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The landscape of human NK cells in homeostasis, tumors, infection, and inflammation

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The landscape of human NK cells in homeostasis, tumors, infection, and inflammation

Thesis for Doctoral Degree (Ph.D.)

By

Nicole Wild

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To my past, present, and future loved ones.

”… to learn is not to know;
there are learners and the learned.
Memory makes the one, philosophy the other.”
- Alexandre Dumas, *The Count of Monte Cristo*

Popular science summary of the thesis

The human body is in constant interaction with the environment – not only through the skin, but also by breathing and eating. Organs such as the lungs and intestines therefore require a particularly effective and quick immune defense to maintain health.

One immune cell type known for its rapid response in eliminating virus-infected and malignant cells are natural killer (NK) cells. NK cells are part of the innate immune system and present in many human tissues. However, it is still only partially understood how they differ between organs – both in health and in disease. This doctoral thesis contributes to addressing some of these questions.

Study I presents an immune cell atlas of the human lung, providing an overview of the distribution of leukocytes across different lobes within the same lung. In addition, we identified that a lung-specific NK cell population was enriched in lower lung regions.

Study II shows that lung-localized NK cells accumulate withing lung tumors compared to the surrounding lung tissue. In addition, signs of immune cell exhaustion varied between tissue regions, while NK cells in the tumor center remained responsive to activation.

Study III describes a strategy of immune evasion employed by the SARS-CoV-2 variant BQ.1 compared to earlier variants. BQ.1 escapes NK cell recognition by dampening activating viral signals while maintaining inhibitory signals toward NK cells.

Study IV investigates whether intestinal NK cells and T cells differ between healthy individuals and people with inflammatory bowel disease (IBD). The results showed that particularly NK cells from the ileum of IBD patients exhibited lower levels of granzyme A compared to patients without IBD. This suggests that granzyme A from NK cells may play a role in the gut.

The findings generated in this thesis expand the current understanding of the human immune system in tissues. They may contribute to the development of new therapeutic strategies for cancer, transplantation, and IBD, while also providing important insights into viral evolution in the context of the human immune response.

Populärvetenskaplig sammanfattning av doktorsavhandlingen

Den mänskliga kroppen står i ständig kontakt med omgivningen – inte bara via kontakt genom huden utan även när vi andas och äter. Organ som lungor och tarmen behöver därför ett särskilt effektivt och snabbt immunförsvar för att hålla oss friska.

En immuncell som är känd för sin snabba respons vid bekämpning av virusinfekterade och cancerartade celler är den naturliga mördarcellen (NK-cellen). NK-celler är del av det medfödda immunförsvaret och förekommer i många av kroppens vävnader. Trots detta är det fortfarande endast delvis förstått hur de skiljer sig åt mellan olika organ – både i hälsa och vid sjukdom. Denna avhandling bidrar till att besvara några av dessa frågor.

I **Studie I** presenteras en immuncellsöversikt över den mänskliga lungan, som ger en överblick över fördelningen av leukocyter i olika lunglober från en och samma lunga. Det visade sig att en lungspecifik population av NK-celler övervägande ansamlas i de nedre delarna av lungan.

Studie II visar att lunglokaliserade NK-celler ansamlas i lungtumörer jämfört med den omgivande lungvävnaden. Dessutom varierade tecken på immuncellsutmattning mellan olika vävnadsregioner, medan NK-celler i tumörens centrum fortsatt svarade på aktivering.

Studie III beskriver en strategi för immunflykt hos SARS-CoV-2-varianten BQ.1 jämfört med tidigare varianter. BQ.1 undgår igenkänning av NK-celler genom att dämpa aktiverande virala signaler samtidigt som hämmande signaler mot NK-celler bibehålls.

Studie IV undersöker hur intestinaler NK-celler och T-celler skiljer sig mellan personer med och utan inflammatorisk tarmsjukdom (IBD). Resultaten visade att särskilt NK-celler från ileum hos individer med IBD uppvisar lägre nivåer av granzym A jämfört med patienter utan IBD. Detta tyder på att granzyme A från NK-celler kan spela en roll i tamen.

De resultat som genererats inom denna avhandling bidrar till en fördjupad förståelse av immunsystemet i kroppens vävnader. De kan nyttjas i framtida utveckling av behandlingsstrategier vid cancer, transplantation och IBD, samt ge viktiga insikter i virusevolution i samspel med det mänskliga immunförsvaret.

Populärwissenschaftliche Zusammenfassung der Doktorarbeit

Der menschliche Körper steht im ständigen Austausch mit der Umwelt – nicht nur über die Haut, sondern auch über die Atmung und die Nahrungsaufnahme. Organe wie Lunge und Darm benötigen daher eine besonders effektive und schnelle Immunabwehr, um uns gesund zu halten.

Eine Immunzelle, die für ihre schnelle Reaktion bei der Abwehr virusinfizierter und entarteter Zellen bekannt ist, ist die natürliche Killerzelle (NK-Zelle). NK-Zellen sind Teil der angeborenen Immunantwort und kommen in vielen menschlichen Geweben vor. Dennoch ist bislang nur begrenzt verstanden, wie sie sich in unterschiedlichen Organen unterscheiden – sowohl im gesunden Zustand als auch bei Erkrankungen. Diese Doktorarbeit erforscht einige dieser Fragen.

In **Studie I** wird ein Immunzell-Atlas der menschlichen Lunge präsentiert, der einen Überblick über die Verteilung von Leukozyten in unterschiedlichen Lungenlappen von ein und derselben Lunge bietet. Dabei zeigte sich, dass sich eine lungenspezifische NK-Zell-Population überwiegend in den unteren Lungenregionen anhäuft.

Studie II zeigt, dass sich in der Lunge ansässige NK-Zellen im Zentrum von Lungentumoren im Vergleich zum umliegenden Lungengewebe anreichern. Darüber hinaus unterschieden sich Anzeichen von Immunzell-Erschöpfung zwischen verschiedenen Geweberegionen, während NK-Zellen im Tumorzentrum weiterhin auf Aktivierung reagieren.

In **Studie III** wird eine Strategie der Immunflucht der SARS-CoV-2-Variante BQ.1 im Vergleich zu früheren Varianten beschrieben. BQ.1 entgeht NK-Zell-Erkennung, indem virale Signale abgeschwächt werden und gleichzeitig NK-Zell-inhibierende Signale erhalten bleiben.

Studie IV untersucht, wie sich NK-Zellen und T-Zellen zwischen Personen mit und ohne chronisch-entzündlicher Darmerkrankungen (IBD) unterscheiden. Die Ergebnisse zeigten, dass NK-Zellen vom Ileum von Betroffenen geringere Mengen Granzym A aufweisen. Deshalb wird vermutet, dass Granzym A von NK-Zellen eine noch unvollständig geklärter Rolle im Darm spielen könnte.

Die im Rahmen dieser Doktorarbeit gewonnenen Erkenntnisse erweitern das bisherige Verständnis des menschlichen Immunsystems in Geweben. Sie können

zur Entwicklung neuer therapeutischer Ansätze bei Krebs, Transplantation und IBD beitragen und liefern zugleich wichtige Einblicke in die virale Evolution im Zusammenspiel mit der menschlichen Immunabwehr.

Abstract

NK cells are critical components of the innate immune system, yet their heterogeneity and functional specialization across human tissues and their role in shaping viral adaptations remain incompletely understood. This thesis investigates NK cell biology across organs and disease contexts, integrating phenotypic, transcriptional, and functional analyses, and explores how these cells are shaped by, and in turn shape, immune responses in cancer, infection, and inflammation.

In **Study I**, immune cell composition was mapped across matched regions of the human lung. While leukocyte distribution was largely uniform within the parenchyma, airway-associated compartments represented distinct immunological niches for adaptive immune cells. In contrast, NK cells displayed marked heterogeneity driven primarily by inter-donor variability. This was partly driven by the presence of adaptive-like trNK cells in a subset of donors. These cells were enriched in peripheral lower lung regions and absent from other tissues, suggesting lung-specific specialization. Together, these findings indicate that immune surveillance in the healthy lung is shaped by the interplay between anatomical context and inter-individual variation, providing a reference framework for future studies of pulmonary immunity across disease states.

Study II extended this analysis to NSCLC, revealing an enrichment of $CXCR3^+CXCR6^+$ $CD8^+$ TRM and trNK cells toward the tumor center. While $CD8^+$ TRM exhibited high levels of immune checkpoint receptor expression, trNK cells expressed comparatively low levels, suggesting retained functional plasticity. Notably, despite reduced perforin, granzyme expression was highest in the tumor center, and trNK cells displayed stronger functional responses than $CD49a^-$ NK cells, indicating preserved effector potential. These findings highlight opportunities to improve immunotherapy by targeting chemokine-driven recruitment, retention, and local suppression of lymphocytes within tumors.

Study III investigated viral NK cell immune evasion, demonstrating that the SARS-CoV-2 BQ.1 variant exploits a single amino acid substitution in the $Nsp13_{232-240}$ epitope to alter HLA-E presentation. Loading of the BQ.1 Nsp13 epitope decreases HLA-E/peptide complex stability, promotes self-peptide loading, and restores inhibitory CD94/NKG2A signaling, thereby suppressing NK cell activation. This mechanism represents a subtle form of immune evasion and provides evidence that viral evolution can target innate immune checkpoints.

Study IV identified an intestinal NK cell subset characterized by high GzmA expression and tissue-resident features. This population was enriched in the ileum and associated with epithelial localization, as well as CD39 and NKG2D expression. In non-inflamed intestinal tissue from IBD patients, these cells showed reduced GzmA expression and frequency while preserving functional capacity. A transcriptionally similar subset was also identified in publicly available datasets. These findings implicate NK cells as contributors to mucosal immune regulation and identify GzmA as a context-dependent mediator of barrier homeostasis and inflammation.

Collectively, this work highlights NK cells as adaptable, tissue-imprinted immune cells whose function is shaped by local environments and disease states. It further demonstrates that both tumors and viruses exploit these regulatory pathways to evade immune control. These insights underscore the importance of studying NK cells in tissue contexts and highlight their potential as targets for next-generation immunotherapies.

List of scientific papers

I. **Wild N**, Brownlie D, Eichhorn, M, Bonaiti E, Schaden J, Nilsén V, Voigt I, Bassett, J, Cai C, Constantz C, Gao Y, Ferreira S, Franklin M, Kammann T, Kaushal J, Kokkinou E, Marchalot A, Akhirunnesa, Mouchtaridi E, Müller TR, Raineri EJM, Sekine T, Stamper C, Tibbitt C, Michaëlsson J, Sandberg JK, Mjösberg J, Jorns C, Birk M, Buggert M, Marquardt N. A Mapping the immune landscape across the human lung. *Manuscript*.

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List of abbreviations

AC	Adenocarcinoma
ADCC	Antibody-dependent cellular cytotoxicity
AI	Artificial intelligence
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
BCRs	B-cell receptors
CCL(i)	C-C motif chemokine ligand (i)
CCR(i)	C-C motif chemokine receptor (i)
CD#	Cluster of differentiation
CD	Crohn's disease
(H)CMV	(Human) cytomegalovirus
COVID-19	Coronavirus disease 2019
CX3CR1	C-X3-C motif chemokine receptor 1
CXCR(i)	C-X-C motif chemokine receptor (i)
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleid acid
DSS	Dextrane sulfate sodium
EOMES	Eomesodermin
FACS	Fluorescence activating cell sorting
FBS	Fetal bovine serum
GSDMB	Gasdermin B
GzmA/B/H/K/M	Granzyme A/B/H/K/M
HLA	Human leukocyte antigen

HCoV	Human coronaviruses
IBD	Inflammatory bowel disease
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IHOPE	Immunology Human Organ Donor Programme
IL-(i)	Interleukin-(i)
ILC	Innate lymphoid cell
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
KIRs	Killer-cell immunoglobulin-like receptors
LAG-3	Lymphocyte activation gene 3
LLN	Lung lymph node
LN	Lymph node
MAIT (cell)	Mucosa-associated invariant T (cell)
MesLN	Mesenteric lymph node
MHC	Major histocompatibility complex
NK cell	Natural killer cell
NSCLC	Non-small cell lung carcinoma
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD-1	Programmed cell death protein 1
pM2I	Single amino acid change from methionine to isoleucine at position 2
PMA	Phorbol-12-myristat-13 acetate

PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
S1PR1	Sphingosin-1-phosphate receptor 1
SARS-CoV-(i)	Severe acute respiratory syndrome coronavirus-(i)
SCC	Squamous cell carcinoma
scRNA-seq	Single cell RNA sequencing
TCM	Central memory T cell
TCRs	T-cell receptors
TEM	T effector memory cell
TEMRA	Effector memory T cells re-expressing CD45RA
TGF-β	Transforming growth factor-β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	Tumor-infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TRM	Effector-memory-like tissue-resident T cell
trNK	Tissue-resident NK cell
UC	Ulcerative colitis
UMAP	Unifold manifold approximation and projection
VDJ (genes)	Variable, diveres and joining (genes)
Very late antigen-1	VLA-1, α1β1 integrin

Introduction

This thesis aims to investigate natural killer (NK) cell heterogeneity across human tissues and disease contexts. NK cells act as sentinels in barrier tissues such as the lung and intestine and are also present within solid tumors. However, their distribution as well as phenotypic and functional adaptation within these tissues remain incompletely understood.

To address this, we first established an anatomically resolved framework of the human lung (**Study I**), defining how leukocytes, and NK cells in particular, are distributed across matched lung lobes as well as airway-associated regions and lymph nodes.

Beyond homeostasis, the lung requires tightly regulated immune surveillance in the context of malignancy. Although NK cells are potent mediators of tumor cell elimination, their function is often modulated by the immunosuppressive tumor microenvironment. We therefore investigated their phenotype and functional potential across tumor center, margin, and tumor-adjacent lung tissue in comparison to tumor-free regions (**Study II**).

NK cells also play a central role in antiviral immunity. The respiratory virus SARS-CoV-2 has been shown to evade NK cell recognition, raising the question of how emerging variants further adapt to modulate these interactions. In this context, the BQ.1 variant was investigated to determine how viral mutations reshape NK cell recognition pathways and contribute to immune evasion (**Study III**).

Finally, these concepts were extended to the human intestine (**Study IV**), another major barrier tissue, to examine how NK cell programs are adapted to mucosal environments and how they are altered in chronic inflammatory conditions.

Together, these studies provide a conceptual framework for understanding NK cells as context-dependent immune cells with identity and function continuously shaped by tissue architecture and disease-specific cues.

1 Background

This thesis will mainly focus on the adult human immune system, in particular NK cells. Although important for immunology and NK cells research, background information and work investigating animal models or fetal tissues will only be discussed in context of relevant articles.

2. The human immune system

The words immune, immunity, and other related terms originate from the Latin words "in" (not) and "munis" (ready for service), which together formed "immunitas" or "immunis", meaning "exempt" or "freed from obligation". Historically, this concept referred to being exempt from disease. The modern scientific use of immunity emerged primarily in the late nineteenth century, paralleling advances in the understanding of infectious diseases and the physiological mechanisms underlying host protection.

The human body is continuously exposed to antigens from microorganisms, food, and the environment through inhalation, ingestion, and physical contact. Effective immune surveillance is therefore essential, particularly at barrier sites. In addition to external challenges, intrinsic factors such as genetic mutations or cellular ageing can give rise to cancer. These threats are counteracted by the immune system which comprises lymphoid tissues, diverse immune cell populations, antibodies, cytokines, and the complement system. By distinguishing from self and non-self, the immune system limits tissue damage while eliminating tumor cells or infected cells, providing both protection and long-term memory against a wide range of pathogens.

The two arms of immunity are referred to as innate and adaptive immunity. Immediate, non-specific defense is generally provided by the innate immune system, while the slower developing adaptive immune system offers a specific and targeted response to pathogens. Both systems interact closely to safeguard health through continuous immunosurveillance.

2.1. The innate immune system

Innate immunity comprises physical barriers, enzymes, the complement system, and leukocytes that provide rapid, unspecific defensive responses. The immune cells involved are of myeloid as well as lymphocytic lineage and can be found in

blood and tissues. They originate from a shared precursor, the hematopoietic stem cell, which is primarily located in the bone marrow (1).

In tissues, mechanisms independent of complement create a primary non-specific inflammatory response driven by immune cells detecting molecular patterns from pathogens and internal cues like cellular stress or damage (2, 3). This is facilitated by macrophages, dendritic cells (DCs), and granulocytes but also epithelial cells, which express pattern recognition receptors (PRRs) (2, 3). PRRs allow detection of pathogen-associated molecular patterns (PAMPs), commonly shared by pathogens, such as lipopolysaccharides from bacteria or viral dsRNA as well as endogenous danger-associated molecular patterns (DAMPs), like extracellular DNA or RNA (2, 3). Pathways triggered by PRRs initiate the production of pro-inflammatory cytokines, cell adhesion molecules, and immunoreceptors (2-4). These molecules collectively coordinate the initial host response to infection or cellular stress.

In this thesis, the focus will be on the cellular compartment of the innate immune system, in particular NK cells.

2.1.1. Myeloid cells

This chapter provides a brief overview of the major myeloid cell types addressed in Study I. Although not the primary focus of this thesis, these cells are essential for maintaining health and tissue homeostasis and are in close interplay with lymphoid cell populations.

During embryonic development, myeloid cell formation occurs in successive waves. The earliest hematopoietic progenitors emerge from the hemogenic endothelium in the yolk sac (5), marking the first wave of blood cell development. From these progenitors, erythron-myeloid progenitors arise and differentiate into primitive macrophages, some of which directly seed developing tissues (6, 7). Others migrate to the fetal liver, where they contribute to fetal hematopoiesis and give rise to monocytes, macrophages, granulocytes, and erythrocytes (6, 7). A subsequent wave originates from hematopoietic stem cells generated in the aorta-gonad-mesonephros region (8, 9). These stem cells colonize the fetal liver and produce additional myeloid populations, although their contribution to early tissue macrophage development appears to be more limited (9, 10).

In postnatal life, myeloid cells continue to derive from hematopoietic stem cells in the bone marrow (11, 12). They differentiate via common myeloid progenitors and

mature through multiple intermediate stages within the bone marrow (12) and in the spleen under steady-state or stressed conditions (reviewed in (13)). After maturation, granulocytes and monocytes are released into the bloodstream and either circulate or are recruited to peripheral tissues, where monocytes can further differentiate into macrophages and DCs (reviewed in (14-16)). Throughout this process, myeloid cell development is strongly shaped by anatomical location, local niche signals, and interactions with neighboring cells (reviewed in (17)), resulting in substantial heterogeneity across peripheral tissues (reviewed in (18)).

Monocytes, macrophages, DCs, granulocytes, and mast cells constitute the core myeloid populations within postnatal human tissues. Circulating monocytes can be recruited to tissues, especially during inflammation, where they differentiate into macrophages or DCs and contribute to pathogen clearance and immune regulation (14, 19). Tissue-resident macrophages are a mix of long-lived embryonically seeded cells and monocyte-derived cells with highly tissue-specific functions in tissue homeostasis, repair and host defense by phagocytosis and orchestration of local immune responses (20, 21). Alongside these myeloid populations, innate lymphoid cells represent a complementary arm of the innate immune system.

2.1.2. Innate lymphoid cells

Innate lymphoid cells (ILCs) are often described as a bridge between innate and adaptive immunity, as they are present from birth, lack antigen-specific receptors, and rapidly produce cytokines that shape adaptive responses (22). The precise embryonic origin of lymphoid progenitor cells that give rise to ILCs remains incompletely defined (23-27), however, it is well established that they arise from hematopoietic stem cells in the bone marrow and continue to do so after birth (23, 24). Functionally, ILCs are classified into three major groups: group 1 ILCs (ILC1), which include NK cells and non-NK ILC1, group 2 ILCs (ILC2), and group 3 ILCs (ILC3) (28-30), each defined by characteristic transcription factors and cytokine profiles. NK cells are found in circulation and tissues, whereas other ILC subsets are predominantly present in tissues (31). In the following chapters, non-NK ILC subsets and NK cells are introduced in greater detail in separate sections.

2.1.2.1. Non-NK ILCs

Group 1 ILCs, including NK cells and ILC1s, produce interferon-γ (IFN-γ) in response to IL-12 and IL-18, thereby contributing to early defense against intracellular

pathogens (32). Non-NK ILC1s rely primarily on the transcription factor T-bet during development and mature locally within tissues, with limited recirculation. They exhibit limited cytotoxicity, and are largely tissue-resident (32, 33), displaying tissue-specific imprinting (34-36), and have been identified across multiple human organs, including the liver, tonsils, lungs, and the small intestine (36-38). In detail, within the human gut, non-NK ILC1s comprise heterogeneous subsets that differ in their cytotoxic capacity (38, 39). Among these, intraepithelial ILC1s represent a population with cytotoxic potential and phenotypic characteristics resembling NK cells and tissue-resident memory $CD8^+$ T cells (38, 40). Non-NK ILC1 subsets, including intraepithelial ILC1s, have been reported to be enriched in individuals with inflammatory bowel disease (IBD) (39-41).

ILC2s secrete type 2 cytokines such as IL-4, IL-5, and IL-13 in response to epithelial-derived signals including IL-25 and IL-33 (42, 43). They play key roles in immunity against helminths and in allergic inflammation and are characterized by high GATA3 expression and CD161 as well as CRTH2 surface expression (44, 45).

ILC3s produce IL-17 and IL-22 in response to bacterial stimuli and depend on the transcription factor RORγt for their development and function (46). Together, these subsets mirror T helper cell polarization programs while functioning as innate, tissue-adapted immune sentinels.

2.1.2.2. NK cells

First discovered in 1975, NK cells were identified as lymphocytes capable of spontaneous cytotoxic activity against murine Moloney leukemia cells (47, 48). Around the same time, similar observations of "natural cytotoxic reactivity" of lymphocytes against tumor cells were reported (49, 50). One decade later, it was demonstrated that NK cells preferentially target cells with absent or reduced expression of classical major histocompatibility complex (MHC) class I molecules, including many tumor and virus infected cells (51-53). These findings provided experimental support for the "missing-self" hypothesis and established a fundamental principle of innate immune surveillance, whereby NK cells complement $CD8^+$ T cell responses by recognizing and eliminating cells that evade T cell detection through MHC class I downregulation (53, 54). These pioneering studies established the existence of a previously unrecognized immune cell type, fundamentally shaping the field of immunology and laying the foundation for future immunotherapeutic strategies (53, 54).

Historically, NK cells are commonly described as $CD3^{-}$ lymphocytes and are distinguished by the expression of the surface markers CD56 and CD16. NK cells with a $CD56^{bright}CD16^{-}$ phenotype are commonly associated with immunoregulatory functions (55), while $CD56^{dim}CD16^{+}$ NK cells are predominantly responsive to target cells and capable of performing antibody-dependent cellular cytotoxicity (ADCC) (56). This paradigm has evolved substantially in recent years, driven by advanced multi-omics approaches and an increasing focus on tissues, revealing additional layers of NK cell heterogeneity and function. Nevertheless, this remains an ongoing process, with many aspects, such as the influence of distinct tissue niches and diverse disease states, still to be uncovered.

NK cells possess the unique ability to detect "missing-self," whereby reduced or absent MHC class I expression, referred to in humans as human leukocyte antigen (HLA) class I, on target cells can trigger NK cell activation (47). In humans, classical HLA class I molecules include HLA-A, -B, and -C, while non-classical molecules such as HLA-E and HLA-G also play important roles (3, 57). NK cells integrate signals from receptors recognizing both classical and non-classical HLA class I molecules, with the non-classical HLA-E emerging as an important regulator of NK cell responses during viral infections (58-60).

Unlike adaptive lymphocytes, NK cells rely on a finely tuned balance of inhibitory and activating receptors to discriminate between healthy and stressed, infected or transformed cells (61). Upon activation, they rapidly release cytotoxic granules such as granzyme B (GzmB) and perforin, as well as cytokines, enabling immediate immune defenses (61). Through these mechanisms, NK cells play a crucial role in early antiviral immunity and tumor surveillance, while also providing broader immunomodulatory functions. Their ability to respond rapidly and integrate activating and inhibitory signals makes them effective in controlling early viral infections and limiting malignant transformation.

Activation of NK cell is regulated through a repertoire of inhibitory and activating receptors that recognize ligands on surrounding cells (61, 62). Inhibitory receptors commonly signal through immunoreceptor tyrosine-based inhibitory motifs (ITIMs), whereas activating receptors often associate with immunoreceptor tyrosine-based activation motifs (ITAMs) (61). Key inhibitory receptors include killer-cell immunoglobulin-like receptors (KIRs), which recognize HLA-A, HLA-B, and HLA-C (63) molecules, as well as CD94/NKG2A, which binds HLA-E (64, 65). NK cells may also express immune checkpoint receptors such as programmed cell

death protein 1 (PD-1) (66) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) (67). Activating receptors include members of the natural cytotoxicity receptor family, such as NKp46 and NKp30, as well as NKG2D and NKG2C (reviewed in (62, 68)). In addition, NK cells can express CD16 (FcγRIIIa), which enables ADCC (61). Through CD16 engagement, NK cells recognize the Fc region of antibodies bound to target cells and initiate signaling cascades that result in target cell killing through the release of perforin and GzmB (69). The integration of inhibitory and activating signals therefore determines NK cell activation and target specificity.

In addition, NK cell responses are further modulated by cytokines produced during immune activation. Cytokines such as IL-12, IL-15, and IL-18 stimulate NK cells and promote the production of pro-inflammatory mediators, including IFN-γ and tumor necrosis factor (TNF) (55, 70).

2.1.2.3. NK cell development and maturation

In the adult body, NK cells develop from common lymphoid progenitors in the bone marrow, where they sequentially acquire NK cell-lineage identity, effector molecules, and education through self-HLA interactions. During this process, NK cells, in contrast to non-NK ILC1s, depend on the transcription factors Eomesodermin (EOMES) and, to a lesser extent, T-bet, which imprints their cytotoxic program (71).

NK cells then undergo distinct maturation stages in the bone marrow and secondary lymphoid organs from $CD56^{bright}$ to $CD56^{dim}$ with progressive KIR and CD57 acquisition and HLA-dependent education. $CD56^{bright}$ NK cells gradually mature into $CD56^{dim}$ cells that upregulate perforin, granzymes, and repertoires of inhibitory receptors such as KIRs and NKG2A (72). During this maturation, stepwise acquisition of KIRs and eventual expression of CD57 marks a trajectory toward terminal differentiation, where $CD57^{+}KIR^{+}CD56^{dim}$ NK cells often display robust cytotoxic function but reduced proliferative capacity and responsiveness to some cytokines (72, 73). In parallel, interactions of inhibitory receptors with self HLA class I "educate" or "license" subsets across these stages, which is addressed in the section below.

In addition, while circulating $CD56^{bright}$ NK cells exhibit potent cytokine-producing capacity and limited cytotoxicity, their roles within tissues are less clearly defined (74). In organs such as the liver (75), lung (76), and gut (77), NK cells comprise both

recirculating CD56^{dim} cells and tissue-resident CD56^{bright} populations that can retain immature-like features and display distinct receptor profiles, including variable expression of KIR and NKG2A (75-78). Adding to the complexity of NK cell developmental states, tissue-resident NK cells are distributed across most human organs, yet developmental pathways and maturation states remain incompletely understood (78). In addition, recently identified subsets further highlight the heterogeneity of NK cells, such as CD56^{bright} lung NK cells with adaptive-like features, characterized by co-expression of KIRs and NKG2C despite their CD56^{bright} phenotype (79).

Together, these developmental processes are increasingly well characterized in circulation but remain incompletely understood in tissues and disease contexts. It remains unclear how specific tissue environments shape NK cell maturation and long-term functional programming in homeostasis as well as infection, inflammation, and cancer.

2.1.2.4. NK cell education

The balance of activating and inhibitory cues of NK cells ultimately determines if the target cell will be attacked or not (80, 81). NK cells undergo a process known as education, tuning, or licensing, to become fully capable of recognizing cells lacking HLA class I (82, 83). In line with current models of NK cell education, interactions between inhibitory receptors such as notably KIRs (killer cell immunoglobulin-like receptors) and CD94/NKG2A and their matching HLA class I ligands calibrate NK cell responsiveness (83, 84). More recently, the rheostat model has been proposed, suggesting that the strength and number of self-specific inhibitory signals generate a graded spectrum of NK cell responsiveness across individuals (85). Educated NK cells display enhanced responsiveness, whereas NK cells that do not engage inhibitory receptors with self-MHC molecules remain hyporesponsive, although this state can be modulated by the surrounding MHC environment. (85). Additional factors, including activating receptors (e.g. NKG2D, NKp46), co-receptors, and cellular organization at the immunological synapse, also contribute to this process (86-88). This ultimately results in a spectrum of NK cell functionality across not only individuals, but also disease states (83, 84).

In humans, NK cell education is shaped by interactions between inhibitory receptors such as KIRs and CD94/NKG2A and self-HLA class I molecules, including HLA-E presenting leader peptides from HLA-A, -B. and -C (65). The inhibitory

receptor complex NKG2A/CD94 recognizes HLA-E and plays a central role in tuning NK cell responsiveness, as strong NKG2A/CD94-HLA-E interactions contribute to NK cell education and enhance responses to “missing-self” targets, while simultaneously limiting activation when HLA-E is sufficiently expressed (89). This balancing function is particularly relevant in pathological settings such as viral infections and cancer, where HLA-E expression is dynamically regulated and thereby shapes NK cell activity while preventing excessive tissue damage (89). These interactions calibrate NK cell responsiveness and, due to high polymorphisms and independent inheritance of HLA (90) and KIR genes (91), generate substantial inter-individual variation in NK cell functional potential with effects on infection and cancer (83, 84). This variability has important consequences in disease. In tumors or virally infected cells that retain or upregulate HLA class I, highly educated NK cells may be strongly inhibited via their self-specific KIRs or NKG2A, whereas in settings of HLA loss these cells are more efficient at detecting missing-self, a balance that is highly relevant in lung cancer and chronic viral infections (84). In IBD, intestinal NK cell compartments show altered KIR-HLA and NKG2A/NKG2D pathways, suggesting that disease-associated changes in NK cell education and checkpoint engagement can modulate chronic mucosal inflammation (77). Conversely, less educated NK cell subsets can retain activity in environments where HLA expression remains relatively intact, and specific HLA–KIR constellations have been linked to distinct outcomes in viral control and cancer risk (83, 84).

Across tissues, NK cell education is further shaped by local environments that influence receptor expression and functional output (92, 93). Factors such as HLA class I expression levels, cytokine milieu, and tissue architecture modulate how education is functionally interpreted, resulting in tissue-dependent activation thresholds. Together, local HLA landscapes and residency-associated programs create distinct niches in which NK cells integrate systemic education with local cues to tailor immune responses. However, the molecular mechanisms underlying NK cell education in tissues, both in homeostasis and disease, remain incompletely understood.

Present in both circulation and peripheral tissues NK cells can adopt distinct phenotypic and functional characteristics (78, 92). Increasing evidence suggests that tissue environments shape specialized NK cell subsets, in part resembling features described for tissue-resident $CD8^{+}$ T cells (76, 94). The following

chapters discuss tissue-specific NK cell populations and their characteristics in greater detail.

2.2. The adaptive immune system

The adaptive immune system consists of two arms, cell-mediated immunity facilitated by T cells and B cells, and antibodies as the main effector molecules of humoral immunity (2, 3). In contrast to innate lymphocytes, T and B lymphocytes express highly specific antigen receptors enabling recognition of distinct pathogen-associated antigens.

Adaptive lymphocytes start their development from the common lymphoid progenitor found in the bone marrow (95). During T cell development, lymphoid progenitors seed to the thymus and differentiate into $CD4^+$ or $CD8^+$ T cells with highly variable T cell receptors (TCRs) (2, 3). B cells undergo early maturation in the bone marrow, progressing to the immature B cell stage, before entering the circulation. They subsequently home to secondary lymphoid organs, like lymph nodes or the spleen, where antigen encounter and additional maturation signals drive their differentiation into specialized subsets, including memory B cells and antibody-secreting plasma cells (2, 3).

2.2.1. T cells

T cells form a highly diverse compartment that can be broadly divided into $CD4^+$ and $CD8^+$ lineages with distinct, non-redundant roles in antigen-specific immunity (3). $CD8^+$ T cells recognize peptides presented by HLA class I molecules expressed by virtually all nucleated cells and differentiate into cytotoxic effectors that patrol tissues and eliminate infected or transformed targets via perforin-granzyme release and death-receptor pathways (3). $CD4^+$ T cells, in contrast, recognize peptides presented by HLA class II molecules on professional antigen-presenting cells (APCs) (3). They give rise to specialized helper and regulatory subsets (e.g. Th1, Th2, Th17, Tfh and regulatory T cells (Treg)) that shape the magnitude and quality of immune responses by providing cytokines, co-stimulatory signals, support of B cells and $CD8^+$ T cells as well as enforcing immune tolerance (96, 97).

T cells can be further categorized into functionally and phenotypically distinct subsets based on CCR7 and CD45RA expression (98). Naïve T cells ($CCR7^+CD45RA^+$) home to lymphoid tissues, where they scan APCs and serve as a reservoir of unprimed antigen-specific clones (98). Central memory T cells ($CCR7^+CD45RA^-$, TCM) also recirculate through lymphoid organs but have high

proliferative capacity and rapidly generate new effector cells upon antigen re-encounter (98). Effector memory T cells ($CCR7^{-}CD45RA^{-}$, TEM) preferentially migrate to peripheral tissues, where they provide rapid on-site effector functions such as cytokine production and cytotoxicity (98, 99). Terminally differentiated effector memory T cells re-expressing CD45RA ($CCR7^{-}CD45RA^{+}$, TEMRA) display strong immediate effector activity but limited proliferative potential (99). Tregs are a specialized $CD4^{+}$ T cell subset that restrain excessive immune activation and maintain self-tolerance through suppressive cytokines, checkpoint molecules, and modulation of APC function (97). Beyond conventional αβ T cells, mucosal-associated invariant T (MAIT) cells represent a specialized subset that is predominantly $CD8^{+}$, although $CD4^{+}$ and double-negative MAIT cells also exist (100, 101). They express a semi-invariant TCR that recognizes microbial riboflavin metabolites presented by the HLA class I-related molecule MR1 and provide rapid antimicrobial responses at mucosal sites (102). Conversely, γδ T cells, enriched at epithelial and barrier surfaces, combine innate-like sensing with T-cell functions to support tissue surveillance, homeostasis, and repair (103).

2.2.2. B cells

B cells complement T cell-mediated immunity by providing a clonally diverse pool of antigen-specific receptors that can be converted into long-lived antibody and memory responses (3). Rather than directly killing infected or transformed cells, B cells integrate signals from antigen, helper T cells, and innate cues to differentiate into antibody-secreting plasma cells and memory B cells. Additionally, B cells also act as professional APCs that shape $CD4^{+}$ T cell activation and fate in germinal centers and beyond (3). Upon encountering antigen, either in soluble form or presented by APCs such as DCs, B cells undergo clonal expansion and somatic hypermutation in germinal centers in secondary lymphoid organs, leading to affinity maturation (3). Differentiation generates antibody-producing cells, including short-lived plasma blasts and long-lived plasma cells (104). Plasma blasts are proliferative, highly antibody-secreting cells with substantial migratory capacity, whereas mature plasma cells predominantly reside in bone marrow or tissues and continuously secrete immunoglobulins (Ig) of various isotypes (IgA, IgD, IgE, IgG, and IgM) (3, 104). Through these processes, adaptive immunity achieves both high specificity and durable protection against reinfection.

2.2.3. Hallmarks of adaptive memory

Adaptive immune memory is defined by unique receptors, clonal dynamics, and long-lived protection. During development, B and T cells generate highly diverse antigen receptors through V(D)J recombination, creating many small highly specific clonal populations (2, 3). This specificity is generated through random somatic recombination, the defining compound of adaptive immunity also known as variable (V), diversity (D), and joining (J) gene rearrangement, which allows for great diversification of antibodies and TCRs and B cell receptors (BCRs) (2, 3). Although T and B cells rely on similar V(D)J gene rearrangement mechanisms, TCR specificity and antigen recognition are dictated by T cell lineage. Upon antigen encounter, antigen-specific T and B cell clones undergo rapid clonal expansion, followed by a contraction phase in which most effector cells die while a subset differentiates into long-lived memory cells. These memory populations persist with enhanced responsiveness, enabling faster and highly specific reactions upon re-exposure (2, 3).

3. Characteristics of human NK cells in blood

Most studies investigating NK cell phenotype and function have been conducted using peripheral blood, where NK cells constitute approximately 5-20% of circulating lymphocytes. In blood, $CD56^{bright}CD16^{-}$ NK cells comprise around 5-10% of circulating NK cells (105). This subset is known for immunoregulatory properties by responding to and producing cytokines like TNF and IFN-γ, whilst displaying lower cytotoxicity as compared to $CD56^{dim}CD16^{+}$ NK cells (105). While not abundant in blood, $CD56^{bright}CD16^{-}$ NK cells are enriched in certain human tissues, including the uterus, intestines, and liver (78, 106).

$CD56^{dim}CD16^{+}$ NK cells are the predominant NK cell subset in human blood, accounting for approximately 90% of all NK cells (55). A substantial proportion of these cells are considered terminally differentiated, characterized by the expression of the differentiation marker CD57 and loss of the inhibitory receptor NKG2A (72, 73). The frequency of $CD57^{+}CD56^{dim}CD16^{+}$ NK cells has been shown to increase with donor age (78).

3.1. Adaptive NK cells

In addition to the classical $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cell subsets, a population of adaptive NK cells has gained increasing attention over the past decade. Also referred to as memory, memory-like, or adaptive-like NK cells, these circulating $CD56^{dim}CD16^{+}$ NK cells are strongly associated with human

cytomegalovirus (HCMV) infection and can re-expand upon reactivation (107, 108). They are found in approximately 30-40% of HCMV-seropositive individuals and are absent in the HCMV-seronegative population (107, 109). $CD56^{dim}CD16^{+}$ adaptive NK cells are characterized by elevated expression of KIRs, co-expression of CD57 as well as the activating receptor NKG2C, which recognizes HLA-E ($CD56^{dim}CD16^{+}CD57^{+}KIR^{+}NKG2C^{+}$). Adaptive NK cells not only differ phenotypically from conventional $CD56^{dim}CD16^{+}$ NK cells but also epigenetically (110, 111) and functionally (109-111). They exhibit prolonged survival (110) and are predominantly found in blood but also non-lymphoid organs and tumors (reviewed in (112)). While HCMV infection is the best-established driver of their expansion, other viral infections as well as environmental or chemical exposures may also contribute (113-115). Notably, latent HCMV infection can persist in the human lung (116). In addition, an expansion of adaptive-like $CD49a^{+}KIR^{+}NKG2C^{+}$ NK cells has recently been identified in both blood and lung of a subset of lung cancer patients (79) and are described in detail in the chapters below. However, how viral exposure shapes local NK cell composition and function within tissues remains incompletely understood.

3.2. Refining blood NK cell classification beyond CD56 and CD16

Recent efforts have tried to address the issue of heterogenous NK cell nomenclature. In peripheral blood, high-dimensional single-cell analyses showed that $CD56^{bright}$ and $CD56^{dim}$ compartments encompass multiple transcriptionally and functionally distinct NK cell states that are not fully captured by traditional surface markers (117). To address this, Rebuffet and colleagues recently proposed a standardized nomenclature based on single-cell RNA-sequencing (scRNA-seq)-derived gene signatures, defining conserved "NK1," "NK2," and "NK3" programs across donors that align with cytotoxic, regulatory, and adaptive-like features rather than simple $CD56^{bright}/CD56^{dim}$ gating (117). In their framework, classical $CD56^{bright}CD16^{-}$ cells largely map to NK2, while most $CD56^{dim}CD16^{+}$ cells are classified as NK1, and adaptive $NKG2C^{+}CD57^{+}$ populations are captured within NK3. This provides a more nuanced classification of human blood NK cells, while its applicability to NK cells in tissues is still being explored (117). **Figure I** illustrates surface and transcriptomic markers characteristic for NK1, NK2, and NK3.

4. Lymphocyte recruitment to tissues and lung tumors

In peripheral tissues, recruitment of lymphocytes relies on a coordinated sequence of homing receptor engagement, chemokine sensing, and

transmigration across specialized vasculature. Tissue-selective expression of adhesion molecules and chemokines on endothelial and stromal cells imprints organ tropism by engaging corresponding receptors on T cells and NK cells, allowing lymphocytes to exit the circulation and accumulate within inflamed or transformed tissue niches.

Within this thesis, immune cell recruitment to tissues is explored in the following sections, with particular emphasis on NK cell infiltration into lung tumors. Later sections examine the composition and characteristics of immune cells within these tumors.

4.1. Recruitment of T cells

T cell recruitment to tissues is orchestrated by combinatorial expression of homing and chemokine receptors that match vascular and stromal cues in each organ (118). Effector and memory T cells first adhere to endothelium via integrins such as LFA-1 (binding ICAM-1) and VLA-4 (binding VCAM-1), then follow gradients of chemokines like CXCL9, CXCL10 and CCL5 into inflamed sites, where local antigen recognition and adhesion to stromal and epithelial cells promote retention and differentiation (118).

In solid tumors, T cell recruitment is a multistep process governed by chemokine gradients, vascular adhesion molecules, and the local tumor microenvironment. In non-small cell lung carcinoma (NSCLC), tumor cells and tumor-associated myeloid cells produce CXCR3-binding chemokines such as CXCL9, CXCL10, and CXCL11, as well as CCL5, which attract $CXCR3^{+}CCR5^{+}$ effector $CD8^{+}$ and $CD4^{+}$ T cells and are associated with "T cell–inflamed" tumor phenotypes (119, 120). Efficient entry of these cells into tumor tissue depends on adhesion molecules such as ICAM-1 and VCAM-1 expressed on tumor vasculature, which engage integrins on T cells. Variability in their expression contributes to differences between "hot" tumors with dense immune infiltration and "cold" tumors with limited or excluded T cell infiltration capacity (120, 121).

In lung tumors, particularly NSCLC, $CD8^{+}$ tumor-infiltrating T cells are a major component of the immune infiltrate, and their abundance and spatial distribution are closely linked to clinical outcome (122). However, recruitment alone is not sufficient for effective tumor control (123). Single-cell transcriptional analyses revealed that $CD8^{+}$ T cells within lung tumors span a continuum from naïve-like and effector states to dysfunctional or exhausted states, indicating that local

microenvironmental cues ultimately shape their functional capacity following infiltration (123-125). However, the characteristics of T cells within tumors, at the tumor margin, and along the tumor–tumor-free axis, as well as their spatial distribution across these regions, remain poorly defined.

4.2. Recruitment of non-NK ILCs

Most non-NK ILC subsets are tissue-resident at steady state, seeded early in life and maintained locally (33, 126). During homeostasis, human non-NK ILCs are enriched in tissues and show features of long-lived, locally adapted populations (33), whereas in chronic inflammation and cancer their accumulation reflects expansion and repositioning of these resident pools together with chemokine-guided recruitment of a limited number of circulating ILC precursors or ILC-like cells (33, 127).

These pathways are reshaped by the tumor microenvironment (TME). In NSCLC, tumor, myeloid and stromal cells produce cytokines and chemokines such as IL-23, IL-1β, IL-33, CCL20 and CXCL16 that shape the local ILC compartment, promoting recruitment, expansion and functional polarization of ILC subsets within and around tumor nests (127-129). IFN-γ-producing ILC1-like cells and NCR^{+} ILC3s accumulate at tumor margins where they sense IL-23 and related inflammatory cues. Subsequently, they can either support anti-tumor immunity by producing TNF and inducing CXCL10-dependent recruitment of T cells and tertiary lymphoid structures, or, when skewed toward IL-17/IL-22 programs, drive neutrophil recruitment, angiogenesis and tissue remodeling that favor tumor progression (130).

In this way, non-NK ILCs in lung tumors are predominantly maintained as locally resident populations, with the capacity to reposition within the tissue. Their spatial distribution and influence on tumor control versus progression are shaped by the tumor chemokine and cytokine milieu, which governs their subset composition and functional polarization, although these processes remain an active area of investigation. Despite significant advances in the field, non-NK ILCs within tumors, at the invasive margin, and across adjacent tumor-free tissue remain insufficiently characterized, both in terms of their phenotypic properties and spatial organization. In addition, how their distribution aligns with or diverges from that of T cells and NK cells within these compartments is still largely unexplored.

4.3. Recruitment of NK cells

NK cells continuously circulate between blood and peripheral tissues, where their localization is tightly regulated by chemokine receptor expression, adhesion molecules, and local microenvironmental factors.

Circulating NK cells typically express CXCR1, CXCR2, CXCR3, CX3CR1, and CCR5, which mediate chemotaxis toward chemokines such as CXCL8, CXCL9, CXCL10, CXCL11, CX3CL1, and CCL5 produced by epithelial, stromal, and resident immune cells during homeostasis and inflammation (131). Additional axes, including CXCR6-CXCL16, CXCR3 ligands, CCR5 ligands, and CX3CR1, guide NK cell positioning within distinct tissue niches (132), such as within the lung (133) during respiratory viral infection (134), and in the gut during inflammatory processes (135).

In addition, NK cell phenotype and functional specialization are further shaped by the tissue microenvironment, which differs from blood by fluctuations in cytokines including transforming growth factor-β (TGF-β) (136), IL-15 (137), as well as metabolic signals (138) and oxygen tension (139). In parallel, the expression of ligands, surface proteins, and other signaling molecules varies according to tissue context and physiological state, forming a complex regulatory network that governs immune cell recruitment, retention, activation, and suppression. Elucidating these dynamic interactions remains an important area of ongoing research.

NK cell entry into solid tumors is similarly chemokine-driven but frequently impaired by tumor-mediated immune evasion. Cancer cells as well as the tumor microenvironment (TME) produce CXCL9, CXCL10, CXCL11, and CCL5, which attract $CXCR3^+$ and $CCR5^+$ NK cells (119, 131). In detail, many tumors recruit less cytotoxic NK cells or regulate NK cell activation by immunosuppressive factors such as TGF-β, prostaglandin E2, and adenosine (131). The TME imposes additional metabolic constraints on NK cells, as hypoxia-driven HIF-1α signaling (139, 140) and tumor-derived lactic acid impair mitochondrial function, reducing cytotoxicity and cytokine production (141), leading to distinct phenotypic and functional features, not only in solid tumors but tissues in general. Detailed tissue-specific NK cell characteristics are discussed in the following sections.

4.4. Hallmarks of tissue residency in NK cells

This chapter provides and overview of the hallmarks of tissue-resident NK (trNK) cells. More detailed descriptions of NK cells in the tissues and conditions relevant to this thesis are described in the sections below.

The first paper describing trNK cells in humans was published in 2013, where CD49a was established as a key marker of tissue residency in the liver (142). Subsequently, trNK cells have been identified in various other tissues, including the uterus (106), small intestines (77, 78), liver (143), tonsils (40), and lung (144). Recent efforts utilizing organ donor cohorts have made it possible to study matched human organs, allowing for direct comparison of NK cell distribution across different tissues (78, 145).

Historically, concepts of tissue residency have been largely derived from T cell studies and applied to NK cells. Hallmarks of tissue residency are the expression of the α1 integrin CD49a, together with other markers such as CD69 and the integrin CD103 (146). In detail, CD49a heterodimerizes with CD29 (integrin β1) to form the integrin very late antigen-1 (VLA-1, α1β1 integrin), which mediates adhesion to collagen IV (147). Type IV collagen is a major component of the basement membrane, an extracellular matrix structure located at the basal side of epithelial and endothelial cells (148). In T cells, CD69 has been shown to interfere with the sphingosine 1-phosphate receptor 1 (S1PR1), inhibiting tissue egress (149-151). CD103 (αE integrin) binds to the integrin β7 subunit of E-cadherin, a protein found on epithelial cells, and forms the heterodimeric integrin αEβ7 (152).

While tissue residency in T cells is acquired during differentiation into memory populations, in NK cells it is primarily associated with less differentiated subsets (76, 94). Of note, trNK cells have so far exclusively been described as $CD56^{bright}CD16^{-}$ NK cells and it is hypothesized that they contribute to tissue homeostasis rather than eliciting classic cytotoxic NK cell functions (92, 153). trNK cells share the expression of tissue-residency markers across different sites of the human body, but also expresses organ-specific characteristics, potentially influenced by local microenvironmental factors (92, 153, 154). Key surface and transcriptomic markers expressed by trNK cells are depicted in **Figure I**.

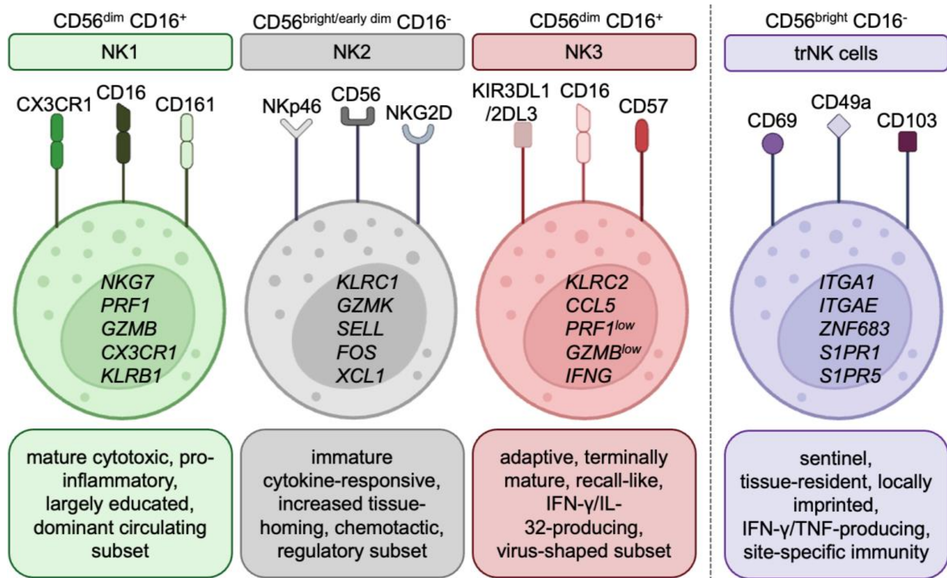

Figure I: Phenotypic and transcriptional features of NK cell subsets. Schematic illustrating characteristics of NK1 (green), NK2 (grey), and NK3 (red) populations, alongside key features of trNK cells (purple).

It is not yet fully understood whether trNK cells originate from circulating NK cells recruited during inflammation (155) or whether they constitute a distinct, self-renewing population established within tissues (76, 149). In mice, fate-mapping and parabiosis studies in the liver, skin, and uterus demonstrated that pre-existing uterine-resident NK cells proliferate locally during decidualization, with minimal contribution from the circulation, supporting the concept that some resident NK pools are sustained *in situ* (149, 156). In contrast, human liver transplantation studies revealed that donor CD56^{bright} liver-resident NK cells are gradually replaced by recipient-derived NK cells that enter the liver and acquire tissue-resident traits, indicating that other resident NK compartments can be renewed from blood-derived precursors (157). In barrier tissues such as the lungs and gut, local chemokines recruit circulating NK cells (132, 146, 158). Upon entering these tissues, NK cells can acquire tissue-resident phenotypes and persist as specialized sentinels with organ-specific cytokines and niches shaping their activation and differentiation (132, 146, 158). In detail, human studies show that CXCR3^{+} and CXCR6^{+} NK cells are preferentially recruited to inflamed airways and lung parenchyma (76, 159), where TGF-β and local stromal cues imprint tissue-resident, functionally restrained NK cell subsets that differ from highly cytotoxic intravascular NK cells patrolling the pulmonary vasculature (160). In the gut, NK

cells adapt to the intestinal microenvironment by upregulating retention molecules such as CD69, CD103, CD49a, and the chemokine receptor CXCR6, which together support their localization within the intestinal epithelium and lamina propria (77). These findings suggest that trNK cells likely arise from a combination of long-lived, locally maintained populations and newly recruited circulating NK cells that acquire tissue-resident characteristics upon entry.

However, the relative contribution of local versus circulatory NK cells to the local trNK cell population appears to vary between organs and disease conditions, and their origin and maintenance remain incompletely resolved. Additionally, it is unclear whether trNK cells represent stable, self-renewing lineages or transiently imprinted circulating NK cells, how plastic these states are, and which local tissue cues are required to establish and sustain tissue residency of NK cells.

5. Immune cells in human tissues

The focus of this thesis is to investigate the heterogeneity of NK cells across different tissues and disease contexts in the adult human body. In addition to NK cells, Studies I, II, and IV also address the broader immune cell composition in various human tissues, with particular emphasis on the lung and intestine. Study I characterizes the pulmonary leukocyte landscape, while Studies II and IV further examine lymphocyte populations in lung tumors and IBD, respectively.

Given this context, additional immune cell types beyond NK cells are introduced in the following sections, where relevant, to provide a comprehensive framework for understanding tissue-specific immune organization and its implications for disease.

5.1. The human lung

The human lungs comprise the left and right lung, consisting of two and three lobes, respectively, and are connected via the trachea and primary bronchi (161). Part of the tracheobronchial tree, the trachea divides into the primary bronchi, which further branch into progressively smaller airways that terminate in alveolar sacks, where gas exchange occurs (161). As a barrier organ with a large surface area exposed to the external environment, the lungs are continuously challenged by inhaled pathogens, including bacteria and viruses. Effective defense therefore requires a rapid and finely tuned local immune response capable of eliminating harmful microbes while maintaining tolerance to harmless particles like pollen. Key players in lung immunity include epithelial cells lining the bronchi and forming

alveoli (162), as well as airway macrophages, commonly referred to as alveolar macrophages (163). While the functions of alveolar macrophages are relatively well characterized, the composition and organization of other immune cells, particularly NK cells, across individual lung areas remain poorly defined.

5.1.1. Myeloid cells in the homeostatic lung

In the lung, alveolar macrophages reside within the airspace and are largely long-lived, self-maintaining cells of embryonic origin that maintain surfactant recycling and clearance of inhaled particles and pathogens (164, 165). In addition, interstitial and perivascular macrophages occupy the lung parenchyma and vessel-associated niches, where they contribute to the regulation of immune and vascular responses (164, 165). DCs populate tissues and act as professional antigen presenting cells (APCs), linking innate and adaptive immunity by priming T cell responses in lymph nodes (166). Granulocytes, consisting of neutrophils and smaller proportions of eosinophils and rarer basophils, are mostly short-lived in blood but can be rapidly recruited to the lung during microbial or parasitic infection or injury (167). Mast cells reside in barrier tissues near the vasculature and nerves, where they regulate tissue damage, host defense, and allergic inflammation (168). Together, these populations form a dynamic network that preserves tissue integrity while enabling rapid responses to danger.

5.1.2. T cells in the homeostatic lung

The homeostatic lung contains a large and phenotypically diverse T cell compartment that must maintain a delicate balance between rapid antimicrobial protection and prevention of excessive inflammation (169, 170). Most T cells in human lung parenchyma are $CD69^+CD103^+$ tissue-resident memory T cells (TRM) that persist long-term in the airway and interstitial niches and are poised for rapid effector responses (169, 170). These cells are enriched for effector-memory phenotypes, display a broad TCR repertoire, and include both $CD4^+$ and $CD8^+$ subsets capable of producing IFN-γ, TNF, IL-17, and other cytokines upon local antigen encounter, providing front-line defense against inhaled pathogens (170). At the same time, lung-resident Treg and specialized helper subsets shape tolerance to inhaled harmless antigens and support tissue repair, illustrating how the resident T cell pool contributes not only to immune surveillance but also to preservation of lung structure and function at steady state (169, 170).

Although substantial progress has been made in understanding lung T cell biology, key aspects remain unresolved. It is still unclear how distinct TRM, Treg, and effector subsets are maintained and renewed over years in different airway and parenchymal niches, and to what extent their exhausted or regulatory states are reversible *in vivo* (169, 170). How environmental factors reshape resident T cell pools over time in individual patients and how this impacts protection versus chronic immunopathology or impaired tumor control remains poorly understood.

5.1.3. NK cells in the homeostatic lung

Eary studies suggested that circulating $CD56^{dim}CD16^{+}$ NK cells, the predominant subset of pulmonary NK cells (144), exhibit reduced functional activity (144, 171). This has been linked to the local production of TGF-β (172) by airway macrophages (173), which potentially inhibits NK cell activation and effector functions.

Additionally, smoking has a profound impact on NK cell function. As early as 1988, smoking was shown to impair NK cell responses in bronchoalveolar lavage fluid (BALF) (174). These findings have been supported by more recent studies demonstrating reduced functional capacity of NK cells in lung tissue from smokers compared with non-smokers (144). Both current and ex-smokers exhibit decreased frequencies of NK cells in lung tissue, accompanied by a relative increase in T cell frequencies (144). The same study reported that the majority of NK cells in the human lung are highly differentiated $CD56^{dim}CD16^{+}$ cells, frequently expressing KIRs and CD57 (144). Despite this mature phenotype, lung NK cells are hyporesponsive to target cell stimulation, even following IFN-α priming, compared with autologous blood-derived NK cells, regardless of smoking status (144). Notably, NK cells from smokers exhibited further reduced responsiveness compared to NK cells from ex-smokers, suggesting that this impaired functional state is at least partially reversible (144). Surface and transcriptomic markers expressed by circulating NK cells in the lung are shown in **Figure II**.

These observations underscore the complexity of pulmonary NK cell regulation and suggest that functional heterogeneity may vary across anatomical compartments. While lifestyle factors such as smoking have been extensively studied (144, 174), the role of anatomical and biomechanical features of the lung have received comparatively little attention. The interplay of gravity, ventilation, and perfusion creates region-specific microenvironments within the lung, with enhanced lymphatic drainage in the lower lobes and relatively increased ventilation in the upper lung regions (175, 176). Such differences may contribute to

localized immune regulation but have largely been overlooked when studying immune cell distribution across lung regions.

Importantly, many pulmonary diseases exhibit pronounced spatial patterns, suggesting that regional microenvironments influence disease susceptibility and immune responses. Pulmonary fibrosis, for example, predominantly affects the lower lobes, whereas emphysema is more commonly observed in the upper lobes (177, 178). Similarly, infectious and inflammatory diseases show distinct localization patterns: pneumonia may present with lobe-specific involvement depending on etiology (179), tuberculosis preferentially affects the upper lobes (176, 180), and asthma and chronic obstructive pulmonary disease differentially involve smaller airways and upper lung regions (181-183). In addition, latent viral infections such as HCMV have been detected in lung tissue (116), raising the possibility that local viral reservoirs further shape region-specific immune landscapes. In lung cancer, tumor subtypes also exhibit characteristic central or peripheral localization within the lung (176). Moreover, clinical interventions such as lung transplantation expose different lobes to varying degrees of ischemia–reperfusion injury and alloimmune responses, potentially further shaping local immune niches (184).

Despite major advances from single-cell and spatial mapping of the human lung, which have revealed regional specialization of epithelial, stromal, and vascular compartments (185-189), the resolution of immune populations, particularly NK cells, remains insufficient. While these studies have outlined broad immune cell classes and provided a valuable framework of pulmonary immune organization (186, 188, 189), a detailed and systematic characterization of NK cell heterogeneity across distinct anatomical areas of the human lung is still lacking.

In general, most human studies investigating pulmonary NK cells have focused on bulk lung tissue (78, 187, 189), BALF (190), or macroscopically defined regions from non–patient-matched samples, (76, 79, 144, 187, 191, 192), with only limited efforts to systematically compare NK cell phenotype and function across individual lung lobes and other matched areas. While matched lung region analyses enable valuable intra-individual comparisons, such studies remain limited and have not comprehensively investigated NK cell characteristics (186, 188). Consequently, the spatial organization of NK cell populations within the human adult lung remains poorly defined.

Addressing this gap will be important for understanding how local NK cell organization may contribute to region-specific susceptibility to infection, tissue

injury, or transplant-related complications. This is particularly relevant given that NK cells have been identified as resident populations in the lung and are increasingly recognized as key regulators of both homeostasis and disease (193).

5.1.4. trNK cells in the human lung

In the human lung, trNK cells were first described as $CD16^-CD69^+$ NK cells infiltrating NSCLC and adjacent parenchyma, where they were enriched compared with blood (194, 195). Shortly after, lung NK cells expressing CD49a and CD103 together with a cytokine-responsive but relatively restrained cytotoxic profile were identified as response to influenza infection (191, 192). Subsequent analyses of macroscopically tumor-free lung tissue from lobectomy donors confirmed a $CD16^-CD69^+CD49a^+$ ($\pm CD103^+$) NK population in healthy parenchyma that segregated transcriptionally from circulating NK cells and non-resident lung NK cells, establishing lung trNK cells as a bona fide tissue compartment outside the tumor context (76, 144). Additionally, trNK cells were found in BALF of healthy individuals (196).

The key ligands for trNK cell-associated integrins in the lung are type IV collagen, which forms a major component of the alveolar and vascular basement membranes (148) and provides a binding partner for CD49a (142), as well as E-cadherin on airway and alveolar epithelial cells (197), which interacts with CD103 (198). In homeostasis, these matrix and epithelial structures are maintained in a relatively quiescent state, whereas in chronic lung disease (199) and remodeling, such as fibrosis (177, 183) or tumor growth (200), basement membrane composition and E-cadherin expression are altered, potentially reshaping local adhesion and retention cues for $CD49a^+$ and $CD103^+$ trNK cells and modifying their distribution and retention within the lung parenchyma.

Compared with conventional $CD69^-$ lung NK cells and blood NK cells, lung trNK cells are enriched for a residency-associated phenotype ($CD16^-CD69^+CD49a^+ \pm CD103^+$ $CXCR3^+CXCR6^+$) and show reduced expression of recirculation markers such as *S1PR5*, *SELL*, and *KLF2/3* (76). At the transcriptional level, trNK cells exhibit higher expression of residency- and activation-linked genes, including *ZNF683*, *ITGA1*, *CXCR6*, and *CD69*, and lower expression of egress-associated genes and some cytotoxicity modules. Functionally, conversely to hypofunctional lung $CD69^-$ NK cells, lung trNK cells remain IL-15 responsive, upregulating Ki-67, perforin, and GzmB upon cytokine stimulation more readily (76, 144). Lung trNK cells also showed enhanced responses to

influenza virus infection (191, 192). An illustration of key markers expressed by pulmonary trNK cells is shown in **Figure II**.

In addition to these phenotypic, transcriptional, and functional characteristics, recent work has identified distinct metabolic features of lung trNK cells (196). Utilizing SCENITH to investigate NK cell metabolism from matched BALF and blood from healthy donors, the study describes that $CD49a^{+}CD69^{+}CD103^{+/-}$ $CD56^{bright}CD16^{-}$ trNK cells have increased glycolytic capacity and glucose dependence compared with non-trNK BALF and blood NK cells, alongside reduced mitochondrial reliance (196). Re-analysis of RNA-seq data further showed enrichment of glycolysis- and activation-related transcripts, suggesting that lung trNK cells are metabolically primed for rapid responses during inflammation (196). Altogether, these findings indicate a tissue-tuned program that is distinct from both circulating NK cells and non-resident lung NK cells and likely reflects adaptation to local stromal and epithelial cues (76, 144, 191, 192, 196).

Despite these advances, key aspects of lung trNK biology remain unresolved. Existing human data are largely cross-sectional and derived from BALF, lobectomy, or tumor-adjacent tissue. Thus, the *in vivo* dynamics, distribution across distinct lung lobes, lifespan, and turnover of $CD16^{-}CD69^{+}CD49a^{+}(\pm CD103^{+})$ trNK cells during pulmonary disease, natural infection, inflammation, hypoxia, or steady state remain unknown, as does the balance between local self-renewal and continuous replenishment from circulating NK cells (76, 144, 191, 192). Furthermore, although type IV collagen and E-cadherin have been identified as ligands for CD49a and CD103, their spatial distribution and regulation across distinct anatomical niches and disease states have not been mapped in a way that directly links matrix and epithelial remodeling to trNK positioning *in vivo* (177, 200, 201). Additionally, knowledge regarding metabolic adaptations of lung trNK cells are limited to findings in BALF and remain unexplored in lung tissue in homeostasis as well as disease (196).

Most studies treat the lung as a single homogeneous organ (76, 144, 191, 192, 196, 201), providing essentially no information on whether lung trNK distribution, phenotype, transcriptomics, metabolism, or function differ between the five lobes or between central and peripheral regions, even though biomechanical features (175, 176) as well as many lung diseases and transplant-related insults show region-specific patterns (176, 178, 179, 181, 182). Finally, although trNK cells differ from conventional lung NK cells, their roles in human antiviral, antibacterial, or

tissue-repair responses remain unclear. It is also not yet understood how pathogen- or microenvironment-specific cues shape durable, dynamically regulated trNK programs (191, 192, 201).

5.1.5. Adaptive-like trNK cells in the lung

Expansions of adaptive-like trNK cells, defined as $CD49a^{+}KIR^{+}NKG2C^{+}CD56^{bright}CD16^{-}$ NK cells with a tissue-resident phenotype ($CD69^{+}$±$CD103^{+}$), have recently been identified in macroscopically normal lung tissue and, at lower frequencies, in matched blood (79). This subset presents a distinct NK cell compartment that integrates tissue residency with features of HCMV driven adaptive differentiation (79). At transcriptomic level, they cluster separately from conventional lung trNK cells and circulating adaptive $CD56^{dim}$ NK cells (79). RNA-sequencing further demonstrated that $KIR^{+}NKG2C^{+}$ trNK cells form a distinct transcriptional cluster, with over 100 genes differentially expressed compared with $KIR^{-}NKG2C^{-}$ trNK cells, while sharing a core adaptive-like gene signature with peripheral blood adaptive NK cells (for example, increased *KIR*, *KLRC2*, *GZMH*, *ITGAD* and decreased *FCER1G*, *IL18RAP*) (79). Notably, the KIR repertoire of lung $KIR^{+}NKG2C^{+}$ trNK cells is skewed toward inhibitory self-KIRs, and their overall KIR expression pattern differs from $CD16^{+}$ NK cells in paired peripheral blood (pb) and lung as well as from adaptive $CD56^{dim}CD16^{+}$ pbNK cells, indicating subset-specific KIR selection and/or differentiation in the lung environment (79). Functionally, $KIR^{+}NKG2C^{+}$ trNK cells exhibit enhanced effector responses, including increased degranulation and production of IFN-γ and TNF upon target cell stimulation, compared with non-adaptive ($KIR^{-}NKG2C^{-}$) trNK cells (79). A graphic summary of receptors and genes expressed by lung adaptive-like trNK cells is shown in **Figure II**.

Beyond non-inflamed lungs, $NKG2C^{+}KIR^{+}$ NK cells have also been observed in bronchoalveolar lavage from lung transplant recipients, particularly in the setting of HCMV viremia, indicating that intubation plus allograft HCMV reactivation can drive expansions of highly KIR-expressing $NKG2C^{+}$ NK cells in the airway compartment, although these studies did not systematically assess CD49a or other residency markers (202). Insights from murine models further support this concept, as murine CMV infection induces long lived, tissue-resident memory-like NK cells that arise from circulating NK cells, upregulate CD49a, and establish durable residency in non-lymphoid tissues, including the lung (155). These findings provide a mechanistic framework for how infection and tissue-driven signals can

generate KIR^+NKG2C^+ trNK cell populations with a self-KIR biased repertoire and high NKG2C co-expression in the human lung.

However, several aspects of KIR^+NKG2C^+ trNK cell biology in the human lung remain unclear. It is not known how and where KIR^+NKG2C^+ trNK cells are initially imprinted, what proportion derives from adaptive $CD56^{dim}$ versus naïve-like $CD56^{bright}$ NK cells or unknown progenitors, or how stable their tissue-resident phenotype and KIR repertoire remain over time, particularly in relation to HCMV exposure (79, 202). Most studies rely on single time points from lobectomy, BALF, or mouse lung tissue, providing limited insight into their turnover, spatial distribution across lung lobes and microanatomical niches, or their functional role during natural infections. It also remains unclear whether this population is specific to the lung or present in other tissues in donors with such expansions. Moreover, it is yet to be determined if factors such as different respiratory viruses, mechanical ventilation, tissue injury, or the transplant microenvironment can independently induce KIR^+NKG2C^+ trNK cells or primarily expand pre-existing HCMV-imprinted populations.

Understanding how tissue environments shape NK cell phenotype, adaptation, and function in the lung is essential for elucidating disease mechanism and may reveal new avenues for therapeutic options of pulmonary infections or cancer.

5.2. Lung cancer

Lung cancer is one of the leading causes of cancer-related mortality worldwide, with smoking as the major risk factor and most patients being current or former smokers older than 65 years (203). NSCLC accounts for approximately 85% of lung cancers and typically grows more slowly than small cell lung cancer, which is less common but more aggressive and rapidly metastatic (204). Within NSCLC subtypes, adenocarcinoma (AC) usually arises from transformed alveolar type II epithelial cells in peripheral lung regions and represents about 40% of NSCLC, whereas squamous cell carcinoma (SCC) originates from bronchial squamous epithelium in central airways and is characterized by frequent genomic alterations and keratinization (205).

AC and SCC differ not only in cell of origin but also in their tumor immune microenvironments. AC generally shows a more "lymphoid-rich" landscape with higher fractions of resting $CD4^+$ T cells, memory B cells, mast cells and active NK cells, and relatively fewer macrophages and neutrophils, immune features that tend to associate with better overall survival (206, 207). In contrast, SCC often

displays denser $CD8^+$ T cell infiltration within tumor nests, higher tumor mutational burden and distinct patterns of PD-L1 expression, suggesting stronger intrinsic immunogenicity but also a microenvironment more skewed toward myeloid and regulatory networks that can shape and sometimes limit effective antitumor responses (207, 208).

In addition to the already complex network of tumorigenesis, mutations in key oncogenes further reshape the lung TME. These mutations alter chemokine production, antigen load, and metabolic stress, which influence the recruitment, activation, and exhaustion of T cells, NK cells, and ILCs (209). *EGFR* (epidermal growth factor receptor) and *KRAS* (Kirsten rat sarcoma viral oncogene homolog) are the most common driver oncogenes in lung AC and critically shape the composition and function of the tumor-infiltrating immune compartment (209). SCC, on the other hand, often presents with a more complex pattern, with higher mutation frequencies compared to AC (210). *EGFR*-mutated NSCLC typically exhibits a relatively "cold" immune phenotype, with reduced lymphocyte infiltration, lower PD-L1 expression (211), diminished $CD8^+$ T cell and $CD56^{dim}$ NK cell content (212), and a more immunosuppressive cytokine milieu, consistent with the limited clinical benefit these patients derive from PD-1/PD-L1 blockade (213). By contrast, *KRAS*-mutant tumors, particularly those arising in smokers, tend to have higher tumor mutational burden and a more inflamed microenvironment, with increases in tumor-infiltrating lymphocytes (TILs), including NK cells and other innate lymphoid cells (214). These tumors are generally more responsive to immune checkpoint inhibition, although co-occurring mutations, for example in other genes like *STK11* or *KEAP1*, can limit T cell and NK cell cytotoxicity (215).

Surgical resection by lobectomy remains the standard of care for early-stage NSCLC, but relapse, inoperable disease and resistance to standard therapy underscore the need for alternative treatment strategies, including those that harness local immune responses against tumor cells (216).

Tumor development in the lung is accompanied by complex patterns of immune cell infiltration shaped by chemokines, adhesion molecules, and local metabolic cues. Chemokine gradients such as CXCL9, CXCL10, and CXCL11 acting through CXCR3, and CX3CL1 acting through CX3CR1, promote recruitment of activated NK cells and $CD8^+$ T cells into NSCLC lesions (217). These trafficking signals act on top of vascular and stromal barriers influenced by hypoxia, acidosis, and extracellular matrix remodeling, which can either permit or exclude effector lymphocytes from

tumor nests (218, 219). Within this milieu, NK cells, ILCs, and T cells infiltrate lung tumors to varying degrees (122) and adopt tissue-imprinted states that range from cytotoxic and protective to exhausted, pro-angiogenic, or immunoregulatory, depending on the balance of activating versus suppressive signals they encounter.

Checkpoint receptor upregulation is a hallmark of TILs and reflects sustained antigen exposure and chronic stimulation within the tumor microenvironment (220). In addition to classical expression of PD-1 (programmed cell death-1), other inhibitory receptors such as LAG-3 (lymphocyte activation gene 3), TIGIT (T cell immunoreceptor with Ig and ITIM domains), and TIM-3 (T cell immunoglobulin and mucin domain 3) are frequently co-expressed on T cells and innate lymphocytes, forming a layered regulatory network that constrains effector function (220, 221). In $CD8^+$ T cells, co-expression of these checkpoints is widely associated with a spectrum of dysfunction often termed "exhaustion," characterized by reduced proliferative capacity, impaired cytokine production (e.g. IFN-γ, TNF), and altered transcriptional and metabolic profiles (222). $CD4^+$ T helper subsets can similarly acquire inhibitory receptor expression, which may skew their differentiation and limit their ability to sustain anti-tumor responses (222). Tregs, in contrast, often express high levels of immune checkpoints like CTLA-4 and TIGIT, which can enhance their suppressive activity and further reinforce an immunosuppressive milieu (222). Within the innate compartment, NK cells also exhibit increased expression of inhibitory receptors in tumors. The enrichment of NK cells within $TIM\text{-}3^{high}$ and $TIGIT^{high}$ populations supports the notion that they, like T cells, undergo functional adaptation or exhaustion in response to persistent tumor-derived signals (223). This state is often accompanied by diminished cytotoxicity and reduced cytokine secretion, limiting their capacity to eliminate malignant cells.

Importantly, these inhibitory programs are not always irreversible. Functional studies demonstrate that *ex vivo* blockade of PD-1 and CTLA-4 can restore IFN-γ and TNF production in specific lymphocyte subsets. Recently, checkpoint blockade of tumor-infiltrating $CD69^+CXCR6^+$ trNK cells rescued their capacity to secrete cytokines to levels comparable to trNK cells in non-tumor lung tissue (223). Similar effects have been extensively described in exhausted $CD8^+$ T cells, where checkpoint inhibition can partially restore effector function and proliferation (224, 225). These findings suggest that while some TILs are terminally

exhausted, others retain functional plasticity and can be functionally "rescued" upon checkpoint blockade.

Despite rapid progress, many aspects of lymphocyte phenotype and function within tumors and adjacent tissue remain poorly defined. The relative contributions and plasticity of circulating versus tissue-resident T cell and NK cell subsets across the tumor–non-tumor axis are not yet fully understood. Moreover, how these populations are reprogrammed by chronic exposure to TGF-β, hypoxia, and nutrient competition has only recently begun to be resolved through single-cell and spatial approaches. It also remains unclear to what extent metabolic constraints within the fibrotic hypoxic lung tumor niche imprint durable exhaustion programs in T and NK cells, and how this impacts long-term tumor control and responses to immunotherapy.

5.2.1. T cells and lung cancer

Beyond innate lymphocytes, T cells form a major component of the immune infiltrate in NSCLC and critically influence patient outcome (122). Tumor-infiltrating $CD8^+$ T cells can differentiate into exhausted states characterized by high PD-1, TIM-3, and LAG-3 expression (226). Conversely, the expression of these markers has been associated with improved prognosis in NSCLC cohorts (226, 227). Additionally, elevated frequencies of $CD103^+CD8^+$ T cells inside and near NSCLC is associated with improved survival and better responses to PD-1/PD-L1 blockade (228, 229). A recent study investigating the impact of co-localization and co-infiltration of NK cells and $CD8^+$ T cells in NSCLC confirmed association with improved patient survival (230).

Histology-specific analyses suggest that SCC often displays higher densities of $CD8^+$ T cells within cancer cell nests than AC, whereas in AC, the prognostic benefit of strong $CD4^+$ and $CD8^+$ T cell infiltration appears more pronounced (231, 232). This is in line with partially distinct immune microenvironments in AC versus SCC, as well as with the varying contributions of T cell subsets to clinical outcomes in these tumor types (206, 207).

It remains unclear how and when T cells are recruited to solid lung tumors, whether they are already present at tumor onset, and how they become impaired or contribute to shaping the TME. Further research is needed to unravel their spatial organization within and adjacent to tumors, and which mechanisms determine effective versus dysfunctional immune responses in NSCLC.

5.2.2. ILCs and lung cancer

Non-NK ILCs are increasingly recognized as modulators of the lung tumor microenvironment, with context-dependent pro- and antitumor roles. They are detectable in human NSCLC but are less well characterized than NK cells.

Peripheral and tumor-associated group 1 ILCs with ILC1-like ($EOMES^{low}$, $T\text{-}bet^{+}$) phenotypes appear reduced or functionally exhausted in NSCLC patients, similar to NK cells, suggesting impaired type-1 antitumor immunity (233). In contrast, pro-tumoral “ex-NK/ILC1-like” cells can arise upon TGF-β exposure in lung tumor microenvironments, displaying diminished IFN-γ production and cytotoxicity (234), and associate with angiogenic activity and more advanced disease (235).

By contrast, ILC2 frequencies are elevated systemically and within NSCLC lesions, where an IL-25-ILC2 axis associates with increased Treg accumulation, enhanced tumor growth and metastasis, and reduced overall survival, indicating that ILC2 can foster an immunosuppressive, tumor-promoting milieu in human lung cancer (236). Additionally, ILC2s were enriched in peripheral blood and lung tumor tissue of NSCLC patients with an increased expression of PD-1 (237). They displayed increased immunosuppressive capacity, characterized by high production of type 2 cytokines IL-4 and IL-13, which promote M2-like macrophage polarization and thereby reinforcing an immunosuppressive TME (237). Other studies further highlight the tumorigenic role of ILC2s in lung cancer. It was shown that IL-33–activated lung ILC2s secrete IL-5, which drives eosinophil accumulation that in turn metabolically suppresses NK cell IFN-γ production and cytotoxicity, creating an innate checkpoint that permits metastasis of lung cancer (238). Disrupting the IL-33–ILC2–eosinophil axis restores NK cell effector function and reduces metastasis, highlighting ILC2s as potential upstream regulators of antitumor NK cell activity in the lung (238).

In NSCLC, tumor-infiltrating NCR^{+}ILC3s accumulate in early-stage disease at the edge of tertiary lymphoid structures, where they produce pro-inflammatory cytokines and lymphoid-organizing factors and may support protective antitumor tertiary lymphoid structure formation (239). Moreover, IL-23-producing pulmonary SCC can drive *in situ* plasticity of the ILC compartment: IL-23 from tumor cells converts ILC1s into ILC3s, increasing IL-17-producing $CD3^{-}ROR\gamma t^{+}$ ILC3s, which in turn promotes IL-17-dependent tumor cell proliferation and is associated with poor prognosis in SCC, but not AC (128).

Non-NK ILC biology differs between lung cancer subtypes. In SCC, tumor-derived IL-23 drives conversion of ILC1s into IL-17–producing ILC3s, which is associated with poor prognosis, a mechanism not evident in AC. In contrast, ILC2 expansion and type-2–skewed responses appear across both NSCLC subtypes, suggesting shared immunosuppressive roles independent of histology (128).

Overall, current lung cancer data and broader cancer ILC literature indicate that non-NK ILCs can either support antitumor surveillance or contribute to an immunosuppressive, tumor-promoting niche, but their precise role in NSCLC remains incompletely defined. Additionally, a comprehensive understanding of the phenotypic features of non-NK ILCs across intratumoral, marginal, and adjacent non-tumor regions has yet to be clearly defined.

5.2.3. NK cells in lung cancer

In NSCLC, $CD16^-$ NK cells are enriched in both tumor and tumor-adjacent tissues, whereas $CD16^+$ NK cells are relatively depleted compared with non-tumor lung tissue and blood (194, 195, 240). These intratumoral $CD16^-$ NK cells often display limited degranulation toward tumor targets (194, 195) but can secrete substantial amounts of IFN-γ, TNF, and pro-angiogenic mediators such as vascular endothelial growth factor, reflecting a shift from classical cytotoxicity toward immunomodulatory and, in some contexts, pro-tumor activity in the NSCLC microenvironment (235). Multiple cohorts have reported that higher overall NK cell or $CD56^{dim}$ NK cell infiltration in NSCLC lesions associates with improved survival, whereas predominance of $CD56^{bright}CD16^-$ or functionally suppressed NK cell subsets can correlate with poorer outcome, emphasizing that the prognostic impact of NK cells depends on subset composition rather than total numbers alone (230, 241, 242). Experimental work also indicates that TGF-β can drive phenotypic conversion of NK cells toward less cytotoxic ILC1-like cells, a process proposed to occur in solid tumors and likely relevant to the accumulation of weakly cytolytic, tissue-resident group 1 ILCs in lung cancer (234, 243).

Several studies have investigated tissue-resident–like NK populations in lungs of patients with NSCLC. In detail, $CD69^+CXCR6^+$ ($\pm CD103^+$) trNK cells are detectable in NSCLC tissue (223) and $CD69^+CD49a^+$($\pm CD103^+$) trNK cells in adjacent tumor-free lung (76). $CD69^+CXCR6^+$ trNK cells accumulate preferentially within NSCLC lesions, exhibit an exhaustion-like phenotype with elevated inhibitory receptors, and yet retain the capacity to rapidly produce IFN-γ and TNF *ex vivo*, albeit at somewhat lower levels than $CD69^-$ NK cells from tumor-free lung (223). Given the

established role of CXCR6–CXCL16 interactions in lymphocyte trafficking, retention, and survival (129), alongside their frequent upregulation in tumors (244, 245), these findings suggest that CXCR6–CXCL16 signaling, together with tumor-associated extracellular matrix remodeling (148), contributes to the positioning and persistence of trNK cells within the lung TME.

Data comparing NK cells between lung AC and SCC are limited, but available studies suggest qualitative differences in how NK cells are skewed by these pathologies. In NSCLC overall, $CD56^{+}CD16^{-}$ NK cells with a pro-angiogenic phenotype predominate in tumors, while conventional cytotoxic $CD16^{+}$ NK cells are relatively depleted (235). Patients with SCC showed markedly higher production of angiogenic factors by $CD56^{+}CD16^{-}$ NK cells in tumor, adjacent lung and even peripheral blood than patients with AC, suggesting a stronger systemic skewing of NK cells toward pro-angiogenic, tumor-supportive functions (235). By contrast, current single-cell and bulk profiling studies indicate that in AC the dominant feature is depletion and functional exhaustion of cytotoxic states, with less pronounced systemic pro-angiogenic reprogramming, implying that AC and SCC may differ in whether NK cells are primarily "silenced" or actively co-opted as pro-vascular helpers (223, 246).

Functionally, however, most intratumoral NK cells in NSCLC appear at least partly suppressed (194, 195, 223). Recent transcriptional and functional investigations showed that tumor-infiltrating NK cells down-regulate cytotoxic molecules and degranulation against K562 target cells or autologous tumor cells, up-regulate inhibitory receptors (including CTLA-4 and NKG2A), and acquire a gene signature consistent with hypofunction and exhaustion (247). This dysfunction has been linked to high local TGF-β levels (234), hypoxia (248), and metabolic stress in the lung TME (194, 247), which collectively impair NK cell mobility, metabolism, and effector function (249). Recent pan-cancer single-cell reference mapping by Netskar and colleagues defined distinct functional states of tumor-infiltrating NK cells across solid tumors, including NSCLC. They showed that specific NK states correlate with patient survival, underscoring that it is the qualitative state of the NK cell compartment, rather than mere presence of NK cells, that shapes clinical outcome (250). An overview of markers associated with intratumoral lung NK cells are shown in **Figure II**.

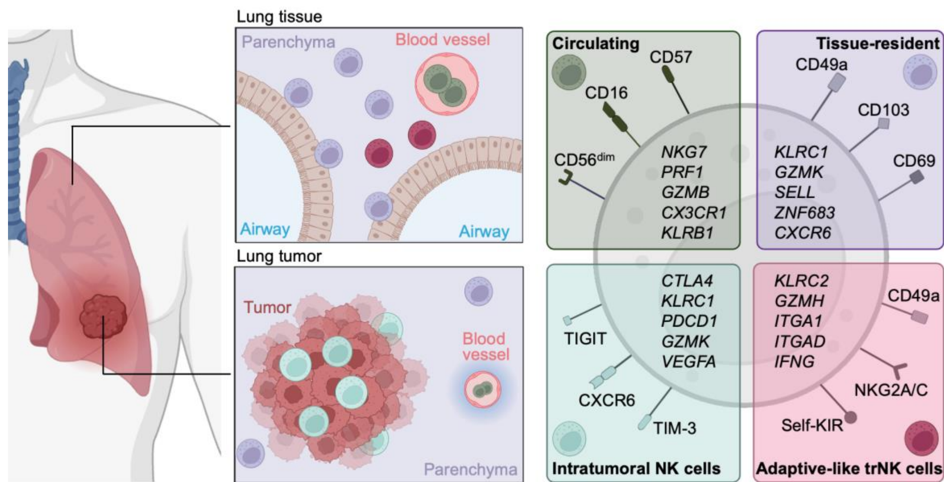

Figure II: Phenotypic and transcriptional characteristics of pulmonary NK cell subsets. Illustration of hallmark genes and surface markers of circulating NK cells within the lungs (green), pulmonary trNK cells (purple), lung adaptive-like trNK cells (pink), and NK cells inside lung tumors (turquoise).

However, whether exhausted or ILC1-like states can be durably reprogrammed back to potent cytotoxicity *in vivo*, and how distinct metabolic, hypoxic, and TGF-β-driven niches within individual tumors differentially imprint long-lived NK cell dysfunction and adaptation is still unclear. Additionally, it remains to be explored how plastic intratumoral and tumor-adjacent NK cells are in human NSCLC, and how their heterogeneity differs across lung AC and SCC. Finally, how NK cells are distributed along the tumor-tumor-free axis and how location shapes their phenotypic and functional characteristics remains undefined.

5.3. NK cells and respiratory viral infections

Lung cancer and respiratory viral infections converge on the same immunological landscape in the lung, where NK cells continuously balance tissue protection and damage. Many of the pathways that shape antitumor immunity, like chronic inflammation, checkpoint receptor engagement, and NK cell exhaustion, are also engaged during acute and chronic respiratory viral infections.

Infections of the respiratory tract are typically categorized as either upper respiratory tract infection or lower respiratory tract infection. Lower respiratory tract infections affect the trachea, bronchi and lungs and generally exhibit greater severity compared to upper respiratory tract infections, which affect the mucosa of the nose, sinuses, pharynx, and larynx. Infections with respiratory viruses, such

as SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), rapidly initiate immune responses, in part through induction of DAMPs by lung epithelial cells and airway macrophages (251). These stress signals are recognized by NK cells, enabling the detection and elimination of virus-infected cells. In response, viruses have evolved strategies to evade immune detection. This thesis focuses on the mechanisms by which SARS-CoV-2 modulated NK cell recognition to avoid elimination of infected host cells.

5.3.1. SARS-CoV-2 immune evasion of NK cell recognition

SARS-CoV-2 is a respiratory virus, first identified in late 2019 in Wuhan, China, and formally described in February 2020 (252). SARS-CoV-2 rapidly spread worldwide, causing the coronavirus disease 2019 (COVID-19) pandemic, a severe disease characterized by viral pneumonia and acute respiratory distress. To date, 779 million cases, out of which around 7 million were fatal, have been reported globally (253). Advanced age, obesity, and smoking are among the major risk factors associated with poor outcomes.

Given its tropism for the lower respiratory tract, SARS-CoV-2 directly encounters immune populations residing in or recruited to the lung, including NK cells, which serve as early responders to virally infected cells. However, accumulating evidence indicates that SARS-CoV-2 has evolved multiple strategies to evade NK cell recognition through two mechanisms. One study showed that the SARS-CoV-2 non-structural protein 13 (Nsp13) contains an HLA-E–restricted epitope, $Nsp13_{232-240}$ (VMPLSAPTL), which stabilizes HLA-E surface expression but does not effectively engage NKG2A/CD94. This creates a "missing self"-like signal and can promote activation of $NKG2A^{+}$ NK cells, highlighting a context in which the HLA-E/NKG2A axis may support antiviral responses rather than act purely inhibitory (254). In contrast, SARS-CoV-2 Nsp1 induces host cell shutoff and selectively reduces expression of NKG2D ligands, limiting NK cell-mediated cytotoxicity despite concurrent loss of classical HLA class I, thereby enabling immune evasion (255).

Consistent with these findings, other studies investigating COVID-19 patients report reduced NK cell numbers, enrichment of inhibitory receptor-high NK cell phenotypes, and impaired effector function (190, 256-258). Together, these findings suggest that viral modulation of HLA-E and NKG2D ligand expression occurs alongside broader systemic NK cell dysfunction. While reviews have framed these processes as prototypical NK cell evasion strategies, direct

evidence that viral evolution adapts specifically to NK cell-mediated pressure remains limited (256, 257, 259, 260).

There is still a major gap in integrating single-cell, spatial, and functional datasets to define when and where NK cells successfully recognize infected cells versus being diverted to bystander targets, and how these evasion mechanisms interact with evolving antibody repertoires and trNK cell populations in the lung.

5.4. The human intestine

The human intestine represents an extensive interface with the external environment, requiring finely tuned immune regulation to maintain tolerance to food and commensals with protection against pathogens across the entire intestine (261). This balance is supported by a complex epithelial barrier and an underlying immune compartment composed of lymphocytes, DCs, macrophages, and innate lymphoid cells, as well as organized lymphoid structures such as Peyer's patches and isolated lymphoid follicles that coordinate antigen sampling and local immune responses (261).

This thesis focuses on the adult small and large intestine, including the ileum, caecum, and colon, with particular emphasis on NK cells and consideration of T cells as a major lymphocyte population shaping intestinal immunity. Together, these populations help elucidate how the gut immune system maintains homeostasis and contributes to disease.

5.5. T cells in the homeostatic gut

The human intestinal immune system maintains a delicate balance between tolerance to commensal microbiota and rapid responsiveness to pathogens, with T cells playing a central role in this equilibrium (262). In steady state, most intestinal T cells reside within the lamina propria and intraepithelial compartments, forming a highly specialized and regionally adapted immune network (262).

Regional differences along the intestine shape distinct T cell landscapes. Across all intestinal areas, TRM constitute a major fraction of the T cell pool (263). These long-lived populations are present throughout lifetime, shaped by local environmental cues (263-266), and provide rapid on-site responses while maintaining tissue integrity (265, 266). The ileum, characterized by a relatively high microbial load and abundant Peyer's patches, harbors T cell populations enriched in effector and memory T cell phenotypes that support barrier integrity and

antimicrobial defense (267). Th17 cells, partly induced by commensal bacteria, produce cytokines such as IL-17 and IL-22 that promote epithelial regeneration and mucosal protection (268). In addition, a gut-specific population of MAIT cells was recently identified, particularly enriched in the ileum and marked by the expression of immunomodulatory markers including CD39 and PD-1 (269). In contrast, the colon contains the highest density and diversity of microbiota (270), which is associated with an increased frequency of Tregs that maintain immune tolerance and prevent excessive inflammation (271). The caecum represents an immunologically intermediate site, sharing features of both small and large intestine. However, its T cell composition in homeostasis remains comparatively underexplored (272, 273).

Collectively, T cells in the homeostatic gut are shaped by regional differences in microbial composition, nutrient availability, and microenvironmental signals, resulting in site-specific functional specialization along the ileum–caecum–colon axis (262). Although substantial knowledge has been gained, key aspects of how local environmental cues shape local T cell phenotypes across intestinal regions, as well as during disease development, progression, and remission, remain incompletely understood.

5.6. NK cells in the homeostatic gut

NK cells are an integral part of the intestinal immune surveillance network and eliminate infected or transformed epithelial cells while maintaining a balance with important commensals along the small and large intestine (77).

Human gut-associated NK cells are found mainly in the lamina propria and intraepithelial compartments of ileum, caecum, and colon (77), but it is still unclear whether they represent a self-renewing tissue-resident pool or are continuously replenished by circulating NK cells that acquire gut-imprinted phenotypes *in situ* (77). NK cell recruitment from peripheral blood is facilitated by CD161 (KLRB1), which is frequently expressed on circulating NK cells. $CD161^+$ NK cells are enriched among intestinal NK cells and $CD103^+$ cells in inflamed lamina propria, suggesting a role for CD161 in gut entry or retention, although direct *in vivo* trafficking data are lacking (274). Less differentiated NK cells express CXCR4, and its ligand CXCL12, produced by stromal and endothelial cells, has been linked to tissue homing in multiple organs, supporting a potential role for the CXCR4/CXCL12 axis in NK cell recruitment to the intestinal mucosa (275).

Phenotypically, gut NK cells differ from blood NK cells across the ileum and colon. Approximately 40–60% of human intestinal NK cells are $CD56^{bright}CD16^{-}$ cells with tissue-resident–like features, whereas $CD56^{dim}CD16^{+}$ NK cells form a minority in lamina propria and intraepithelial compartments (77, 78). The lamina propria of the ileum, caecum, and colon is rich in collagen and extracellular matrix, and intestinal NK cells use CD49a to adhere to collagen-containing structures, supporting their retention in the mucosal stroma (77). Intraepithelial NK cells in both small intestine and colon commonly express CD103, enabling binding to E-cadherin on epithelial cells and positioning them at the epithelial barrier (77, 276).

Compared with peripheral blood NK cells, $CD103^{+}$ intraepithelial NK cells show reduced baseline expression of perforin and GzmB but higher CXCR6 expression, and can produce IFN-γ when stimulated with epithelial-derived danger signals, consistent with a barrier-sentinel role rather than purely cytotoxic behavior (40). As first line of defense, NK cells induce regulated cell death in target cells through perforin-mediated pore formation and delivery of serine proteases such as GzmB, which triggers apoptosis (61). In the gut, however, this cytotoxic pathway is likely constrained by the hypoxic and metabolically challenging microenvironment of the lamina propria and epithelium (277). Another mechanism of regulated cell death induction through NK cells is mediated via granzyme A (GzmA) (278). GzmA released by NK cells has recently been shown to trigger pyroptosis through direct cleavage of gasdermin B (GSDMB), highly expressed in intestinal epithelial cells (279, 280), leading to regulated cell death (280, 281).

In addition, GzmA, expressed by intestinal cytotoxic lymphocytes, including NK cells, contributes to extracellular matrix remodeling and facilitates lymphocyte migration across basement membranes (282, 283), suggesting a role in modulating epithelial adhesion and tissue architecture (284). Notably, GzmA has been implicated in promoting epithelial differentiation, including increased occludin expression, thereby enhancing barrier integrity and restricting ferroptosis in experimental models of colitis (286). This finding suggests that GzmA-expressing lymphocytes, such as NK cells, may contribute not only to controlled cytotoxicity but also to the maintenance of intestinal barrier homeostasis. GzmA can also act extracellularly as a pro-inflammatory mediator in IBD, which is addressed in detail in the following section (287). The role of GzmA in the homeostatic gut is illustrated in **Figure III**.

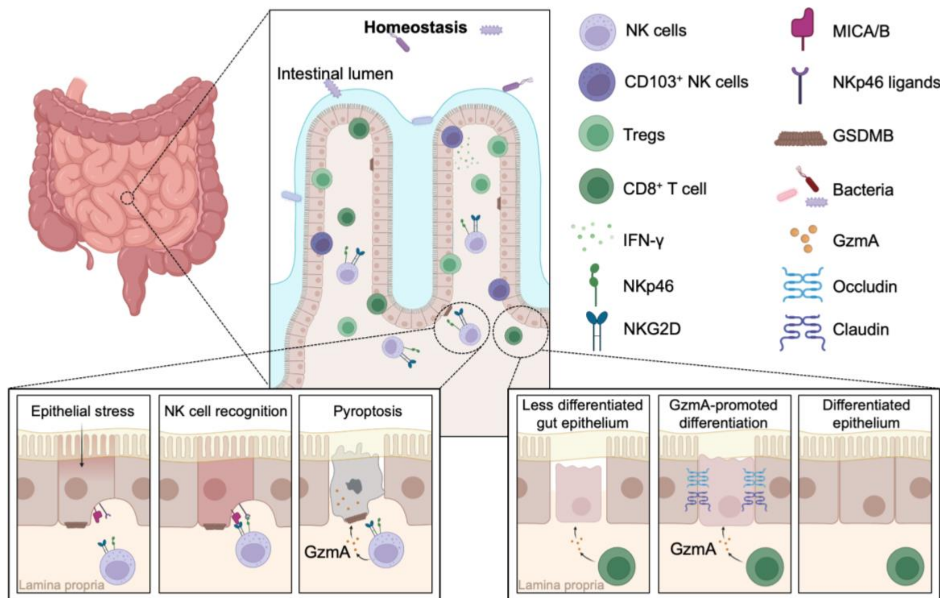

Figure III: The role of intestinal immune cells and GzmA in homeostasis. Illustration of the steady state small intestinal epithelium, highlighting the contribution of GzmA to tissue homeostasis. CD103⁺ NK cells (dark purple) localize within the epithelial layer, whereas other NK cell (purple) and T cell subsets (green) reside in the lamina propria.

It is unclear under which conditions intestinal NK cells preferentially employ perforin–GzmB-mediated apoptosis versus GzmA-GSDMB-dependent pyroptosis, and whether these programs are stably imprinted or dynamically shaped by local cues such as microbiota, hypoxia, and cytokines. While NK cell-derived granzymes can mediate both apoptotic and pyroptotic cell death and potentially influence epithelial repair, their relative contribution in steady state versus inflammation in the human intestine remains poorly defined. In particular, distribution and protein-level expression of GzmA in humans, across homeostasis, chronic inflammation, and IBD patients, are largely unexplored.

5.7. Inflammatory bowel disease

IBD, a collective term for ulcerative colitis (UC) and Crohn's disease (CD), is characterized by a chronic inflammatory state and excessive cytokine production (288, 289). CD commonly targets the terminal ileum but may extend throughout the entire gastrointestinal tract, whereas UC is confined to the colon and rectum (288). The pathogenesis of IBD is not yet fully understood, but the dysregulation of the adaptive as well as innate immune system are suspected drivers of inflammation (288, 289).

In general, CD is characterized by exaggerated Th1 and Th17 responses, with elevated IFN-γ, IL-17, and IL-23 signaling sustaining macrophage activation and chronic inflammation (288-291). In contrast, UC has been linked to an atypical Th2-skewed response, although Th17 pathways also contribute to barrier disruption and neutrophil recruitment (288-290). In both conditions, altered NK cell function may further amplify these cytokine networks and will be addressed in the sections below. Together, dysregulated immune responses promote persistent epithelial damage, microbial imbalance, and chronic inflammation.

5.7.1. T cells in IBD

T cells play a central role in IBD pathogenesis. Compared to homeostasis, T cells in IBD exhibit altered activation states, subset composition, and effector functions that contribute to sustained tissue inflammation and barrier disruption (290).

In CD, these changes are largely associated with Th1 and Th17 responses, accompanied by increased production of cytokines such as IFN-γ, IL-17, and IL-23 observed in inflamed tissue (288-291). These responses contribute to macrophage activation, neutrophil recruitment, and epithelial damage, although their precise influence may vary depending on disease stage and anatomical location (288-291). In contrast, UC has historically been linked to a Th2-like cytokine profile, including IL-5 and IL-13 production, although this model is now considered an oversimplification given the heterogeneous and overlapping T cell responses observed in patients (292, 293). More recent studies indicate that both CD and UC involve a complex interplay of multiple T helper subsets, including Th1, Th17, and Tregs, rather than discrete polarization states (294-296).

Beyond $CD4^+$ T cells, $CD8^+$ T cells and TRM have also been implicated in IBD pathogenesis, where they may display enhanced cytotoxicity and contribute to epithelial injury (295, 297). Emerging evidence from single-cell and spatial transcriptomic studies suggests that T cells in IBD undergo context-dependent reprogramming driven by inflammatory cytokines, microbial signals, and metabolic cues within the intestinal microenvironment (296, 298). However, the extent to which these changes represent stable lineage commitment or reversible functional states remains incompletely understood.

Collectively, T cells in IBD are characterized by a dysregulated balance between pro-inflammatory and regulatory programs, shaped by both intrinsic and environmental factors within the gut. Despite substantial scientific progress, the

influence of intestinal anatomy, local tissue cues, and different disease stages, including onset and remission, remain insufficiently understood.

5.7.2. NK cells in IBD

NK cells have been reported to contribute to CD as well as UC and deciphering their role in pathogenesis remains an active area of research. In CD, immune dysregulation has been associated with specific KIR2DL3/HLA-C1 genotypes in NK cells (299). In human mucosal tissues, a distinct NKp46^{+} NK subset expresses activating receptors such as NKG2D and responds acutely to IL-23 with robust IL-22 secretion, and under certain cytokine conditions can also produce IFN-γ (46, 300). Through their production of IL-22 and IFN-γ and interactions with monocytes and epithelial cells, these mucosal NK cells can modulate the IL-23/Th17 inflammatory milieu, which supports the expansion and survival of pathogenic Th17-lineage CD4^{+} T cells (46, 135, 300, 301). Elevated frequencies of intraepithelial CD16^{-} NK cells were found within the mucosa of patients with CD compared to healthy controls, which was linked to increased IFN-γ levels and subsequently intestinal inflammation (40, 302). Another hallmark of CD is the elevated expression of MHC class I chain-related protein A and B (MICA and MICB), which are recognized as stress-induced molecules by NKG2D and contribute to NK cell activation and induction of cytolytic activity, ultimately contributing to the overall inflammation (303).

UC primarily manifests as an aberrant Th2 immune response, associated with a specific KIR2DL5/2DS1 genotype in NK cells (299). In active UC, the frequency of peripheral blood NK cells is lower than in inactive UC (304). In addition, NKG2D has been identified as a potential key mediator of NK cell-associated cell damage, since NKG2D expression is increased in severe UC as compared to milder UC stages (305). NK cells expressing NKG2D have been hypothesized to contribute to production of pro-inflammatory Th1 cytokines to outweigh the overproduction of IL-13, by aberrant natural killer T cells (306). Recent single-cell analyses have identified a UC-specific BAG6–NCR3 (NKp30) axis interaction linking DCs and NK cells in the inflamed colon. BAG6, a stress-induced ligand expressed by DCs as well as epithelial and stromal cells, engages NCR3, which is upregulated on mucosal NK cells in UC (307). NCR3^{+} NK cells exhibit increased IFN-γ expression and cytotoxicity compared to NCR3^{-} NK cells and those from healthy colon, and this signaling persists even after treatment, suggesting a source of ongoing

inflammation (307). Sustained NK cell activation may contribute to epithelial damage, tissue remodeling, fibrosis, and increased risk of colorectal cancer (308).

Despite growing interest in NK cell phenotype and function in IBD, their subset-specific immunoregulatory roles across different intestinal regions remain insufficiently defined. Moreover, their contribution to pathogenesis and disease persistence is still unclear, as most studies rely on cross-sectional analyses of inflamed tissue rather than longitudinal or functional approaches that capture dynamic changes over the course of disease.

5.7.3. The role of granzyme A in IBD

Several studies have linked GzmA to IBD, although with inconclusive associations. One of the first articles investigating GzmA in patients with active IBD show an enrichment of $GzmA^+$ cytotoxic lymphocytes in comparison to healthy controls, particularly in the upper crypt regions oriented toward the intestinal lumen of the colon (309). In a murine study, GzmA was shown to be elevated in a dextrane sulfate sodium (DSS) colitis model, linking GzmA to development and persistence of disease (310). Recent research further supports a role for GzmA as a driver of IBD, as extracellular GzmA has been linked to increased colonic inflammation and a higher risk of colorectal cancer (311).

Conversely, GzmA has also been linked to beneficial effects, including support of intestinal epithelial cells and improved outcomes. IBD patients with higher levels of intestinal *GZMA* messenger RNA showed an improved response to etrolizumab treatment (312). In line with this, pediatric IBD patients exhibit a reduced frequency of $CD8^+$ T cells co-expressing *GZMA* and *ENTPD1* (encoding CD39) compared to healthy controls (285), suggesting that reduced *GZMA* expression may be linked to IBD pathogenesis. Furthermore, GzmA may contribute to intestinal homeostasis and barrier integrity maintenance by inducing regulated cell death. Mouse studies demonstrate that GzmA and GzmB expressed by intraepithelial lymphocytes cooperate to protect the small intestinal epithelium during enteric infection, with the balance between GzmA-mediated and GzmB-mediated cell death shaping pathogen control versus epithelial damage (313). Recent work further shows that GzmA promotes epithelial differentiation and tight-junction protein expression, including increased occluding expression, thereby improving barrier integrity and limiting ferroptosis in experimental models of colitis (286).

As introduced in chapter 5.6, GzmA released by NK cells and cytotoxic T cells can induce pyroptosis in intestinal epithelial cells through direct cleavage of GSDMB (280). GSDMB is emerging as a key epithelial regulator in IBD, with multiple genetic and expression studies linking risk alleles to disease susceptibility and demonstrating marked upregulation in inflamed ileal and colonic epithelium in both CD and UC (314-316). Its expression is particularly enriched in intestinal epithelial cells at sites of active inflammation (316). Under homeostatic conditions, GSDMB has been associated with epithelial repair and migration, whereas its dysregulation or disease-associated variants can promote barrier disruption and spontaneous enterocolitis in experimental models (316).

In summary, the role of intestinal GzmA is likely context dependent. Reduced GzmA levels may impair controlled cell death mechanisms and epithelial differentiation, potentially contributing to disease progression, whereas elevated extracellular GzmA may exert pro-inflammatory effects that drive intestinal inflammation. t also remains unclear which cell types represent the main source of intestinal GzmA and whether these are impaired in IBD, potentially representing a novel therapeutic target.

Despite scientific efforts, the immunoregulatory roles of different immune cell subsets, including NK cells and T cells, as well as GzmA in both health and IBD are not yet fully understood. Current knowledge is largely derived from colonic tissue, with comparatively limited investigation of other intestinal regions, such as the ileum and caecum, potentially overlooking site-specific phenotypic and functional immune cell diversity. Moreover, most studies focus on patients with active disease, whereas analyses of asymptomatic individuals or those in remission are scarce, limiting insight into baseline immune regulation and early disease-driving mechanisms.

2 Research aims

In this thesis, we unravel the phenotype, transcriptome, and function of human NK cells in the lung and intestine across different physiological and pathological conditions, thereby contributing to the understanding of tissue-specific immune homeostasis and disease.

Study I: The composition and distribution of immune cells, as well as the phenotypic, transcriptional, and functional characteristics of NK cells, were assessed across matched lung lobes and airway-associated regions, with particular emphasis on defining site-specific NK cell features.

Study II: The distribution as well as immune checkpoint and effector profiles of tumor-infiltrating lymphocytes were examined across tumor and adjacent tumor-free tissues, including the functional properties of NK cells within the TME.

Study III: The impact of a point mutation in the BQ.1 SARS-CoV-2 $Nsp13_{232-240}$ epitope on escape from HLA-E/NKG2A-mediated NK cell recognition was investigated.

Study IV: Comprehensive mapping of effector molecules in NK cells across human tissues, with a focus on distinct anatomical regions of the intestine, and definition of region-specific phenotypes and functional adaptations of these cells in individuals with and without IBD.

Collectively, these studies provide an in-depth characterization of NK cells across lung and intestinal tissues in homeostasis and disease, advancing the understanding of mechanisms underlying tissue tolerance, immune evasion, and inflammation. These insights establish a framework for future research and may inform the development of improved diagnostic and therapeutic strategies.

3 Materials and methods

This chapter provides a summary of the scientific methods used across the studies compiled in this thesis. Detailed information about protocols, materials, reagents, and samples are listed in the methods section of each study.

3.1 Ethical considerations

Having access to human samples like blood, tissue-biopsies, and patient-matched whole organs is a unique resource and an incredibly valuable opportunity to advance our knowledge about tissue-specific immunology. Similarly, investigating human specimen of tumors and inflamed tissue allows for in-depth characterization of immune cell phenotypes at the site of disease progression or persistence. Clinical data such as age, sex, weight, comorbidities, medication, and smoking status are equally valuable, especially in combination with the biological material. All sample collection and associated data handling were conducted in accordance with strict ethical regulations and established consent procedures. The studies included in this thesis were approved by the Swedish Ethical Review Board.

Immune cells derived from peripheral blood were utilized across all studies. Peripheral blood was collected from buffy coats from healthy blood donors at Karolinska University Hospital transfusion medicine with informed consent for research use. Blood components like buffy coats not required for clinical purposes are routinely made available for research. Isolated peripheral blood mononuclear cells (PBMCs) were utilized for experimental assays and served as reference sample and staining controls in parallel with tissues obtained from the cohorts included in this thesis.

For **Studies I and IV**, material from deceased patients, which provided consent for research through registering in Socialstyrelsen's donation register or through consent given by their next of kin, was used. Only tissues considered unsuitable for transplantation were collected for research, ensuring no impact on the availability of organs for clinical use. All clinical and biological data was pseudonymized already in the hospital, protecting the privacy of the organ donor postmortem.

In **Study II**, lung tumor tissue and non-tumor tissue were obtained from lung cancer patients undergoing lobectomy. Importantly, the non-tumor tissue would otherwise have been discarded during routine clinical procedures, and its use for

research therefore did not impose additional risk or burden to the patient. Additionally, sampling of a small amount of peripheral blood involved only minimal risk.

In **Study IV**, intestinal punch biopsies were obtained during routine colonoscopy procedures, with low additional risk for complications for the patients. In addition, up to 20 mL of peripheral blood were collected, which is a minimal and low-risk procedure.

For all studies, information was provided at the clinic by physicians and other medical personal to the patients, or in case of the organ donor cohort, to the next of kin. All participants, including the organ donors or their next of kin, were required to provide written consent before samples or blood were taken.

Across all cohorts, all personal information was pseudonymized at the day clinic or hospital, and only the respective physician has access to the key code and the patient's personal data. Researchers involved in any study exclusively receive the coded information. Potential risks to patient confidentiality may arise from the sharing of high-dimensional datasets, such as scRNA-seq data. To mitigate this, only processed and de-identified count matrices are shared, which do not contain direct identifiers and reduce the risk of re-identification.

3.2 Human sample collection

Processing of all tissues as well as details for cultured cells are described in detailed in each method section of the respective manuscripts and papers.

Study I: Whole human lungs and lung lymph nodes as well as mediastinal lymph nodes, liver, spleen, intestines and blood were collected from the "Immunology Human Organ Donor Programme" (IHOPE). Only organs deemed unsuitable for transplantation were used. Organs were procured at collaborating hospitals, preserved on ice, and transported to the Karolinska Institutet immediately following surgery. Tissues were stored on ice and were processed after maximum of 10 hours after surgery.

Study II: Lung tissue und lung tumor tissues were obtained from patients undergoing lobectomy for suspected lung cancer. Tissue and blood collection did not affect surgical or diagnostical procedures. Different regions (peritumoral region, tumor margin, tumor center as well as non-matched tumor-distal tissue) were defined by macroscopic assessment, validated by the pathologist in charge.

Study III: In this study, PBMCs obtained from buffy were frozen, thawed, and rested overnight prior to experiments. Additionally, established cell lines were used to investigate the immune-evasive properties of different SARS-CoV-2 strains, including Caco-2 cells (colorectal AC), A549 cells engineered to express angiotensin-converting enzyme 2 (ACE2; A549-hACE2, lung AC), and K562 cells expressing HLA-E*01:03 (K562/HLA-E; bone marrow-derived lymphoblasts).

Study IV: Patients with and without inflammatory bowel disease (IBD) were recruited at Capio Gastro Center Stockholm. For this study, peripheral blood as well as matched intestinal punch biopsies from non-inflamed areas were collected from patients with and without IBD diagnosis. Blood was kept at room temperature and biopsies were kept on 4°C after retrieval and during transport. Tissues and blood were processed within 3 hours after sampling.

A graphical summary of the tissue sources and cell lines used in each study is provided at the end of this chapter (**Figure IV**).

3.3 Flow cytometry and fluorescence-activated cell sorting

All studies included in this thesis utilized flow cytometry analysis. In brief, monoclonal antibodies conjugated to fluorescent molecules, or fluorochromes, enable the detection of specific antigens, commonly referred to as markers, present on the cell surface and within intracellular compartments. Each individual fluorochrome is excited by a specific wavelength and, upon excitation, absorbs light and re-emits it at a different wavelength. This wavelength can be detected at its peak emission wavelength in conventional flow cytometry or as total emission wavelength in spectral flow cytometry.

Inside a flow cytometer, single cells labeled with fluorochrome-tagged antibodies pass through a fluidics system, where light emitted from lasers excite the fluorochromes, which in turn re-emit light. This light is directed through a series of lenses and filters to detectors, which forward the photocurrent to the electronical system for conversion to digital output. In fluorescence-activated cell sorting (FACS), cells can be separated based on the presence or absence of specific markers, enabling targeted analysis of defined cell populations post sorting.

Flow cytometry can be broadly divided into conventional and spectral flow cytometry. Conventional flow cytometry detects fluorochromes using discrete bandpass filters assigned to specific channels, whereas spectral flow cytometry captures the full emission spectrum of each fluorochrome and applies

computational unmixing to resolve highly multiplexed signals. Spectral flow cytometry therefore allows the simultaneous detection of a greater number of markers compared to conventional approaches.

Spectral flow cytometry was used in **Study I** to unravel the leukocyte distribution across different lung lobes, LLN, and primary bronchus, using freshly isolated cells. Conventional flow cytometry was used in all studies to investigate phenotype and function of lymphocyte subsets or cell lines.

FACS was used in **Study I** to isolate live leukocytes from frozen samples and to further enrich this population for $CD16^{+}$ and $CD16^{-}$ NK cell subsets. This allowed enabled representation of otherwise scare NK cells for downstream transcriptomic analysis.

3.4 Single-cell RNA-sequencing and publicly available datasets

Transcriptomic analysis like scRNA-seq are powerful tools that allow in-depth characterization of cells and allows profiling of yet uncharacterized subsets.

In **Study I**, leukocytes enriched with $CD16^{+}$ and $CD16^{-}$ NK cells were sorted from different lung areas and sequenced by the National Genomics Infrastructure (NGI) located at SciLifeLab in Stockholm. Files for further analysis were provided after CellRanger sequence alignment by the NKGI via UPPMAXX NAISS. Downstream QC and analysis of total gene expression data and TCR sequence analysis were performed in RStudio using R.

In **Study IV**, publicly available data from the human Crohn's disease atlas (317) was utilized to investigate if the $GzmA^{high}$ NK cell phenotype can be identified on transcript-level.

3.5 NK cell functional assays

In **Studies I, II, and IV**, NK cells were stimulated with either K562 target cells or phorbol 12-myristate 13-acetate and ionomycin (PMA/ionomycin) to investigate their ex vivo functional capacity after isolation from different tissues. In **Study III**, different SARS-CoV-2 peptides and self-peptides presented by HLA-E on K562 target cell were used to assess their impact on NKG2A-mediated NK cell inhibition.

K562 target cell-based assays used in **Studies I-III** capture physiologically relevant cytotoxic responses through receptor-ligand interactions, whereas

PMA/ionomycin stimulation in **Study IV** bypasses these pathways to assess the maximal, receptor-independent, functional capacity of NK cells.

3.5.1. K562 target cell assay

Functional responses of NK cells derived from different lung areas (**Study I**) and from lung peritumor and center (**Study II**) were assessed using K562 target cell stimulation. NK cells from tissue mononuclear cells were co-cultured with K562 target cells, and their activity was evaluated by measuring the expression of CD107a as well as TNF using flow cytometry.

3.5.2. NK cell inhibition assay

In **Study III**, NK cells were enriched from frozen PBMCs by negative selection and rested overnight prior to the assay. Briefly, NK cells were co-cultured with K562 target cells expressing HLA-E*01:03 (K562/HLA-E) that had been loaded with either BA.5 or BQ.1 $Nsp13_{232-240}$ peptides or a mix of viral and self-peptides. Functional responses of $NKG2A^{+}$ NK cells were assessed by measuring the expression of CD107a, TNF, and INF-γ via flow cytometry, alongside simultaneous evaluation of HLA-E surface stability on K562 target cells.

3.5.3. PMA/ionomycin stimulation

In **Study IV**, NK cells from peripheral blood and intestinal tissue mononuclear cells were stimulated with PMA (phorbol 12-myristate 13-acetate) and ionomycin to determine their functional potential. NK cell responses were determined through flow cytometry by assessing the expression of CD107a and INF-γ.

3.6 *In silico* screening of SARS-CoV-2 immune evasive characteristics

In **Study IV**, *in silico* methods were applied to estimate the immune-evasive properties of BQ.1 at the population level, including mutation fitness and prevalence, and to model and compare its sequence and characteristics with other SARS-CoV-2 variants.

3.6.1. Screening of SARS-CoV-2 variant frequency

Frequencies of SARS-CoV-2 variants BA.2.75, BA.5, XBB.1.5, and BQ.1, including BQ.1.1, were compared between week 37 of 2022 and early 2023, from publicly available data obtained from the European Centre for Disease Prevention and Control. Further, reported infections and hospitalizations associated with BA.5,

BQ.1, or other variants were collected from publicly available databases from the Swedish Intensive Care Registry.

3.6.2. Viral fitness estimation and comparison to other viruses

Peptide binding of BA.5 Nsp13$_{232-240}$ to HLA-E was predicted using lysine scanning along the epitope sequency. This enabled identification of positions relevant for HLA-E interaction by comparing relative binding affinities of lysine-substituted peptide variants.

Viral fitness was estimated by contrasting the observed prevalence of mutations in large-scale SARS-CoV-2 datasets with their expected frequency under a neutral mutation rate. The generated scores reflected whether specific substitutions are enriched or depleted in the viral population.

In parallel, Nsp13$_{232-240}$ sequence conservation across coronaviruses, including sarbecoviruses, SARS-CoV-1, SARS-CoV-2 BA.5 and BQ.1, as well as endemic human coronaviruses (HCoV) was evaluated, with particular emphasis on variations at position 2.

Finally, Hamming distances were calculated by quantifying HCoV and BQ.1 Nsp13$_{232-240}$ sequence divergence relative to a BA.5 reference, providing a framework to assess evolutionary variation within the Nsp13$_{232-240}$ epitope.

3.7 *In vitro* analysis of BQ.1 Nsp13$_{232-240}$ pM2I on HLA-E binding and stability

In **Study IV**, several *in vitro* approaches were employed to investigate the immune evasion mechanisms of BQ.1. These included peptide-loading assays using K562/HLA-E cells to assess peptide-dependent stabilization, viral infections, crystallography of HLA-E/peptide complexes, and NKG2A binding assays to evaluate receptor engagement.

3.7.1. Peptide presentation and stability assay with K562 cells

Peptide presentation was investigated with K562/HLA-E cells loaded with synthetic peptides at different target concentrations. After incubation, cells were either used for co-culture experiments or analyzed for their HLA-E surface levels via flow cytometry

The stability of HLA-E loaded with synthetic peptides was investigated with K562/HLA-E cells. Cells were co-incubated with peptides present in media

followed by peptide deprival across different time points. Subsequently, HLA-E surface expression was determined with flow cytometry.

3.7.2. SARS-CoV-2 infection

Human cell lines Caco-2 and A549-hACE were infected *in vitro* with ancestral SARS-CoV-2 or SARS-CoV-2 BQ.1 to investigate the surface expression of HLA-E. Infections were performed in the BSL-3 laboratory and cells were stained and analyzed with flow cytometry.

3.7.3. Pre-processing, crystallization, and assessment of HLA-E/peptide complexes

HLA-E and β2-microglobulin were recombinantly expressed in bacterial systems, isolated, and solubilized under denaturing conditions. The individual components were refolded in the presence of specific peptides to assemble stable HLA-E–peptide complexes of HLA-E*01:03 in complex with BQ.1 $Nsp13_{232-240}$ or BA.5 $Nsp13_{232-240}$. Subsequently, the complexes were purified by size exclusion chromatography and biochemically validated with SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). Finally, these complexes were crystallized and subjected to structural analysis with nano differential scanning fluorimetry to evaluate the melting point of each HLA-E*01:03/$Nsp13_{232-240}$ complex to determine complex stability.

3.7.4. NKG2A binding assay

To assess receptor binding, mixtures of viral and self-peptide-loaded K562/HLA-E cells were incubated with recombinant NKG2A/CD94. Receptor binding was detected via labeled secondary antibodies. To enable comparison across conditions, peptide mixtures (BQ.1 or BA.5 with self-peptide, or self-peptide alone) were adjusted to achieve comparable HLA-E surface levels, allowing evaluation of how different peptide repertoires influence NKG2A engagement.

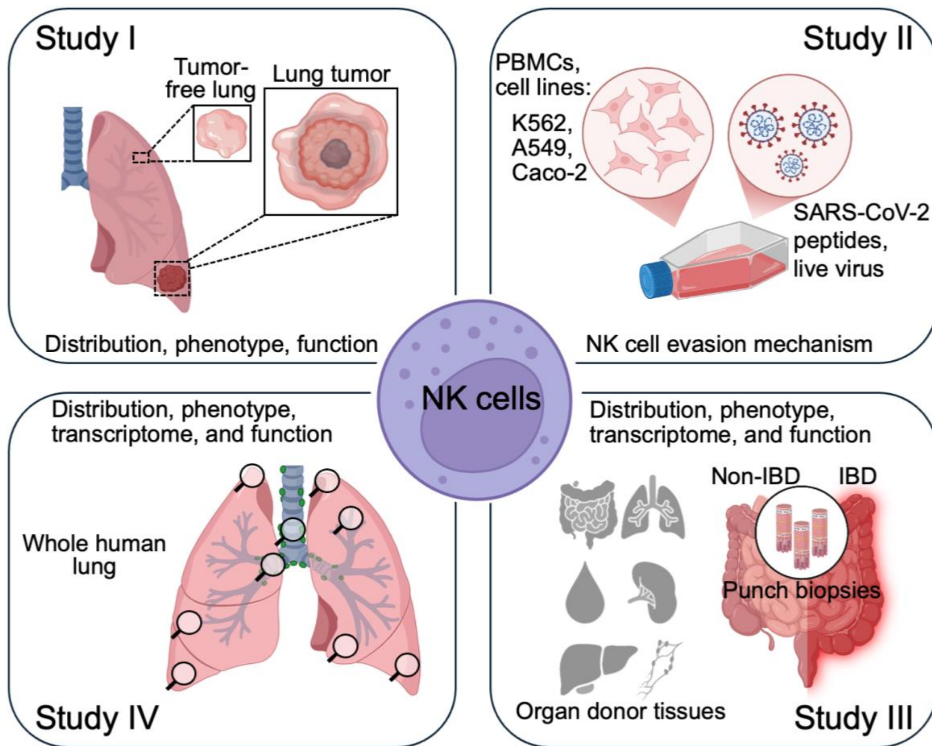

Figure IV: Overview of the biological material used in each study. Schematic illustrating the tissues analyzed in **Studies I, III, and IV**, together with the cell lines and viruses used in **Study IV**. NK cells were the primary focus across all studies.

4 Results and discussion

In this thesis, we investigated the distribution of leukocytes, with a particular focus on NK cells, across the human lung, identifying an enrichment of adaptive-like trNK cells in the lower lung regions (**Study I**). Furthermore, trNK cells with comparatively low immune checkpoint receptor expression and preserved ex vivo functional capacity were found to accumulate in human lung tumors (**Study II**). In parallel, mechanisms of BQ.1 SARS-CoV-2 evasion of NK cell recognition and killing were investigated *in vitro* and *in silico* (**Study III**). Finally, NK cell effector molecule expression was analyzed across human tissues, alongside characterization of intestinal NK cells in patients with and without IBD (**Study IV**).

4.1. Study I: Mapping leukocytes across the human lung with particular focus on NK cells

The heterogeneity of the human lung remains insufficiently characterized with respect to immune cell composition across different lobes and bronchial regions. Given the anatomical organization of the lung into distinct lobes and the tracheobronchial tree, along with clinical evidence of region-specific disease manifestation, local immune cell populations may vary in their distribution, phenotype, and functional potential. Although studies have examined matched lung areas from whole lungs obtained from organ donors, the resolution is often insufficient to enable detailed analysis of NK cell subsets (186, 188).

The same applies to large transcriptomics-based atlases combining several studies, where NK cells are readily present; however, rarer phenotypes such as adaptive-like trNK cells are often underrepresented. This likely reflects limited inclusion of donors with substantial expansions of this NK cell subset (185-189).

To further expand the knowledge of pulmonary immunity, we employed comprehensive phenotypic, transcriptional, and functional analyses of leukocytes, with particular focus on NK cells, across matched human lung regions (**Study I, Fig. 1A**). Proteomic and transcriptional analysis revealed a largely uniform immune composition within the lung parenchyma, contrasted by distinct organization in airway-associated compartments such as the primary bronchus and lung lymph nodes (LLN) (**Study I, Fig. 1D, F-H, 2C**). These findings align with recent lung atlas studies reporting broadly stable immune cell frequencies across distal lung regions and suggest that, under homeostatic conditions, large-scale anatomical

differences across the parenchyma have only limited influence on overall immune cell abundance (318-321).

While T cells were broadly distributed and dominated the leukocyte pool, B cells were enriched in airway-associated sites (**Study I, Fig. 1D-G, 2C**). Single-cell transcriptomics further demonstrated that inter-donor variability exceeded regional differences, although subtle location-dependent pathway programs were detectable across lymphocyte populations (**Study I, Fig. 2D, 4C**). The regional enrichment of B cells in airway-associated regions may reflect underlying pulmonary anatomy, with LLN preferentially located along the hilum and larger bronchi (322). Although LLN were dissected from both the primary bronchus and lung parenchyma, the increased frequencies of B cells may partly originate from bronchus-associated lymphoid tissue (BALT) within the bronchial wall. While BALT is typically absent under homeostatic conditions in humans, it can be induced by exposure to pathogens, pollutants, or inflammatory stimuli. Whether mechanical ventilation contributes to inducible BALT formation remains unclear (323).

TCR profiling identified clonally expanded T cells that were preferentially confined to specific anatomical compartments, including airway-associated regions in certain donors (**Study I, Fig. S4**). This is in line with other research suggesting localized antigen-driven expansion (324). These observations support the concept that the bronchial compartment can act as a distinct immunological niche for T cell clonal diversification in a subset of individuals. However, this pattern was not consistently observed across all donors, highlighting substantial inter-individual variability in pulmonary immune organization (**Study I, Fig. S4**).

4.1.1. Enrichment of adaptive-like trNK cells in lower lung areas

Within our dataset, NK cells represented as a heterogeneous population with pronounced donor-dependent transcriptional and functional diversity (**Study I, Fig. 4B, C, 5D, 6A-F; Fig. S6C, S7A**). Distinct NK cell states were identified, including cytotoxic, regulatory, proliferative, and tissue-adapted subsets, with one subset displaying combined tissue-residency and adaptive-like (NK3) features (**Study I, Fig. 4A, B, D, 5C, D; Fig. S6C)**. These adaptive-like trNK cells were enriched in the lung parenchyma, particularly in lower peripheral regions (**Figure V**), and were largely independent of T cell residency patterns or circulating adaptive NK cells (**Study I, Fig. 3F, G; Fig. S5E-G**). Adaptive-like trNK cells were absent in other matched tissues, including LLN, the intestines and mesLN, liver, spleen, and blood (**Study I, Fig. S5D**).

This extends previous findings from cancer patients to tumor-free lungs (79), suggesting that this phenotype is not solely driven by malignancies but may also be shaped by donor-specific factors and prior infection history.

The preferential enrichment of these cells in peripheral and lower lung regions likely reflects regional differences in ventilation, perfusion, microbial exposure, and local epithelial and stromal cues that shape NK cell phenotype and persistence (175). Cytokine signals are also expected to contribute, although the spatial distribution of key NK cell-modulating cytokines such as IL-12, IL-15, and IL-18 remains poorly defined.

In addition, infection-associated processes may further influence these patterns. Latent HCMV infection can persist in the lung (116), and respiratory infections, including influenza and SARS-CoV-2, induce localized cytokine responses in the airway mucosa (325). Epithelial-derived factors such as TGF-β, known to promote tissue-residency programs in T cells and NK cells, may further support tissue retention and functional adaptation (40, 124, 326-332). Together with host-specific variation in immune responses, these factors may drive donor- and region-specific NK cell phenotypes. However, how lifelong viral exposure shapes the local immune landscape, and in particular the phenotype and function of trNK cells in the lung, remains poorly understood.

To further investigate this, NK cell functional capacity was assessed across lung regions (**Study I, Fig 5D**). Despite transcriptional signatures indicative of activation and cytotoxic potential (**Study I, Fig. 5C, Fig. S7**), *ex vivo* functional assays revealed overall low NK cell responsiveness to target cells, with marked variability between donors and localized region-specific activity in some cases (**Study I, Fig. 6**). This heterogeneity may reflect differences in prior immune exposure, local microenvironmental conditioning, or the relative abundance of tissue-resident versus circulating NK cell subsets, highlighting the complexity of translating transcriptional states into functional outcomes in human lung tissue.

Collectively, the findings from this study suggest that, although lung immune architecture exhibits regional organization, overall leukocyte distribution is largely homogeneous across parenchymal regions, with the exception of adaptive-like trNK cells. Expansion of this subset was donor-dependent and, when present, exhibited preferential enrichment in the lower parenchymal areas. Further research is needed to identify the triggers of this expansion, determine its persistence over time, and clarify how it is spatially coordinated across different

lung lobes. Such heterogeneity may have important implications for regionally restricted immune responses, for example during respiratory infections or in the early establishment of lung tumors, where localized immune niches could influence disease initiation and progression.

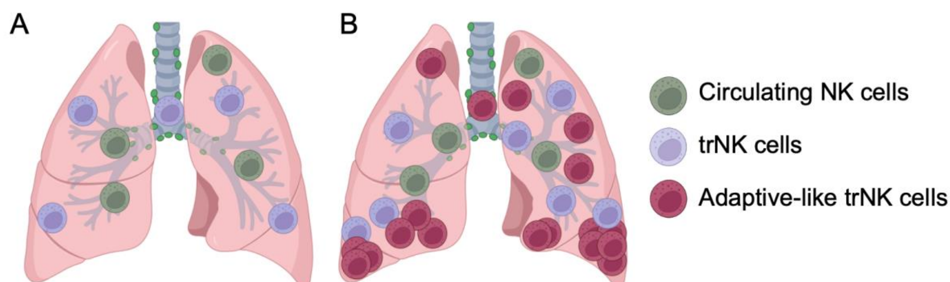

Figure V: Distribution of pulmonary NK cell subsets. (**A**) Illustration of a donor without expansion of adaptive-like trNK cells and (**B**) a donor with such expansion. Adaptive-like trNK cells (pink) are preferentially enriched in lower lung regions in a donor-dependent manner, whereas other NK cell subsets in the lung, including circulating (green) and non-adaptive-like trNK cells (purple), are more uniformly distributed and show no clear donor dependency.

4.2. Study II: Investigation of lymphocyte distribution in and around solid lung tumors

To extend our understanding of immune cell distribution beyond homeostasis, we investigated lymphocyte composition within lung tumors as well as in tumor-adjacent and -distal lung tissue (**Study II**). Lung cancer is a leading cause of cancer-related mortality worldwide, with smoking as the primary risk factor and most patients over 65 years of age. The majority of cases are classified as NSCLC (~85%), which can be subdivided into AC and SCC, differing in anatomical origin, histology, and genetic profile (204, 216, 333-335). Despite current treatment strategies, overall survival remains limited, highlighting the need for a better understanding of tumor-immune interactions.

While recent advances in tumor immunology have primarily focused on circulating T cells and their tumor infiltration, the spatial organization of tissue-resident lymphocytes within lung tumors remains poorly defined. Although trNK cells have been identified in both tumor-free and cancerous lung tissue (38, 194, 195), their distribution and role in the TME, alongside other resident populations such as other ILCs and TRM, remain insufficiently characterized. Defining the localization and functional states of these lymphocytes may provide important insights for improving immunotherapeutic strategies.

Consistent with previous findings (38, 194, 195), frequencies of $CD16^-$ NK cells and ILCs were higher toward the tumor center compared to the peritumor and tumor-distal tissue, while those of T cell subsets were consistent across lung tumor areas (**Study II, Fig. 1b, c**). Markers of tissue residency, including CD69, CD49a, and CD103, were enriched on $CD16^-$ NK cells and $CD8^+$ T cells within the tumor core, whereas ILCs were uniformly $CD69^+$ across regions, indicating a preferential accumulation of tissue-resident cytotoxic populations in the tumor center (**Study II, Fig. 1d-g**). No apparent difference in enrichment along the tumor-free to tumor-center axis was observed between AC and SCC (**Study II, Fig. 1h; Fig. S1H-J**), a pattern potentially influenced by limited sample size.

4.2.1. Chemokine and checkpoint receptor expression and localization along the tumor-tumor-free axis

To link the accumulation of NK cells within the tumor center with trafficking and retention, chemokine receptor expression on trNK cells, ILCs, and $CD8^+$ TRM was assessed across the tumor center, margin, peritumor, and tumor distal areas (**Study II, Fig. 2**). CXCR3 was broadly expressed on trNK cells and $CD8^+$ TRM (**Study II, Fig. 2b, c**) in both AC and SCC (**Study II, Fig. S2B, C**), whereas higher CXCR6 expression was observed toward the tumor center, specifically in SCC (**Study II, Fig. 2b, c; Fig. S2B, C**). This was accompanied by an increase of $CXCR3^+CXCR6^+$ populations (**Study II, Fig. 2c**), and lower CCR2 expression (**Study II, Fig. 2b, c**). In contrast, ILCs showed minimal surface chemokine receptor expression, except for CCR2, which was observed across all regions and cancer types (**Study II, Fig. 2b; Fig. S2B, C**).

These findings suggest that CXCR6 contributes to the accumulation of tissue-resident T and NK cell subsets within the tumor center. More broadly, they support a model in which CXCR3- and possibly CCR5-mediated recruitment precedes CXCR6-dependent retention (247, 336), resulting in preferential enrichment of $CXCR3^+CXCR6^+$ trNK and $CD8^+$ TRM cells in the tumor center (**Study II, Fig. 2c)**. Together with previous studies identifying CXCR6 as a hallmark of intratumoral tissue-resident NK and T cells (223, 247, 337), this points to CXCR6 as a central organizer of lymphocyte localization and persistence in NSCLC. In contrast, the largely chemokine-receptor-poor ILC compartment may instead reflect local repositioning of pre-existing resident cells rather than ongoing recruitment.

Although lymphocytes have the capacity to infiltrate tumors, their intratumoral activity is often limited (223, 247, 249). NK cells and T cells within the TME were

reported to upregulate the expression of immune checkpoint receptors, also referred to as exhaustion markers, including PD-1 (338), TIGIT (339), TIM-3 (340), an CD39 (341). In contrast, the immune checkpoint receptor landscape of trNK cells and CD8+ TRM remains less well defined.

Analysis across tumor regions revealed that CD8+ and CD4+ TRM expressed high levels of these receptors, with frequencies increasing toward the tumor center (**Study II, Fig. 3; Fig. S3A**). In contrast, trNK cells and ILCs displayed low surface expression of checkpoint receptors across all sites, apart from a slight increase of TIM-3 and TIGIT expression on trNK cells and elevated CD39 expression on ILCs towards the tumor center (**Study II, Fig. 3b**). No significant difference of immune checkpoint receptor expression was observed between AC and SCC (**Study II, Fig. S3B**).

Thus, whereas tumor-infiltrating CD4+ and CD8+ TRM progressively acquire a more inhibitory phenotype, trNK cells exhibited relatively limited checkpoint receptor expression. This pattern is consistent with the idea that intratumoral tissue-resident lymphocytes are checkpoint-sensitive but not uniformly terminally exhausted. Therefore, it is suggested that trNK cells may preserve a potentially rescuable functional potential within the TME (223, 247, 342). This relatively low checkpoint receptor expression on trNK cells (**Study II, Fig. 3b**) therefore raised the question of whether this phenotype is accompanied by retained effector potential.

4.2.2. Functional profiling of tissue-resident cytotoxic lymphocytes in the tumor microenvironment

These observations prompted us to investigate whether this distinct phenotypic profile translates into functional differences of cytotoxic cells within the TME. In the tumor center, trNK cells and CD8+ TRM expressed higher levels of GzmA and GzmB, but not perforin (**Study II, Fig. 4b, c**). Notably, perforin was largely absent in tissue-resident T and NK cells, whereas CD16+ NK cells consistently expressed perforin across all tumor sites (**Study II, Fig. S4C, D**). CD4+ TRM and ILCs displayed low or absent expression of granzymes and perforin (**Study II, Fig. S4C, D**). Comparing NSCLC subtypes, only GzmB expression was elevated in CD8+ TRM in the tumor center in SCC, but not in AC (**Study II, Fig. S4B**). Additionally, lower frequencies of non-tissue-resident CD8+ T and NK cells expressed GzmA and GzmB (**Study II, Fig. S4C, D**). A graphical summary of the distribution of NK cells, T

cells, and ILCs from the peritumoral region to the tumor center is presented in **Figure VI**.

Finally, to validate whether the phenotypic profile of trNK cells translates into functional capacity, we performed degranulation assays with K562 target cells on NK cells isolated from tumor-adjacent tissue and the tumor center (**Study II, Fig. 4d, e**). $CD49a^{+}CD16^{-}$ NK cells from the tumor center exhibited higher degranulation compared to both peritumoral counterparts and tumor center-derived $CD49a^{-}$ $CD16^{-}$ NK cells (**Study II, Fig. 4d, e**). These results indicate that trNK cells retain functional competence despite low perforin expression within lung tumors.

Although NK cells in NSCLC have historically been considered functionally impaired (194) and are still often reported to show limited degranulation against target cells (247), more recent studies suggest that trNK cells are not terminally exhausted but instead represent a potentially responsive immune population. $CD69^{+}CXCR6^{+}$ trNK cells in NSCLC can rapidly produce IFN-γ and TNF *ex vivo* but become functionally restrained upon direct tumor contact (223). This inhibition is at least partly rescued by PD-1 and CTLA-4 blockade, indicating a reversible dysfunctional state rather than terminal exhaustion (223). In parallel, intratumoral NK cells can suppress DC activation in a partially CTLA-4–dependent manner, and CTLA-4 expression correlates with CXCR6 at the transcriptional level (247).

Together, these observations support the hypothesis that intratumoral trNK cells are not irreversibly impaired but instead represent a poised, tissue-resident effector pool whose cytotoxic function may be re-invigorated by targeted cytokine, metabolic, or checkpoint-based interventions in solid tumors. This raises the broader question of how external challenges shape NK cell functionality, and whether similar mechanisms of immune modulation are exploited in other pathological contexts, including viral infections.

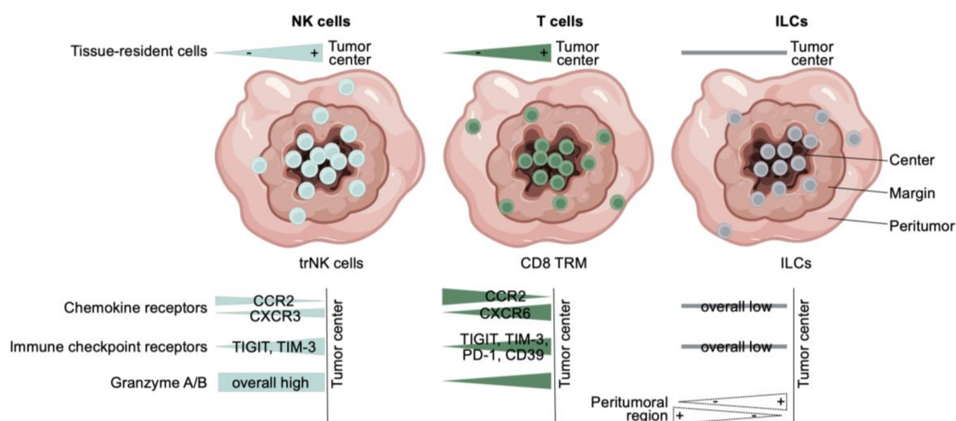

Figure VI: Distribution of lymphocytes in and around lung tumors. Illustration of the distribution of NK cells (left), T cells (middle), and ILCs (right) along the peritumoral-tumor center axis. Tissue-resident NK and T cell populations accumulate toward the tumor center. Expression of chemokine receptors, immune checkpoint receptors, and GzmA/B is depicted by arrows indicating relative increases or decreases, or by horizontal bars indicating stable expression, depending on location.

4.3. Study III: SARS-CoV-2 evasion of NK cell-mediated killing

Infection with SARS-CoV-2 primarily targets the respiratory tract and can manifest across a wide clinical spectrum, from asymptomatic disease to severe, life-threatening outcomes. In response to infection, the innate and adaptive immune systems act to eliminate infected cells, while the virus evolves strategies to evade immune recognition. In this setting, **Study III** investigates how the SARS-CoV-2 Omicron sub-lineage BQ.1 modulates NK cell-mediated recognition and cytotoxicity to promote immune escape.

BQ.1 is distinguished from the previously dominant BA.5 variant by six amino acid substitutions. Notably, a mutation associated with the increased virulence of BQ.1 affects the $Nsp13_{232-240}$ epitope, where a methionine-to-isoleucine substitution at position 2 (pM2I) arises from a single nucleotide change (G-to-A) (**Study III, Fig. 1A, B, D; Fig. S1**). BQ.1 rapidly became the dominant circulating variant toward the end of 2022 (**Study III, Fig. 1A**), indicated by an increase in reported infections, and a higher burden of local COVID-19-related intensive care unit admissions (**Study III, Fig. 1B**).

4.3.1. Nsp13 pM2I shapes HLA-E presentation and NK cell inhibition

The SARS-CoV-2 $Nsp13_{232-240}$ epitope is restricted to HLA-E, which is recognized by NK cells via the inhibitory receptor complex CD94/NKG2A (254).

The BA.5 Nsp13$_{232-240}$ epitope (VMPLSAPTL) has remained conserved throughout SARS-CoV-2 evolution from the ancestral variant and functions as a viral NKG2A antagonist by stabilizing HLA-E without effectively engaging CD94/NKG2A on NK cells (254) (**Study III, Fig 2B–D, Fig 3A–C, Fig 4B–D, Fig. 5B, C;**). Further, by outcompeting self-peptides, it shifts the HLA-E repertoire toward non-inhibitory viral complexes, thereby reducing inhibitory signaling to NK cells and functionally mimicking a "missing-self" state (**Study III, Fig 4B–D, Fig 4H–J**).

Recent work has highlighted that SARS-CoV-2 exploits HLA-E to fine-tune NK cell responses during acute infection (254). In particular, infection of primary lung epithelial cells leads to increased HLA-E expression, creating a platform for peptide-dependent tuning of NK cell activity. In this context, NK cell subsets expressing NKG2A showed enhanced activation when encountering targets presenting the Nsp13$_{232-240}$ peptide (encoding VMPLSAPTL), whereas NKG2A^{-} NK cells remain largely unaffected (254). At the same time, adaptive NKG2C^{+} NK cells, which are typically activated by HCMV-derived peptides presented by HLA-E (343), did not respond to the Nsp13$_{232-240}$ epitope, indicating that this viral peptide selectively modulates inhibitory rather than activating HLA-E–dependent pathways (254).

The pM2I mutation in the BQ.1 Nsp13$_{232-240}$ epitope (encoding VIPLSAPTL) reverses this effect. VIPLSAPTL binds and stabilizes HLA-E less efficiently, as shown by reduced affinity and shorter complex half-life (**Study III, Fig 2B-H**). Position 2 was further confirmed as a critical determinant of HLA-E/Nsp13$_{232-240}$–complex stability (**Study III, Fig. S2A, B**). This feature is supported by differences in the HLA-E/peptide complex melting point of BA.5 versus BQ.1 as well as structural analysis confirming alterations in B-pocket interactions (**Study III, Fig 3A-C; S3A, B**). In detail, introducing isoleucine at p2 in the BQ.1 Nsp13 peptide adds an extra side-chain branch (CG2) that bumps into HLA-E residue Y7, forcing Y7 to tilt toward the N-terminus of the binding groove (**Study III, Fig 3A-C**). This small steric crowding makes the contacts between p2I-Y7 and Y7-G26 suboptimal, leaving the B-pocket in an "overpacked" state, with the HLA-E/Nsp13$_{232-240}$–complex becoming less stable (**Study III, Fig 3A-C**). In turn, this structurally explains why the BQ.1 peptide is presented less efficiently by HLA-E, facilitating replacement by self-leader peptides and thereby strengthening NKG2A-mediated inhibition of NK cells (**Study III, Fig. 4B-D**). This increases the proportion of inhibitory HLA-E complexes, thereby restoring NKG2A signaling and reducing NK cell degranulation, cytokine production, and polyfunctionality in mixed peptide settings (**Study III, Fig**

4B–D, Fig 4F–L). Thus, BA.5 transiently disrupts NKG2A signaling, whereas BQ.1 re-establishes a self-dominated inhibitory landscape (**Study III, Fig 4M–N**) (**Figure VII**).

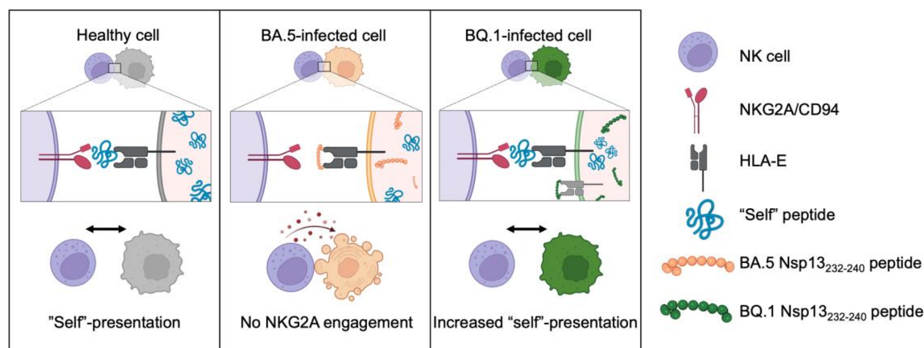

Figure VII: BQ.1 evades NK cell recognition by favoring self-presentation. Model comparing presentation of "self" versus viral peptides by HLA-E and their effects on NKG2A/CD94 signaling in NK cells. In healthy cells, self-peptide-HLA-E complexes engage NKG2A/CD94 and suppress NK cell activation. In BA.5-infected cells, the Nsp13$_{232-240}$ peptide disrupts this interaction, reducing inhibitory signaling and promoting NK cell activation. In contrast, the BQ.1 Nsp13$_{232-240}$ peptide destabilizes viral peptide–HLA-E complexes, favoring presentation of host self-peptides and thereby restoring NKG2A/CD94-mediated NK cell inhibition.

Additionally, live virus infection experiments comparing ancestral SARS-CoV-2 (encoding VMPLSAPTL) and BQ.1 (encoding VPLASAPTL) confirmed these findings, showing reduced HLA-E surface expression in BQ.1-infected Caco-2 and A549 cells (**Study III, Fig 2I–K; Fig. S2C, D**).

4.3.2. SARS-CoV-2 BQ.1 fitness, HCoV-like convergence, and host genetics

Across millions of SARS-CoV-2 genomes, pM2I is consistently associated with fitness advantage and is conserved in BQ.1 lineages (**Fig 1D, Fig 5A–B**). Comparisons of Nsp13-like peptides from wildlife sarbecoviruses, SARS-CoV-1, prior SARS-CoV-2 variants, and endemic HCoVs revealed a unique p2MI mutation in BQ.1 and descendants (**Study III, Fig 5C**). Evaluation of HLA-E loading and stabilization characteristics revealed BA.5 VMPLSAPTL as a strong HLA-E binder, BQ.1 VIPLSAPTL as intermediate, and HCoV peptides as generally poor HLA-E stabilizers (**Study III, Fig 5D–E**). The drop in HLA-E presentation from BA.5 to BQ.1 is disproportionately large relative to the underlying minimal sequence change,

moving BQ.1 closer to a "low HLA-E footprint," resembling endemic coronaviruses (**Study III, Fig 5F-G**).

Integrating these results, the fitness advantage of pM2I likely reflects an accumulation of effects mostly associated with decreased viral HLA-E binding of the BQ.1 Nsp13 peptide and allowing self-peptide presentation to dominate. This allows BQ.1 to evade recognition by both NKG2A^{+} and NKG2C^{+} NK cells. Conversely, the pM2I mutation confers only partial immune evasion, as presentation of the BQ.1 Nsp13 epitope failed to suppress NKG2A^{+} NK cell responses, which were comparable to responses to BA.5 (**Study III, Fig. S4E, F**).

Host immunogenetic variation in KIR, HLA class I, and the NKG2A–HLA-E axis critically shapes NK cell activation thresholds by tuning the balance between inhibitory and activating receptor signaling during NK cell responses. In relation to severe COVID-19, activating KIR2DS2 in combination with HLA-C1 has been associated with relative protection, whereas enrichment of inhibitory receptors such as KIR2DL1 and KIR2DS4 on CD16^{-} NK cells is linked to more severe disease (344-346). In this context, the pM2I-driven shift toward a self-dominated HLA-E repertoire further reinforces inhibitory signaling. By promoting presentation of self-derived peptides on HLA-E, this mutation enhances engagement of CD94/NKG2A, effectively strengthening an already inhibitory signaling environment. These effects likely act on top of inter-individual differences in HLA class I signal peptides, which have been shown to shape the strength of NKG2A-mediated inhibition through variation in HLA-E–presented self-peptides. Individuals whose signal peptide repertoire favors strong NKG2A engagement may therefore be more susceptible to the pM2I-driven shift toward a self-dominated HLA-E landscape, resulting in a more pronounced inhibition of NK cell activity.

These effects likely interact with inter-individual differences in HLA class I signal peptides, which have recently been shown to predict the strength of NKG2A-mediated inhibition through variation in HLA-E–presented self-peptides (89). Individuals with signal peptide repertoire that favor strong NKG2A engagement may therefore be more susceptible to the pM2I-driven shift toward a self-dominated HLA-E repertoire. As a result, NK cells are exposed to converging inhibitory inputs from both classical HLA class I/KIR and non-classical HLA-E/NKG2A pathways.

Simultaneously, activating signals in the periphery may be reduced, as SARS-CoV-2 downregulates ligands for activating receptors such as NKG2D (347). These

effects are likely even more relevant at the site of infection. Lung-resident NK cells, including adaptive-like CD16$^-$ trNK cells, often display distinct receptor repertoires, including increased NKG2A expression (76, 79). In such a setting, restoration of HLA-E–mediated inhibition by BQ.1 could disproportionately limit local NK cell responses, further limiting early antiviral control. Together, the combined effect induces a multi-layered suppression of NK cell activity.

Collectively, these findings highlight the dynamic adaptation of SARS-CoV-2 to host immune pressure. The BQ.1 Nsp13$_{232-240}$ pM2I mutation shifts the HLA-E peptidome toward a self-dominated state, restoring NKG2A-mediated inhibition and reducing NK cell recognition. Together, this underscores NK cells as a potential driver of SARS-CoV-2 evolution.

4.4. Study IV: Tissue-dependent organization of effector molecule expression

Having characterized NK cells and T cells in the human lung under both homeostatic and disease conditions, this approach was extended to the human intestine, with a focus on the ileum, caecum, and colon. In addition, the impact of underlying subclinical intestinal inflammation on the composition of the immune cell compartment was investigated.

Effector molecules are central to the elimination of malignant and infected cells, yet their distribution across the human body remains incompletely defined, particularly in paired tissues settings (78, 145). This has direct clinical relevance, as tissue-specific effector profiles may influence disease progression and therapeutic responses. Therefore, effector molecule expression patterns in immune cells were investigated across matched human tissues (**Study IV, Fig. 1**). Effector molecule expression across CD45$^+$ leukocytes was strongly tissue-dependent, with highest frequencies in liver and minimal granzyme/perforin expression in lymph nodes (**Study IV, Fig. 1A–D**). Intestinal tissues were dominated by GzmA single-positive (GzmAsp) cells, whereas blood, lung, liver, and spleen showed broader co-expression patterns (**Study IV, Fig. 1B–D**), indicating microenvironment-driven shaping of effector profiles.

Across lymphocyte subsets, effector-positive cells were present among CD4$^+$ and CD8$^+$ T cells, CD16$^-$ and CD16$^+$ NK cells, and ILCs in all tissues, but with site-specific contributions (**Study IV, Fig. 2A, B**). CD8$^+$ T cells predominated overall, while CD16$^-$ NK cells contributed disproportionately in ileum, spleen and liver (**Study IV, Fig. 2A, B; Fig. S2A, B**). Co-expression analysis showed enrichment of

$GzmA^+GzmB^+GzmK^-perforin^+$ $CD16^+$ NK cells from blood and perfused organs, whereas $CD16^-$ NK cells displayed large GzmAsp fractions in the intestine, as well as in liver and spleen (**Study IV, Fig. 2D**). Ileal $CD16^-$ NK cells exhibited the highest GzmA expression levels across all subsets and tissues, without similar enrichment of GzmB, GzmK or perforin (**Study IV, Fig. 2E, F; Fig. S2C, D**), defining a tissue-restricted $GzmA^{high}$ $CD16^-$ NK population (**Study IV, Fig. 2E, F**).

4.4.1. trNK cells in the ileum express high levels of GzmA

The tissue-restricted presence of $GzmA^{high}$ cells in the ileum led us to investigate the distribution of tissue-resident signatures across lymphocytes and organs. Phenotypic analysis showed that ileal $CD16^-$ NK cells expressed the highest levels of the tissue-residency markers CD49a and CD103 and of CD69 across all organs examined, with tight co-expression of CD49a and CD103 and co-enrichment specifically in the ileum (**Study IV, Fig. 3A–C; Fig. S3A**). Although correlations between GzmA MFI and tissue-residency and -homing markers (CD49a, CD69, CD103, CCR5, CXCR6) were modest at the ileal level, the overall frequency of $NKG2D^+CD16^-$ NK cells was highest in the ileum and strongly correlated with the proportion of $GzmA^+CD16^-$ NK cells across intestinal sites (**Study IV, Fig. 3D, F-H; Fig. S3B-C**).

In addition to the higher expression levels of GzmA, CD39 surface expression was markedly higher on $CD16^-$ NK cells in intestinal tissues, with around 70% of ileal $CD16^-$ NK cells expressing CD39. Further, CD39 was strongly co-expressed on ileal $GzmA^{high}CD16^-$ NK cells but not on $CD16^-$ NK cells in other organs (**Study IV, Fig. 3J–K; Fig. S3D**). Ileal $CD39^+CD16^-$ NK cells also showed high co-expression of CD49a/CD103, further supporting the definition of a $GzmA^{high}CD39^+NKG2D^+$ trNK subset in the ileum (**Study IV, Fig. 3L–M; Fig. S3E**).

Re-analysis of terminal ileum scRNA-seq data (317) identified 21 immune clusters, with *GZMA* being strongly enriched in clusters 8 and 13 and largely confined to epithelial-compartment immune cells, consistent with an intraepithelial phenotype (**Study IV, Fig. 4A–D; Fig. S4A–B**). Cluster 8 displayed a canonical NK-like signature (*KLRC1/2*, *KLRD1*, *KLRF1*, *NKG7*) with concentrated *GZMA* expression, while ILC-associated genes localized to a separate cluster (**Study IV, Fig. 4D; Fig. S4H**). This supports the notion that the $GzmA^{high}$ phenotype is not an artifact of protein-level gating but a stable transcriptional state. Within cluster 8, *GZMA* expression was highest in $CD3D^-$ cells, which retained an NK-like profile and co-expressed *ITGA1* (CD49a) and *ITGAE* (CD103) with low *FCGR3A* (CD16), closely

matching the GzmA^{high}CD16^{-} trNK phenotype (**Study IV, Fig. 4E; Fig. S4E–H**). *ENTPD1* (CD39) was also highly expressed, while stress-related gene signatures were not broadly elevated, suggesting a specialized but not overtly exhausted state (**Study IV, Fig. 4E–F; Fig. S4I–J**). Additionally, cluster 8 expressed chemokines such as *CCL5*, *XCL1*, and *XCL2* suggesting that, beyond direct cytotoxicity, these cells may orchestrate local immune recruitment and positioning at the epithelial barrier (**Study IV, Fig. S4F**). Identification of an NK-like cluster with high *GZMA* expression at the transcriptional level supports the protein-level findings. Consistent with this, *GZMA* levels were lower in non-inflamed compared to inflamed tissue.

4.4.2. GzmA^{high} trNK cells are reduced in individuals with IBD

Stratification by donor/tissue status revealed that cluster 8 was predominantly derived from non-inflamed tissue, with highest *GZMA* expression and a trend toward increased *GZMA*$^{+}$ cells in non-inflamed samples from IBD patients, whereas *GZMB* and *PRF1* were enriched in inflamed tissue (**Study IV, Fig. 4G–I; Fig. S4K–L**). This supports an association of the *GZMA*-high intraepithelial NK-like population with homeostasis or early, pre-inflammatory states.

In IBD donors who are in remission, the frequency of ileal trNK cells is preserved, but their effector wiring and receptor expression appear remodeled (**Study IV, Fig. 5B–F; Fig. S5B-F**). In detail, GzmA levels and frequencies of GzmA-expressing cells are decreased, NKG2D is expression is lower, and GzmK and perforin expression are elevated in both CD16^{-} and CD16^{+} NK subsets (**Study IV, Fig. 5B, F; Fig. S5C–E**). Additionally, the co-expression of GzmA and CD49a was decreased in ileal CD16^{-} NK cells from individuals with IBD compared to non-IBD donors (**Figure VIII**).

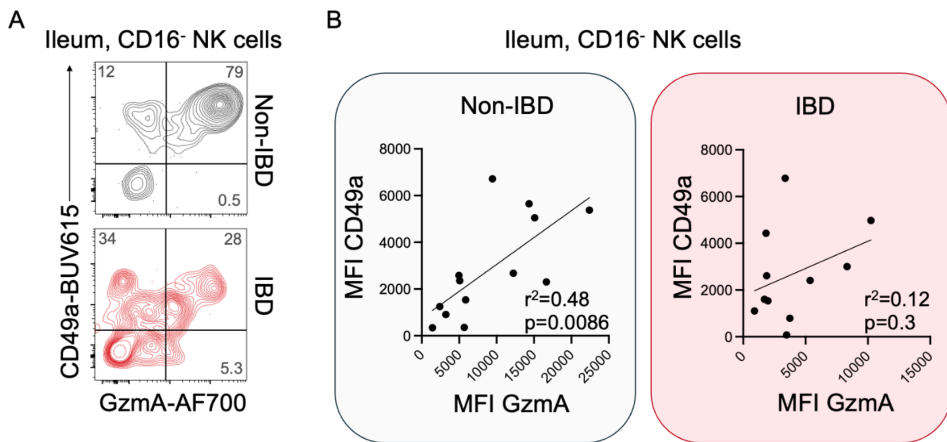

Figure VIII: Reduced co-expression of GzmA and CD49a in ileal CD16⁻ NK cells from IBD patients. (**A**) Representative contour plots showing CD49a and GzmA expression of ileal CD16⁻ NK cells from donors with (bottom) and without IBD (top). (**B**) CD16⁻ NK cells from the ileum of non-IBD individuals show significant co-expression of GzmA and CD49a, a pattern that is diminished in IBD patients (simple linear regression). Samples were taken from non-inflamed areas.

One potential mechanism linked to pro-inflammatory features suggests that the GzmA^{high}CD39^{+}NKG2D^{+} trNK cells may be partially reprogrammed toward a more classical cytotoxic profile, with increased perforin and GzmB/GzmK utilization and reduced stress-sensing via NKG2D. Given that NKG2D-ligand interactions are central for detecting epithelial stress and are dysregulated in IBD (305, 306), loss of NKG2D may limit surveillance and allowing micro-lesions or dysregulated repair to persist.

Concurrently, reduced GzmA expression may indicate loss of functions beyond direct cytotoxicity. NK cell- and T cell-derived GzmA have been linked to epithelial regulation, including GSDMB-dependent pyroptosis in epithelial targets, matrix remodeling, and barrier maintenance (278-280, 286). Thus, reduction in GzmA could attenuate non-cytolytic programs that support epithelial differentiation and barrier integrity, as shown for GzmA^{+}CD39^{+} T cells, where GzmA restrains GPX4-mediated ferroptosis and promotes tight junction and CDX2 expression in the intestinal epithelium (286). Consistent with a beneficial, tissue-protective role, combined *GZMA*/*ITGAE* (encoding CD49a) expression has been associated with favorable responses to anti-integrin therapy (312). Ileal NK cells may represent relevant contributors in this context, as they strongly co-express CD49a and GzmA (**Study IV, Fig. 3D, E**).

Conversely, other reports link elevated extracellular GzmA activity to intestinal inflammation and propose GzmA as a disease-associated biomarker (311, 348). In mouse models, GzmA plays similarly context-dependent roles: in DSS/AOM (dextran sulfate sodium/Azoxymethane) and DSS colitis models, high local or extracellular GzmA exacerbates inflammation and colitis-associated cancer, and GzmA deficiency or pharmacological inhibition improves disease burden (278, 311). In contrast, systemic administration of recombinant GzmA in DSS colitis improves weight loss, colon length, and histologic scores by protecting epithelial cells from ferroptosis (286). Together, these data support a model in which cell-associated GzmA in defined lymphocyte-epithelial circuits is barrier-supportive, whereas uncontrolled extracellular GzmA in the inflamed mucosa can fuel cytokine production, pyroptotic cell death, and chronic intestinal inflammation.

Importantly, our findings point to NK cells as a previously underrecognized source of GzmA in the intestine and indicate that their GzmA-associated programs are potentially altered in IBD, underscoring their potential contribution to disease-associated immune remodeling.

4.4.3. Intestinal NK cells from IBD patients remain functional

To assess functional differences between the patient groups, NK cells were stimulated with PMA/ionomycin. Intestinal NK cells from IBD patients remained functionally competent in standard stimulation assays, with preserved degranulation and cytokine production (**Study IV, Fig. 6A-B, Fig. S6A–B**). Comparing the functional profile of GzmA^{+} and GzmA^{-} NK cells, greater frequencies of intestinal GzmA^{+} NK cells expressed IFN-γ upon activation compared the GzmA^{-} counterpart, an effect that was not observed in GzmA$^{+/-}$ NK cells derived from donor-matched peripheral blood (**Study IV, Fig. 6C-D, Fig. S6C–D**). This suggests that IBD-associated changes reflect a rewiring of effector and receptor programs within a functionally competent NK cell pool, rather than global exhaustion, and that alterations in GzmA and NKG2D expression in ileal CD16^{-} trNK cells may mark early or subclinical shifts in mucosal immune organization. Phenotypic differences between ileal CD16^{-} NK cells from IBD and non-IBD individuals are characterized by patterns of surface and intracellular marker expression as shown in **Figure IX**.

Taken together, the combination of high degranulation capacity, spontaneous cytokine production, and co-expression of CD39 and NKG2D suggests a model in which ileal GzmA^{high} trNK cells may contribute to mediate stress-responsive,

contact-dependent effector functions, such as editing aberrant epithelial cells and modulating barrier integrity. The localization to epithelial layers, together with parallels to GzmA⁺/CD39⁺ TRM and MAIT subsets (269, 286), supports a role in barrier surveillance and mucosal homeostasis. However, this does not preclude the pro-inflammatory capacity of GzmA (311, 348), suggesting that NK cells may contribute to both protective and inflammatory processes depending on context.

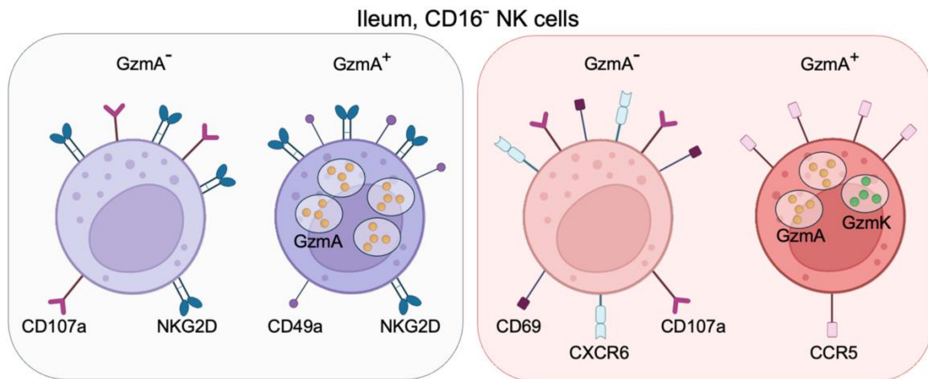

Figure IX: Phenotypical changes of ileal CD16⁻ NK cells in IBD. Illustration of trends in differential surface and intracellular marker expression at the protein level of unstimulated GzmA⁻ and GzmA⁺ CD16⁻ NK cells from the non-inflamed ileum of patients without IBD (left) and IBD patients (right).

5. Conclusions and points of perspective

Studies I, II, and IV expand our understanding of immune cell heterogeneity, particularly of NK cells, in tissue homeostasis, as well as in lung tumors and intestinal inflammatory states. In addition, **Study III** investigates mechanisms of viral immune evasion, demonstrating how emerging SARS-CoV-2 variants modulate NK cell recognition to escape immune control.

Study I provides a detailed map of immune cell composition across matched regions of the human lung, revealing that leukocyte distribution within the parenchyma is largely uniform, while airway-associated compartments represent distinct immunological niches for T and B cells. In contrast, NK cells displayed pronounced heterogeneity, with strong inter-donor variation exceeding regional differences. Notably, a subset of adaptive-like trNK cells was found to be enriched in peripheral lower lung regions and absent from other tissues, indicating lung-specific specialization.

These findings refine current concepts of pulmonary immunity by showing that regional organization is less defined by gross anatomy and more by microenvironmental cues and donor-specific factors. Further, NK cell diversity in tissues, in this context in the lung, is not fully captured by existing classification frameworks. In particular, the NK1, NK2, NK3 nomenclature largely derived from circulating NK cells may be insufficient to describe tissue-derived NK cell states, emphasizing the need for revised definitions that incorporate residency, adaptation, and local context (117).

From a broader perspective, this work underscores the importance of studying immune cells in matched human tissues to resolve spatial organization and functional specialization. In particular, the identification of adaptive-like trNK cells enriched in lower lung regions suggests the presence of a distinct, tissue-adapted NK cell subset with potential relevance for local immunity. These cells may be of interest for therapeutic applications, as tissue-derived adaptive-like NK cells could possess enhanced capacity for tissue infiltration – an important limitation in current immunotherapies targeting solid tumors (reviewed in (349)).

However, several questions remain. The origin and maintenance of adaptive-like trNK cells, whether driven by local expansion, environmental imprinting, or prior viral infections, are not yet understood. Likewise, the functional implications of their regional enrichment, including their roles in tissue homeostasis, antiviral

responses, or long-term immune equilibrium, remain unclear. This is particularly relevant in the context of lung transplantation, where NK cell receptor-ligand interactions, including KIR-HLA combinations, have been linked to graft outcomes (350, 351). Regional differences in NK cell phenotypes may therefore have clinical implications that are not captured by peripheral blood analyses alone.

Study II supports a model in which the NSCLC tumor center represents a specialized immune niche enriched for $CXCR3^{+}CXCR6^{+}$ $CD8^{+}$ TRM and trNK cells, shaped by coordinated recruitment and retention signals. While a substantial fraction of $CD8^{+}$ TRM cells display expression of immune checkpoint receptors, most trNK cells and non-NK ILCs showed lacked such expression, suggesting that these innate populations retain a degree of functional plasticity within the tumor microenvironment. This distinction points to a potentially underexploited compartment of lymphocytes that may be more amenable to functional reinvigoration.

These findings highlight several therapeutic opportunities. Modulation of chemokine axes such as CXCR3, CXCR6, and CCR5 may enhance recruitment and retention of beneficial lymphocyte subsets, while targeting suppressive pathways, including TGF-β and hypoxia, could restore cytotoxic activity in effector-primed populations. In parallel, strategies to improve NK cell infiltration, such as engineering chemokine receptor expression, may help overcome current limitations of NK cell-based therapies in solid tumors. The observed CXCR6 upregulation further supports a retention-associated, tissue-adapted phenotype, consistent with previous studies linking CXCR6 to intratumoral lymphocyte persistence and functional potential (223).

A deeper understanding of how tumor histology, chemokine-driven positioning, local imprinting, and checkpoint regulation affect local immune cells may inform the development of more effective immunotherapies that harness both adaptive and innate immune compartments at the tumor site.

Study III demonstrates that the BQ.1 variant exploits a minimal amino acid change within the $Nsp13_{232-240}$ epitope to reshape peptide presentation by HLA-E. In contrast to the ancestral and BA.5 $Nsp13_{232-240}$ peptide, which stabilize HLA-E in a manner that fails to engage inhibitory NKG2A signaling, the pM2I substitution

reduces peptide-HLA-E stability and facilitates replacement by self-peptides. This shift restores inhibitory signaling through CD94/NKG2A and dampens NK cell activation, representing a subtle yet effective mechanism of immune evasion.

At the same time, this mechanism is not absolute. The BQ.1 $Nsp13_{232-240}$ epitope remains detectable by NK cells, and cells presenting this peptide can still be targeted, indicating that immune evasion is partial rather than complete. This nuanced adaptation highlights the importance of peptide-level changes in shaping host–pathogen interactions and suggests that NK cell surveillance contributes to selective pressure during viral evolution. More broadly, this study suggests that viral evolution can target innate immune checkpoints, with BQ.1 showing reduced HLA-E presentation similar to endemic coronaviruses, consistent with a shift toward immune invisibility.

However, several questions remain regarding the extent to which NK cell-mediated pressure drives viral evolution *in vivo* and how these mechanisms integrate with adaptive immunity and tissue context. In particular, the spatial dynamics of NK cell responses in the lung, especially the role of tissue-resident subsets, remain poorly defined. Host and tissue immunogenetic variation in KIR, HLA, and NKG2A is also likely to modulate these effects and contribute to variability in disease outcomes.

Together, these considerations highlight the need to better resolve NK cell activity at sites of infection, to understand how viral variants shape tissue-resident immunity, and to define interactions between innate and adaptive responses. In this context, targeting the HLA-E/NKG2A axis may represent a promising strategy to restore NK cell activity.

Study IV identifies a previously underappreciated intestinal NK cell subset characterized by high GzmA expression. Across matched human tissues, effector molecule expression was strongly tissue-dependent, and the ileum presented as a site enriched for a $GzmA^{high}$ $CD16^{-}$ trNK cell population with immunomodulatory features indicated by the expression of CD39 and NKG2D. Transcriptomic re-analysis independently supported this phenotype. Further, this population was altered in remission-phase IBD. Although ileal trNK cell frequencies were preserved, their effector and receptor programs were remodeled, with reduced GzmA and NKG2D expression. Notably, functional capacity remains intact,

suggesting rewiring of effector functions rather than global exhaustion and pointing to early immune alterations even in clinically quiescent disease.

More broadly, the findings highlight the context-dependent biology of GzmA. In the intestine, GzmA may support barrier integrity and epithelial homeostasis in defined lymphocyte-epithelial circuits (223), yet under inflammatory conditions extracellular GzmA has also been linked to cytokine amplification and tissue damage (348, 352) The present work adds NK cells to this axis as a previously underrecognized source of intestinal GzmA and suggests that their contribution may differ between homeostasis, remission, and active inflammation.

Key questions remain regarding whether ileal GzmA^{high} trNK cells represent a stable self-renewing population or are continuously replenished and shaped by local cues, and under which conditions they adopt barrier-supportive versus inflammatory programs. Recent human and murine studies support a role for GzmA in intestinal immunity, and the identification of NK cells as a prominent source suggests that this subset may represent a promising target for modulating mucosal immune responses.

This thesis explored the heterogenic landscape of NK cells in tissues, in health, cancer, and inflammation, as well as in the context of SARS-CoV-2 immune evasion. In summary, the studies suggest that NK cells act as tissue-imprinted, context-dependent immune cells shaped by local microenvironments across organs and disease states. Rather than uniform effectors, they adopt distinct roles in homeostasis, tumor immunity, and inflammation, while also being targeted by viral and tumor immune evasion strategies such as HLA-E/NKG2A signaling. Together, these findings highlight NK cells as central regulators at the interface of immunity and immune escape, and as promising targets for future immunotherapeutic approaches.

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7. Declaration about the use of generative AI

The AI-assisted tools ChatGPT-5.3 and Perplexity GPT-5.1 (both OpenAI) were used in the writing of the comprehensive summary ("kappa") of this thesis. ChatGPT was primarily utilized for language refinement and improving clarity and flow of text. All suggestions and modifications generated by the AI were critically assessed and carefully reviewed by me before being incorporated into the text. Perplexity was used to support targeted literature searches and help identify key primary references. All scientific conclusions, interpretations, and the overall content were developed independently by me.

I take full responsibility for the content of the comprehensive summary of the thesis.

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