shown in **Fig. 1D**, a significant increase of ILC1s was found in melanoma patients compared with HDs. To further characterize the ILC subset composition in human melanoma, the frequency of ILC subsets was assessed in the LNs obtained from melanoma patients during LN dissection surgery. Based on routine histologic assessment, these LN were classified as TILN and non–tumor-infiltrated LN (NLN). An increase in the frequency of total ILCs was found in TILN when compared with NLN, although this was not statistically significant (**Fig. 1E**). However, similar to the findings in the PBMC, we observed a significant increase in ILC1s in TILN compared with NLN (**Fig. 1F**). Next, we assessed the functionality of ILCs by evaluating the cytokine production in PBMCs from HDs and in PBMCs and TILN from melanoma patients after *ex vivo* stimulation. ILCs from PBMC and TILN of melanoma patients were substantially impaired in IFNγ production. This reduction was accompanied by a trend in the increase in the production of Th2-associated cytokines in PBMCs from melanoma patients (i.e., IL5 and/or IL13; **Fig. 1G**). Collectively, these results suggested that ILC frequency, subset, and function were dysregulated in melanoma patients. In particular, ILC1s were enriched in both PBMCs and TILN and had impaired IFNγ production.

ILC1s were enriched in tumor and LN of B16-F10 melanoma–bearing mice

To investigate the role of ILCs in melanoma development, we used the most widely acknowledged experimental model to study melanoma *in vivo* (26), the subcutaneous B16-F10 murine melanoma model. Analysis of LN from tumor-bearing mice compared with control mice did not show any difference in the frequency of total ILCs (Supplementary Fig. S1A and S1B). However, similar to the results obtained in human samples, ILC1s were enriched in both TILN and dissociated tumor (Supplementary Fig. S1C–S1E).

Coculture of ILCs with human melanoma cells mimicked observations made in patients

To extend our evaluation to mechanistic analyses, an *in vitro* coculture assay was performed. In-house–generated Me290 melanoma cells were plated with fresh PBMCs from HDs in a ratio of 1:10 and incubated for 48 hours to evaluate ILC frequency and function by flow cytometry. Exposure of HD PBMCs to the Me290 melanoma cell line induced the reduction of total ILCs (**Fig. 2A** and **B**). Quantifying the distinct ILC subsets, we found a significant enrichment of ILC1s at the expense of ILCPs (**Fig. 2C** and **D**). In addition, we also assessed IFNγ and TNFα secretion. As shown in **Fig. 2E**, ILCs cocultured with the Me290 melanoma cells had significantly reduced IFNγ production. Collectively, these data suggest that the contact between ILCs and melanoma cell lines induced the change in ILC frequency and function as observed in melanoma patients *ex vivo*.

Kynurenine- and adenosine-producing enzymes were expressed in Me290 melanoma cells

IDO1 upregulation accompanied by kynurenine production and ADO production are two well-known immunosuppressive mechanisms in malignant melanoma (27–30). We thus investigated the possible involvement of these two mediators in the observed ILC dysfunction. First, we characterized the Me290 melanoma cell line for the expression of IDO1 and two of the adenosine-producing enzymes, CD39 and CD73. Because IDO1 can be induced by proinflammatory

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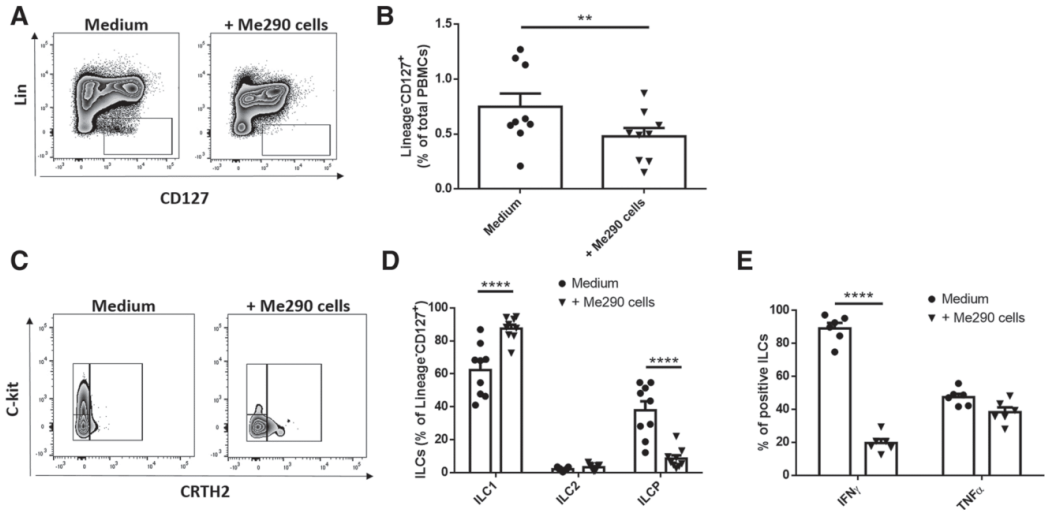

Figure 2.

Coculture of HD PBMCs with human melanoma cells mimicked the *ex vivo* observations in melanoma patients. **A,** Representative example of flow cytometry analysis of ILCs upon coculture with Me290 melanoma cells. **B,** Frequencies of total ILCs ($n = 9$) from **A**. **C,** Representative examples of flow cytometry analysis of ILC subsets upon coculture with Me290 melanoma cells. **D,** Frequencies of ILC subsets ($n = 9$) among total ILCs from **D**. **E,** Frequencies of IFNγ$^+$ and TNFα$^+$ in total ILCs ($n = 6$). In all box charts, data, mean ± SEM (**, $P < 0.01$; ****, $P < 0.0001$). Wilcoxon and/or two-way ANOVA tests.

signals in both healthy and tumor cells (31), Me290 cells were incubated for 24 hours in the presence of IFNγ and TNFα prior to the evaluation of IDO1 expression by flow cytometry (**Fig. 3A**). Purified CD14$^+$ monocytes stimulated with IFNγ were used as a positive control. As summarized in **Fig. 3B**, Me290 melanoma cells do not express IDO1 in their resting condition. By contrast, incubation of Me290 melanoma cells with IFNγ and TNFα significantly induced IDO1 expression and activity (**Fig. 3C**) and stimulated cells were able to produce kynurenines in the presence of L-tryptophan. This production was reversed by the addition of the IDO1-specific inhibitor 1-MT-L (**Fig. 3D**). In addition, we assessed the expression of CD39 and CD73 by the Me290 melanoma cell line by flow cytometry analysis (**Fig. 3E**). Me290 cells constitutively express CD39, whereas only 10% to 20% of Me290 cells express CD73 (**Fig. 3F** and **G**). Lastly, we also evaluated IDO1, CD39, and CD73 expression on human ILCs (Supplementary Fig. S2). Low IDO1 expression could be detected in all ILC subsets (Supplementary Fig. S2A and S2B). As opposed to the observations made in the Me290 cell line, ILCs, particularly ILC1s, expressed high CD73 and low CD39 (Supplementary Fig. S2C and S2D). These findings suggested that the IDO1 and ADO pathways were active in melanoma cells and that combined with the presence of CD73 on ILCs, this might further contribute to sustaining the ADO–immunosuppressive axis.

Impact of kynurenines and adenosine on ILC phenotype and function

In order to determine the effect of kynurenines on ILC subtype composition and function, fresh PBMCs were incubated with L-kynurenines for 48 hours and then stained for ILC subset phenotypic markers. Although not statistically significant, we observed a trending increase in the ILC1 population after treatment with kynurenines compared with the control. No differences were observed in ILC2 and ILCP populations (**Fig. 4A**). Next, we investigated whether L-kynurenines could affect ILC function in terms of cytokine production. PBMCs were incubated with L-kynurenine for 48 hours, stimulated with PMA/ionomycin and then stained for IFNγ and TNFα production. As shown in **Fig. 4B** and **C**, IFNγ production was completely abrogated and TNFα significantly reduced in the presence of kynurenines. Similar observations were obtained using a melanoma cell line transduced with a lentiviral vector expressing the full-length IDO protein (Supplementary Fig. S3). Next, fresh PBMCs were cultured with Cado, a stable form of adenosine, to assess ILC frequency and function after overnight incubation. Similar to the kynurenine treatment, we did not find any difference in ILC frequency and subset distribution (**Fig. 4D**). However, treatment with C-adenosine significantly reduced IFNγ production, whereas only a minor decrease in TNFα production was observed (**Fig. 4E** and **F**). These results suggested that kynurenines and adenosine produced by melanoma cells may directly contribute to the phenotypic and functional ILC shift observed in patients.

The ADO-mediated inhibitory effects on ILCs were partially mediated by the A2A receptor

Given the remarkable interest in targeting the adenosine pathway in melanoma clinical trials (17) and based on our findings, we sought to characterize the expression of the ADORA receptors on *ex vivo* sorted total ILCs from HD PBMCs. As expected, ADORA receptors were prominently expressed in total ILCs (except for A3 receptor). The A2A receptor was the most highly expressed followed by comparable expression of A1 and A2B receptors (**Fig. 5A**). To address the functional importance of the A2B receptor in ILCs, we analyzed the effect of the A2A-selective antagonist ZM241385 on cytokine

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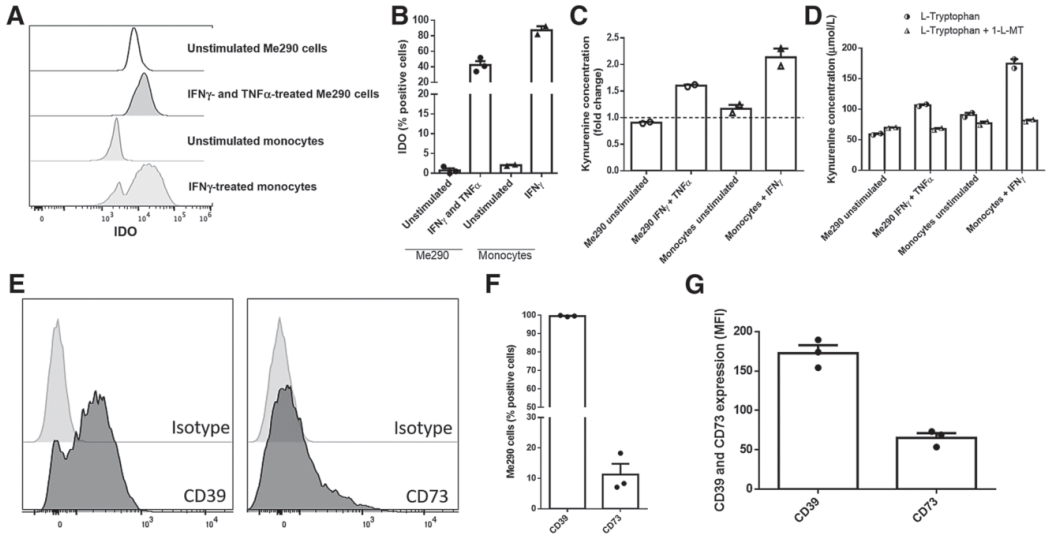

Figure 3.

Expression of kynurenine- and adenosine-producing enzymes in Me290 melanoma cells. **A,** Representative examples of flow cytometry analysis of the expression of IDO in monocytes and Me290 melanoma cells. **B,** Frequency of IDO-positive cells in Me290 cells ($n = 3$) or monocytes ($n = 2$). **C** and **D,** Concentration of kynurenines in the supernatant after culture with 250 µmol/L of L-tryptophan in the absence (**C**) or presence (**D**) of the IDO1 inhibitor 1-L-MT for 4 hours ($n = 3$). **E,** Representative examples of flow cytometry analysis of the expression of CD39 and CD73 adenosine-producing enzymes in Me290 melanoma cells. **F,** Frequency of CD39- and CD73-positive cells in Me290 cells ($n = 3$). **G,** Mean fluorescence intensity (MFI) of CD39 and CD73 expression in Me290 cells ($n = 3$).

production. IFNγ production was consistently upregulated by A2A antagonism (**Fig. 5B**). These findings suggest that the adenosine-mediated inhibitory effects on ILCs were, at least in part, governed by the A2A receptor.

Discussion

Despite advances in our knowledge of ILC biology (e.g., subset distribution and plasticity), the role of these cells in cancer, especially in patients, remains poorly characterized (reviewed in refs. 19, 20, 32). In this study, we characterized ILCs in human melanoma and observed an enrichment of ILC1s, accompanied by an impairment in their proinflammatory cytokine secretion. Previous work from our group and others report expanded, but dysfunctional ILC1s in different types of human hematologic malignancies (19, 33–35). Whether this is also a common hallmark of human solid cancers remains to be confirmed. The hypothesis of ILC1 dysfunction in malignancy is supported by the observation that unconventional type 1–like ILCs, together with type 1 innate–like T cells, locally ensure immune surveillance in oncogene-driven murine prostate and breast cancer models (36). Conversely, two independent studies show that TGFβ in the TME can convert NK cells into ILC1-like populations, which are unable to control tumor growth and metastasis (37, 38). Further work is needed to identify the human correlates of these observations.

Melanoma progression and resistance to therapy are promoted by the presence of a potent immunosuppressive microenvironment, including both neoplastic and nonneoplastic cells (reviewed in refs. 39, 40). Similar to the well-known immunosuppressive effect of the TME on adaptive immune cells, complex mechanism(s) might also modulate the distribution of ILC populations and their function. This complex network is at least partly dependent on the presence of different soluble factors, such as cytokines, vitamins, amino acids, and lipids, present in the tumor milieu. Interestingly, some of these mediators directly modulate ILC polarization in non-tumor settings (41–43). Based on this, we hypothesized that the immunosuppressive mechanisms described in the TME of melanoma patients could be involved in the shifts observed in ILC subset composition and in impaired ILC1 function. IDO1 is broadly activated in human cancer, including malignant melanoma, in an IFNγ-dependent manner (44, 45). However, although the suppressive effect of kynurenine accumulation has been largely studied on T and NK cells in the TME (46), there have been no reports of its impact on ILCs. Kynurenine is a potent agonist of the Aryl hydrocarbon receptor (AHR), a transcription factor expressed by ILC3s/ILCPs (47). In turn, AHR positively regulates IDO1 expression and subsequent kynurenine production (8). Despite the low expression of IDO1 observed in ILCs, we cannot exclude that, in the TME, kynurenine may have induced IDO1 in ILCs, resulting in an AHR/kynurenine/IDO1-positive feedback loop. Moreover, in T cells, AHR activation regulates type 1 regulatory T (Tr1) cell generation. Whether ILC3s/ILCPs may become a regulatory subset upon AHR activation by kynurenine in the TME needs addressing (48). Our *ex vivo* and *in vitro* data support a direct role of kynurenines in functional ILC impairment in melanoma, which is of particular interest in the context of current immunotherapy trials targeting IDO1 with selective inhibitors. It would be highly informative if biomarker analyses on samples from the ECHO-301 trial included ILC profiling (11). Moreover, the assessment of expression of the other tryptophan catabolic enzymes IDO2 and TDO in tumor-

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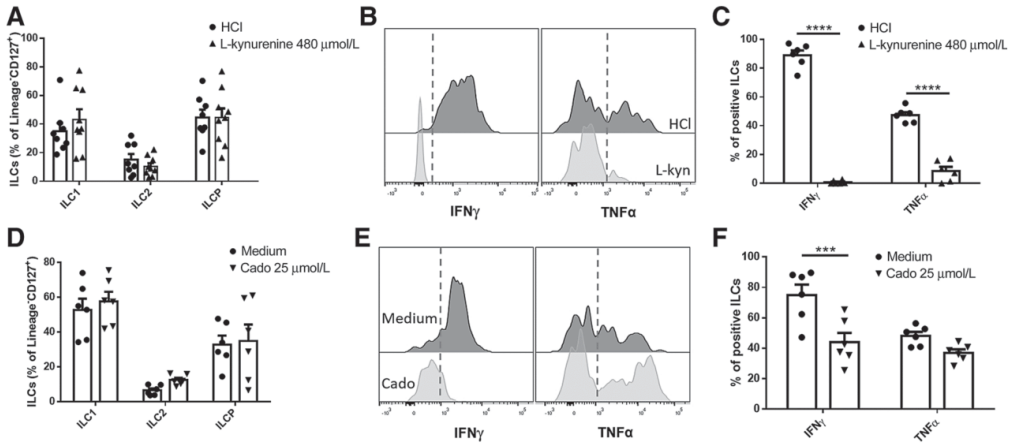

Figure 4.

Impact of kynurenines and adenosine on ILCs. **A,** Frequencies of ILC subsets among total ILCs after L-kynurenine treatment 480 µmol/L for 36 hours (HCl $n = 8$, L-kynurenine $n = 9$). **B,** Representative example of flow cytometry analysis of ILCs upon treatment with L-kynurenine (L-kyn). **C,** Frequencies of IFNγ$^+$ and TNFα$^+$ in total ILCs after treatment for 36 hours with L-kynurenine 480 µmol/L ($n = 6$). **D,** Frequencies of ILC subsets among total ILCs after overnight treatment with Cado 25 µmol/L ($n = 6$). **E,** Representative example of flow cytometry analysis of ILCs upon treatment with Cado. **F,** Frequencies of IFNγ$^+$ and TNFα$^+$ in total ILCs after overnight treatment with Cado 25 µmol/L ($n = 6$). In all box charts, data, mean ± SEM (***, $P < 0.001$; ****, $P < 0.0001$). Two-way ANOVA tests.

infiltrating ILCs might unravel potential compensatory mechanisms operating during immunotherapy with selective IDO1 inhibitors. Understanding the impact of IDO/TDO biology on innate lymphocytes may provide new targets in the blockade of tryptophan catabolism in cancer.

The adenosinergic signaling axis has emerged as a powerful immune checkpoint in the TME (17). Elevated ATP is observed in different tumors (reviewed in ref. 49), and ATP catabolism is primarily mediated by CD39 and CD73 (50). Whereas the expression of these two ectoenzymes has been reported on several immune and stromal cell types, their expression on ILCs remains unknown. Our observation that CD73 was detected on ILCs advocates for a contribution of these cells in the generation of ADO, a metabolite known to potently restrict immune responses via pleiotropic effects in the TME (51). Besides participating in ADO generation, ILCs might also be directly inhibited by ADO. We observed expression of the high-affinity A2A receptor on human ILCs, which is one of the 4 known ADO receptors. ADO signaling through A2A and A2B receptors is the major pathway mediating the ADO-immunosuppressive effect in immune cells (52, 53). We speculated that the reduction of IFNγ production by ILCs after ADO treatment was mediated by the activation of the A2A receptor, as previously shown for T and NK cells (54, 55). Using the selective A2A receptor antagonist ZM241385, we confirmed significant upregulation of IFNγ production. Thus, our results indicated that the reduced production of IFNγ is partially mediated by ADO through the A2A receptor. Further work will be needed to determine the exact impact of ADO on ILCs as opposed to its action on their adaptive counterparts in antimelanoma responses.

In conclusion, we have demonstrated that ILCs were dysregulated in human melanoma, which was due, at least in part, to the presence of kynurenines and adenosine in the TME. We also demonstrated that the inhibition of one of the adenosine receptors reverts the production of IFNγ. In the current milieu of promising preclinical and clinical approaches targeting the IDO and adenosinergic immunosuppressive axes, we have generated new evidences to support blocking these pathways in melanoma patients.

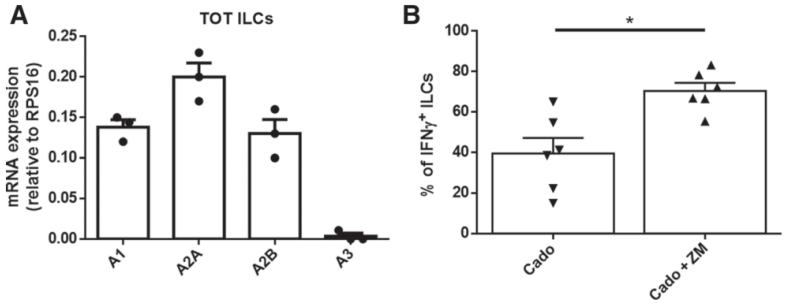

Figure 5.

ADO inhibitory effects were in part mediated by the A2A receptor. **A,** Expression of A1, A2A, A2B, and A3 adenosine receptors assessed by qPCR in freshly sorted total (TOT) ILCs ($n = 3$). **B,** Frequencies of IFNγ$^+$ production in total ILCs after overnight treatment with Cado or Cado/ZM241385 (Cado + ZM) 20 µmol/L ($n = 6$). In all box charts, data, mean ± SEM (*, $P < 0.05$). Wilcoxon and one-way ANOVA tests.

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Disclosure of Potential Conflicts of Interest

P. Romero is a consultant/advisory board member for Immatics Biotechnologies, reports receiving other commercial research support from Roche pRED, Zurich, and reports receiving speakers bureau honoraria from Bristol-Myers Squibb, AstraZeneca, and Roche. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Ercolano, A. Garcia-Garijo, B. Salomé, A. Gomez-Cadena, B. Mastelic-Gavillet, A. Ianaro, P. Romero, S. Trabanelli, C. Jandus
Writing, review, and/or revision of the manuscript: G. Ercolano, B. Salomé, A. Gomez-Cadena, B. Mastelic-Gavillet, A. Ianaro, D.E. Speiser, P. Romero, S. Trabanelli, C. Jandus
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.E. Speiser
Study supervision: A. Ianaro, S. Trabanelli, C. Jandus

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