INNATE LYMPHOID CELLS IN CANCER: FRIENDS OR FOES?

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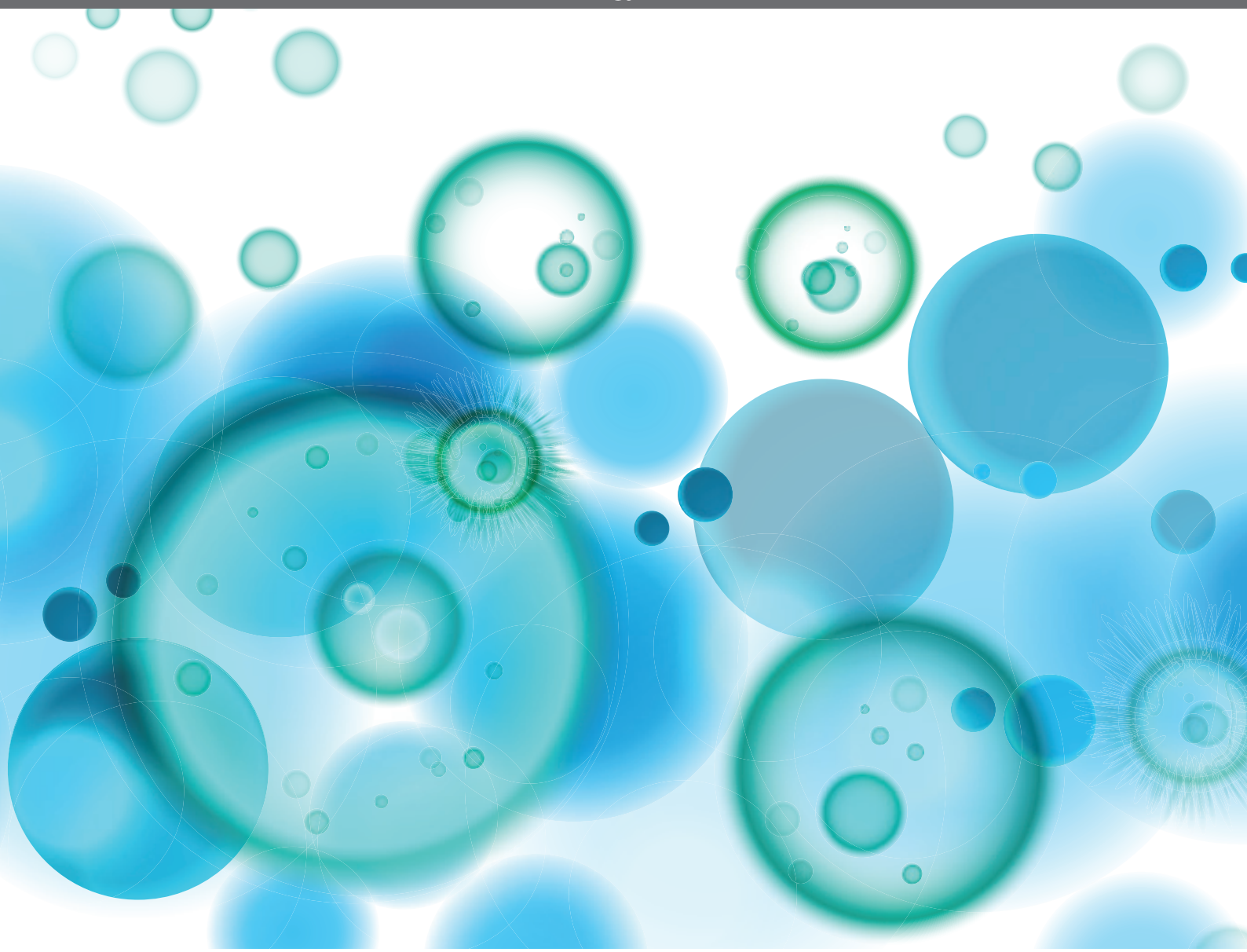

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INNATE LYMPHOID CELLS IN CANCER: FRIENDS OR FOES?

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Editorial: Innate Lymphoid Cells in Cancer: Friends or Foes?

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Editorial on the Research Topic

Innate Lymphoid Cells in Cancer: Friends or Foes?

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Innate lymphoid cells (ILCs) constitute the innate counterpart of T lymphocytes. They are devoid of antigen specific receptors and instead express a myriad of surface molecules allowing them to actively sense, and appropriately respond to, their tissue environment. This family of cells is classified into five subsets based on their developmental trajectories, the transcription factors and cytokines they express, and their effector functions. While type 1 ILCs (ILC1) only express the transcription factor T-bet, NK cells additionally express Eomes. Both subsets produce high levels of interferon (IFN)-γ and cytotoxic molecules, key features involved in the elimination of virus-infected and tumor cells. Type 2 ILCs (ILC2) express the transcription factors Gata3 and Rorα and secrete type 2 cytokines, such as IL-4, IL-5, IL-13 and GM-CSF, which are critical mediators of helminth expulsion and parasite elimination. Complementary to other ILC subsets, LTi cells and type 3 ILCs (ILC3) express the transcription factor Rorγt and produce the cytokines IL-17 and IL-22. LTi cells drive lymphoid organ development during embryogenesis whereas ILC3 contribute to intestinal homeostasis after birth through the promotion of epithelial cell growth and repair as well as the elimination of harmful extracellular pathogens.

ILCs can circulate around the body but predominantly reside within organs to maintain tissue homeostasis, potentially impacting the development and progression of every tumor type. In this regard, the review by An et al. extensively discusses the bidirectional immunomodulation of ILCs with the tumor microenvironment. This includes interactions with the extracellular matrix, other immune and non-immune cell types which express cytokines and growth factors that deeply influence the function of ILCs and their impact on cancer prognosis and treatment. In their review, they further highlight how targeting the heterogeneity and plasticity of the ILC family holds potential for cancer immunotherapy. Complementary to this work, Ducimetière et al., detail the bidirectional communication that exists between ILCs and the tumor microenvironment and discuss the impact of ILC migration *versus* local expansion in the tumor microenvironment in addition to the pathways and molecules involved in ILC interactions with innate and adaptive immunity, endothelial and tumor cells.

Numerous ILC tissue specificities have been reported. Remarkably, ILCs are enriched at mucosal sites, particularly in the intestinal tract, where they engage in activities essential for the preservation of epithelial barrier integrity. In this regard, Huang et al. highlight the dual role played by the

different ILC subsets in the development, prognosis and treatment of colorectal cancer. ILC functions are dictated by their tissue environs, and it is now well appreciated that the tumor microenvironment largely differs according to tumor type. This tumor heterogeneity influences ILC activity, potentially driving divergent outcomes in cancer. This is particularly evident for ILC2 activation pathways in cancer. With this in mind, Ercolano et al. summarized our current knowledge of the role and impact of ILC2s in cancer, comprehensively detailing the pro- and anti-tumorigenic functions of this innate immune cell subset in hematological malignancies, urogenital and gastrointestinal cancers, melanoma, breast and lung tumors. Adding to this, Tumino et al. summarized the role of ILC1, ILC2 and ILC3 in human tumors. Similar to T cells, ILCs express many immune checkpoints at their membrane, further regulating their function. Tumino et al. reviewed the currently reported immune checkpoints expressed by ILCs in various settings including tumor development. Even though the contribution of ILCs in malignancy is only now beginning to emerge, these reviews highlight that, in addition to NK cells, ILCs clearly impact cancer development, progression, prognosis, and treatment.

Discovered more than 45 years ago, seminal studies in mice and humans have demonstrated that NK cells exhibit strong anti-tumor activities. NK cells mediate anti-tumor immune responses through the secretion of cytokines, chemokines and growth factors and the direct lysis of tumor cells. Their activity is dictated by a fine balance between activating and inhibitory receptors. Heightened engagement of these activating receptors promotes NK cell function and the release of effector molecules that kill target cells. In contrast, NK cells are equipped with inhibitory receptors which, when engaged, impair NK cell activity. For example, NK cells express the polymorphic Killer cell immunoglobulin-like receptors (KIR) that recognize HLA class I molecules, which are often downregulated by cancer cells. Consequently, these activating and inhibitory receptor family educate NK cells to recognize and kill target cells while sparing healthy cells. By analysing large cohorts of healthy individuals and leukemic patients from Southern China, Deng et al. observed major differences in their KIR haplotypes influencing NK cell cytotoxic function and patient prognosis. The genotyping of the *KIR A* haplotype has revealed higher homozygosity in healthy donors than in cancer patients. *KIR A* homozygosity was associated with increased NK cell cytotoxicity against leukemogenic cells having altered HLA expression, conferring increased protection against leukemia development.

NK cell anti-tumor function is also influenced by other components of the tumor microenvironment, including the extracellular matrix (ECM). Rossi et al. summarized in detail how the ECM and its components regulates NK cell activity. In particular, the ECM is enriched in TGF-β, known to promote the transdifferentiation of NK cells into ILC1-like cells. In non-small cell lung cancer patients, Verma et al. observed a downregulation of Eomes expression in circulating NK cells that is associated with disease progression. They recapitulated this phenotype in mice and further demonstrated that NK cells that have downregulated Eomes expression exhibit an ILC1-like phenotype with reduced anti-tumor effector function. Thus, in addition to activation and inhibitory receptors, there is a growing interest in this field to better understand the characteristics of the ECM and its impact on ILC function with the aim of designing new cancer therapies.

Malignant brain tumors are difficult to treat due to the unique anatomy of the brain, an immune privileged site that is poorly immunogenic. Consequently, tumors of the central nervous system often exhibit an immunosuppressive tumor immune microenvironment, representing a major therapeutic challenge to overcome. Sedgwick et al. review the emerging evidence for ILCs in brain tumor immunosurveillance, with a specific emphasis on NK cells. In their review, Sedgwick et al. further described various NK cell-based therapies currently under investigations in malignant brain tumors, potentially offering new therapeutic perspectives to patients. Patients with NK cell-enriched tumors often exhibit a more favourable prognosis. Moreover, different NK cell phenotypes may be associated with differential patient outcomes. Using NK cell transcriptional signatures and computational analyses of The Cancer Genome Atlas, Sun et al. demonstrated that low-grade glioma patient tumors enriched for a PDGF-DD activated NK cell phenotype correlates with a more favorable prognosis. PDGF-DD is a ligand for the activating receptor NKp44. Thus, PDGF-DD engagement of NKp44 may promote NK cell anti-tumor function and represent a clinically relevant pathway in low-grade glioma. In contrast to low-grade glioma, Sun et al. found that an IL-2 expanded NK cell phenotype was associated with a more favorable prognosis in bladder cancer. Together, these results indicate that NK cell signatures may be used as prognostic markers to further stratify patients in the clinic.

Collectively, this Research Topic covers the diverse roles and functions of ILC subsets in tumors and their impact on cancer patient prognosis and treatment. While the anti-tumor potential of NK cells in cancer is now well demonstrated, how other ILC subsets impact patient prognosis and treatment efficacy and whether it is feasible to harness their function in cancer remains, to date, largely unknown. This exciting new research field aims to better understand the role of ILCs in cancer and to harness the anti-tumor functions of these innate immune cells for the development of innovative next generation anti-cancer immunotherapies.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Natural Killer Cells Offer Differential Protection From Leukemia in Chinese Southern Han

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Interactions of human natural killer (NK) cell inhibitory receptors with polymorphic HLA-A, -B and -C molecules educate NK cells for immune surveillance against tumor cells. The *KIR A* haplotype encodes a distinctive set of HLA-specific NK cell inhibiting receptors having strong influence on immunity. We observed higher frequency of *KIR A* homozygosity among 745 healthy Chinese Southern Han than 836 adult patients representing three types of leukemia: ALL (OR = 0.68, 95% CI = 0.52–0.89, $p = 0.004$), AML (OR = 0.76, 95% CI = 0.59–0.98, $p = 0.034$), and CML (OR = 0.72 95% CI = 0.51–1.0, ns). We observed the same trend for NHL (OR = 0.47 95% CI = 0.26–0.88 $p = 0.017$). For ALL, the protective effect of the *KIR AA* genotype was greater in the presence of KIR ligands C1 (Pc = 0.01) and Bw4 (Pc = 0.001), which are tightly linked in East Asians. By contrast, the C2 ligand strengthened protection from CML (Pc = 0.004). NK cells isolated from *KIR AA* individuals were significantly more cytotoxic toward leukemic cells than those from other *KIR* genotypes ($p < 0.0001$). These data suggest KIR allotypes encoded by East Asian *KIR A* haplotypes are strongly inhibitory, arming NK cells to respond to leukemogenic cells having altered HLA expression. Thus, the study of populations with distinct *KIR* and *HLA* distributions enlightens understanding of immune mechanisms that significantly impact leukemia pathogenesis.

Keywords: leukemia, AML, ALL, CML, NHL, HLA, KIR, Chinese Southern Han

INTRODUCTION

Natural killer (NK) cells can detect and eliminate leukemia cells (1). To perform this function, NK cells express multiple inhibitory and activating receptors specific for the HLA class I proteins that are expressed on tissue cells (2–4). Polymorphic inhibitory receptors educate NK cells, enabling them to kill tumors or infected cells having altered or reduced HLA class I expression. In turn, the activating receptors can complement this function by identifying foreign or neo-antigens (5–7).

Study of NK cell receptor polymorphism is thus critical for understanding the anti-tumor effects of NK cells, and to facilitate their subsequent manipulation for use in immunotherapy (8).

Killer cell immunoglobulin-like receptors (KIR) are the most polymorphic of the human NK cell receptors, and are characterized by extreme variation in gene number (9). The *KIR* locus comprises up to 13 functional genes, *KIR2DL1-5*, *KIR2DS1-5*, *KIR3DL1/S1,* and *KIR3DL2-3*, where those designated with an "L" suffix encode inhibitory receptors and those with an "S" suffix encode activating receptors. Previous population, transplantation, and disease association studies have identified two functionally distinct groups of *KIR* haplotypes that are present in all populations (10, 11). *KIR A* haplotypes express four inhibitory receptors specific for polymorphic HLA class I (KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2), plus zero or one activating receptor (KIR2DS4) also specific for HLA class I. Multiple distinct *KIR B* haplotypes express fewer inhibitory receptors but more activating receptors than the *KIR A* haplotypes (12).

Although previous examinations of the role of KIR diversity in leukemia control have yielded potentially conflicting results (13), a common feature spanning multiple different populations and leukemia subtypes (14–19) is they are consistent with the *KIR A* haplotype being more frequent in healthy controls than in the patient groups. Because *AA* homozygous individuals possess all four inhibitory receptors and few activating receptors specific for HLA class I, their NK cells may therefore be strongly educated to detect any changes in HLA expression that can occur on leukemia cells (20). However, both the frequency of the *KIR A* haplotype, and the abundance of KIR ligands are highly variable across human populations (21, 22). In this context, examination of specific well-defined human populations is critical for understanding the role of KIR in leukemia control. Here we interrogate such effects in three leukemia subtypes sampled in a large cohort from Southern China, where the *KIR A* haplotype is very frequent.

MATERIALS AND METHODS

We studied 836 adult ALL, AML or CML patients, 225 pediatric leukemia patients and 745 healthy adult blood donors. We also studied 46 adult NHL patients, all having diffuse large B-cell lymphoma. The mean age of the adult patients analyzed was 31.4 years, controls 32.7 years, and pediatric cases 11.2 years. The patients were recruited from the hematopoietic stem cell transplantation (HSCT) program in the Shenzhen Blood Center, from August 1999 through June 2015. Leukemia diagnosis followed the French-American-British (FAB) classification, based on morphological observations. CML patients were not included if they had elected to take TKI (Imatinib) therapy instead of transplant. The CML patients included were from a study published in China (23). The controls were all unrelated healthy blood donors from Shenzhen Blood Center. All subjects are self-identified as Chinese Southern Han. Written informed consent was obtained from all study participants, and from the parents of all pediatric leukemia patients. The cohorts are described in **Supplemental Figure 1**.

KIR and HLA Genotyping

The presence or absence of all 13 functional *KIR* genes and two pseudogenes was determined from genomic DNA using the PCR-SSP based *KIR* Ready Gene kit (Inno-Train Diagnostik GmbH, Kronberg Im Taunus, Germany). It is difficult to distinguish *KIR AB* heterozygotes from *KIR BB* homozygotes, but the *KIR AA* genotype is readily identifiable (9, 24). Thus, individuals were designated as homozygous for the *KIR A* haplotype if they tested positive for *KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR2DS4*, *KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1, KIR3DP1,* and no other *KIR*. *KIR2DL3* and *KIR3DL1/S1* alleles were determined by Sanger sequencing from a subset of 306 controls, as described (25–27). *HLA class I* genes were sequenced as previously described (28), and assigned genotypes at two field (distinct polypeptide sequence) resolution using the ASSIGN4.7 software (Conexio Genomics, Applecross, Australia).

Statistical Analysis

For comparison of *KIR AA* frequencies, data from other populations were obtained from Allelefrequencies.net (21). The database was accessed in August 2018 and all populations having *KIR AA* genotype frequency data were used ($N = 154$). The populations were divided into major geographic origins as indicated (Amerindian, East Asian, European, Oceanian, sub-Saharan African) (21). The frequencies of *KIR AA* genotypes were compared across these major population groups using ANOVA, and, when comparing two populations, using unpaired *t*-tests for comparison of means. The tests were performed using GraphPad software (https://www.graphpad.com/). Odds ratio and confidence intervals were calculated using MedCalc (https://www.medcalc.org/calc/). Where appropriate, the analyses were corrected for multiple comparisons using the Bonferroni method.

NK Cell Cytotoxicity Assays

A total of 15 healthy Chinese Southern Han subjects (six of them with *KIR AA* genotype) were recruited from Shenzhen Blood Center. PBMC were separated by density gradient from 10 ml of peripheral blood and cultured for 13–14 days using the HANK cell *in vitro* preparation kit (Hank Bioengineering, Shenzhen, China). This process uses irradiated feeder cells expressing IL15 to promote NK cell expansion as described (29) for use in immunotherapy (30, 31), and we use it here to produce NK cells for *in vitro* assay. The expansion process increases the number and activity of NK cells but does not affect KIR expression (29, 32, 33). After this process, the mean purity of $CD56^{+}CD3^{-}$ cells was $86 \pm 4.5\%$. Antibodies used were: UCHT1 mouse anti-human CD3-FITC and B159 mouse anti-human CD56-APC (BD Biosciences, CA, USA). Target cells (1×10^6) were incubated for 10 min at 37°C in 1 mL PBS containing 2.5 μmol/L CFSE and then washed in PBS. Target cells were K562, which is a leukemia derived cell line that lacks HLA class I expression, and seven primary leukemic blasts from ALL and AML patients.

NK cells and target cells were combined at an effector-target-ratio of 20:1, and incubated in RPMI-1640 complete medium for 4 h at 37°C with 5% CO_2. Dead cells were stained using 7-AAD (BD Biosciences, CA, USA) for 15 min as described (34) and measured using a BD ACCURI C6 flow cytometer (BD Biosciences, CA, USA). Spontaneous lysis of target cells was measured by including a control without NK cells, and this value was subtracted from the total percentage of specific target cell lysis. All experiments were performed in duplicate.

Detection of CD107a Expression

A total of 18 healthy Chinese Southern Han individuals (11 of them with *KIR AA* genotype) were recruited from Shenzhen Blood Center. NK cells were isolated as above and incubated with K562 cells at a ratio of 5:1 for 5 h at 37°C. Optimal expression of CD107a on the cell surface of degranulating NK cells occurs between 4∼6 h post stimulation (35). After the first 1 h, 4 μL monensin (BD Biosciences, CA, USA) was added to each 6 mL culture. The cells were then incubated with 2.5 uL human Fc block (BD Biosciences, CA, USA) at 25°C for 15 min, then stained with CD56, CD3, and CD107a mAbs. Antibodies used were: UCHT1 mouse anti-human CD3-FITC, H4A3 mouse anti-human CD107a-PECy5, and B159 mouse anti-human CD56-APC (all BD Biosciences, CA, USA). Flow cytometric analysis was performed using an ACCURI C6 machine (BD Biosciences, CA, USA) Surface expression of CD107a was assessed in $CD56^{+}CD3^{-}$ cells (36). To detect spontaneous degranulation, a control sample without target cells was included in every experiment and this information was used for background subtraction of the results.

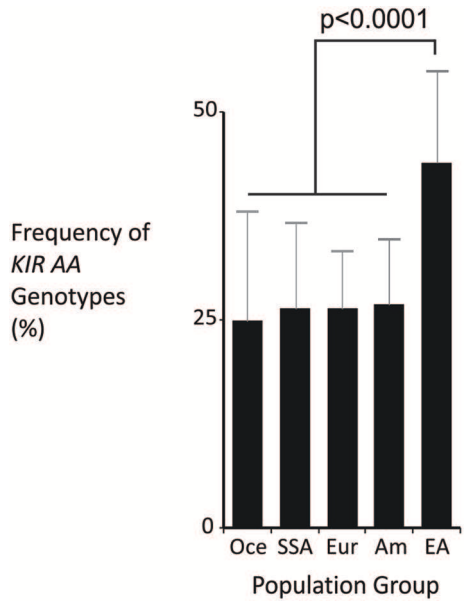


FIGURE 1 | The *KIR AA* genotype is most frequent in East Asia. Mean frequencies of the *KIR AA* genotype from multiple populations representing major world groups: Oce—Oceanian (13 populations), SSA –sub-Saharan African (13 populations), Eur—European (60 populations), Am—Amerindian (24 populations), EA—East Asian (31 populations). Data were obtained from allelefrequencies.net (21). ANOVA test for between group differences $p = 2.1 \times 10^{-16}$. The *p*-value shown is from *t*-test of comparison of means: East Asian is significantly different to every other group, with $p < 0.001$ for each comparison. Vertical bars are S.D.

RESULTS

KIR A Homozygosity Protects Chinese Han From Leukemia and Non-Hodgkin's Lymphoma

The *KIR A* haplotype is found in all human populations to varying frequency and is associated with NK cell function in protection from infectious diseases and in susceptibility to preeclampsia (10, 37). Because NK cells also function in tumor control, we tested whether *KIR A* affects the probability of developing leukemia. We first demonstrated that the frequency of individuals who are homozygous for the *KIR A* haplotype is significantly greater in East Asians than in other populations ($p < 0.0001$: **Figure 1**). We therefore examined a Chinese Southern Han cohort. In this cohort, the frequency of the *KIR AA* genotype is significantly higher among healthy individuals (55.3%) than adult leukemia patients (47.1%: OR = 0.72, 95% CI = 0.59–0.88, $p = 0.0012$) or pediatric leukemia patients (44.9%: OR = 0.6, 95% CI = 0.48–0.89, $p = 0.006$) (**Figure 2A**). When analyzed by disease subgroup, the *KIR AA* genotype was more frequent in healthy controls than in ALL, AML or CML patients (**Figure 2B**). For adult ALL (OR = 0.68, 95% CI = 0.52–0.89, $p = 0.004$) and AML (OR = 0.76, 95% CI = 0.59–0.98, $p = 0.034$), and pediatric ALL (OR = 0.64, 95% CI = 0.45–0.92, $p = 0.017$) the differences are statistically significant (**Figure 2B**). Similar trends were observed for CML and pediatric AML but did not reach statistical significance ($p = 0.052$ and $p = 0.1$, respectively), likely due to smaller sample sizes. We also analyzed non-Hodgkin's lymphoma (NHL) and again observed a protective effect of the *KIR AA* genotype (OR = 0.47, 95% CI = 0.26–0.88, $p = 0.017$), which was statistically significant in spite of the small sample size ($N = 46$: **Figure 2A**). The consistent reduction of frequency in all the disease groups analyzed strongly suggests Chinese Southern Han individuals having the *KIR AA* genotype are protected from developing leukemia or lymphoma.

In *KIR A* Homozygotes, KIR Ligands Differentially Protect From Leukemia Subtypes

To further explore our findings, we analyzed the HLA class I ligands for KIR. *HLA class I* allele frequencies can vary significantly between the ethnic groups and geographic regions of China, for example between Northern and Southern Han (38, 39). Validating the equivalent compositions of our disease and control subjects, there were no significant differences in *HLA-A*, *-B* or *-C* allele or haplotype frequency spectra between controls and the full patient group (**Supplemental Figures 2A,B**) or from previously published frequencies of *HLA class I* alleles of Chinese Southern Han (40). Neither was there a difference between groups in the frequencies of the −21M variant of

A

Group	N	*KIR AA* (%)	OR	95% CI	*p*
Controls	745	55.3			
Adult Leukemia	836	**47.1**	**0.72**	**0.59-0.88**	**0.001**
Pediatric Leukemia	225	**44.9**	**0.6**	**0.48-0.89**	**0.006**
Non-Hodgkin's Lymphoma	46	**37.0**	**0.47**	**0.26-0.88**	**0.017**

B

Group		N	*KIR AA* (%)	OR	95% CI	*p*
Controls		745	55.3			
Adult	ALL	321	**45.8**	**0.68**	**0.52-0.89**	**0.004**
	AML	345	**48.4**	**0.76**	**0.59-0.98**	**0.034**
	CML	170	47.1	0.72	0.51-1.01	0.052
Pediatric	ALL	151	**44.4**	**0.64**	**0.45-0.92**	**0.017**
	AML	88	45.8	0.68	0.43-1.10	0.100

C

	Group	Control	ALL					AML				CML					NHL			
	N	745	321					345				170					46			
	Genotype	%	%	OR	95% CI	*p*	p_C	%	OR	95% CI	*p*	%	OR	95% CI	*p*	p_C	%	OR	95% CI	*p*
KIR	*AA*	55.3	**45.8**	**0.68**	**0.52-0.89**	**0.0044**		**48.4**	**0.76**	**0.59-0.98**	**0.034**	47.1	0.72	0.51-1.0	0.052		**37.0**	**0.47**	**0.26-0.88**	**0.017**
	A3/11	60.4	53.0	0.74	0.57-0.96	0.02		58.3				52.4					54.3			
HLA	*Bw4*	73.4	70.7					72.2				72.4					76.1			
	C1	98.1	98.1					97.7				99.4					100			
	C2	29.2	24.6					23.5	0.74	0.55-0.99	0.047	19.4	0.58	0.38-0.88	0.01		26.1			
KIR	*AA+Bw4*	42.4	**30.2**	**0.59**	**0.44-0.77**	**0.0002**	**0.001**	34.8	0.72	0.55-0.94	0.017	35.3	0.74	0.52-1.0	0.09		32.6			
+	*AA+C1*	54.5	**44.5**	**0.67**	**0.51-0.87**	**0.0029**	**0.012**	47.5	0.76	0.58-0.98	0.033	47.1	0.74	0.53-1.0			37.0	0.49	0.26-0.91	0.023
HLA	*AA+C2*	16.8	12.1	0.69	0.47-1.0			11.6	0.65	0.44-0.95	0.027	**6.5**	**0.34**	**0.18-0.65**	**0.001**	**0.004**	8.7			

D

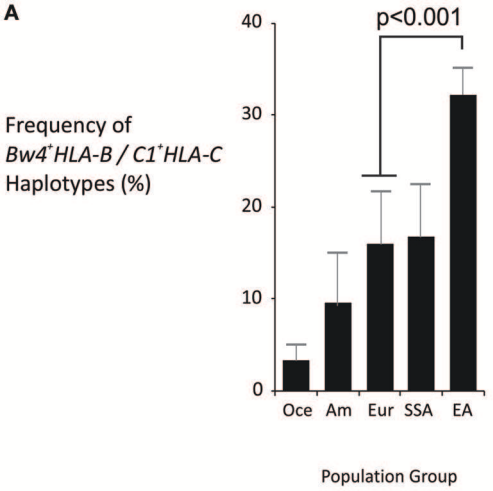
	Group	Control	Pediatric ALL					Pediatric AML			
	N	745	151					88			
	Genotype	%	%	OR	95% CI	*p*	p_C	%	OR	95% CI	*p*
KIR	*AA*	55.3	**44.4**	**0.6**	**0.45-0.92**	**0.017**		45.8			
	A3/11	60.4	59.2					63.9			
HLA	*Bw4*	73.4	72.5					74.7			
	C1	98.1	98.0					82.0			
	C2	29.2	28.9					25.3			
KIR	*AA+Bw4*	42.4	**31.0**	**0.6**	**0.41-0.89**	**0.011**	**0.022**	32.5			
+	*AA+C1*	54.5	43.7	0.7	0.45-0.93	0.018		44.6			
HLA	*AA+C2*	16.8	12.7					10.8			

FIGURE 2 | The *KIR AA* genotype associates with protection against leukemia. **(A,B)** Odds ratios and statistical significance obtained by comparing the *KIR AA* genotype frequencies of healthy controls with **(A)** adult and pediatric leukemia cases and non-Hodgkin's lymphoma, and **(B)** with the subtypes of leukemia. Statistically significant values are indicated in bold. **(C)**. Genotype frequencies, odds ratios and statistical significance obtained by comparing healthy controls (left), with three leukemia types, ALL, AML and CML (center), and NHL (right). The genotypes are: *KIR AA* genotype (top), the four KIR ligands (center) and *KIR AA* genotype in combination with each ligand (bottom). Only those results having 95% CI that do not encompass 1 are shown. P_C–Bonferroni correction factors used were x4 for ligands and x3 for *ligand+KIR AA*. Bold indicates *p*-value remains significant after this correction. **(D)** Shows the equivalent values obtained for pediatric ALL and AML.

HLA-B (**Supplemental Figure 2C**), which can influence NK cell education (41).

KIR bind differentially to specific amino acid motifs present on non-overlapping subsets of HLA class I variants (allotypes). The HLA allotypes are HLA-A*03 and -A*11 (the A3/11 motif: KIR3DL2 ligands), HLA-A and -B allotypes that contain the Bw4 motif (KIR3DL1 ligands), HLA-B and -C allotypes containing the C1 motif (primarily KIR2DL2/3 ligands), and HLA-C allotypes containing the C2 motif (primarily KIR2DL1 ligands). We observed trends for reduction of A3/11 frequency in the adult ALL patients (60.4 vs. 53%, $p = 0.02$: **Figure 2C**) and reduction of C2 frequency in the adult AML (29.2 vs. 23.5%, $p = 0.047$) and CML (29.2 vs. 19.4%, $p = 0.01$) patients relative to controls. However, these findings did not remain significant after accounting for multiple testing by correction for the four ligands analyzed (**Figure 2C**). By contrast, combinatorial analysis of KIR and their HLA class I ligands revealed clear distinctions between the three leukemia subsets (**Figures 2C,D**). The *KIR AA* plus *Bw4* compound genotype was more frequent in healthy individuals (42.4%) than in subjects with ALL (30.2% adult, $p = 0.0002$, and 31.0% pediatric $p = 0.011$) or AML (34.8% adult, $p = 0.017$ and 32.5% pediatric, ns). Similarly, the *KIR AA* plus *C1* compound genotype was significantly more frequent amongst the healthy controls (54.5%) than either the ALL (44.5% adult, $p = 0.003$ and 43.7% pediatric, $p = 0.018$) or AML (47.5% adult, $p = 0.033$ and 44.6% pediatric, ns) patients. For adult ALL, the strength of the effect was consistently greater when considering *KIR* in combination with *HLA class I* than when considering *KIR* genotype alone. These associations remained significant after correction for multiple testing (Pc = 0.001 *Bw4*$^+$*HLA*, and Pc = 0.012 *C1*$^+$*HLA*: **Figure 2C**). A reduced frequency of each of the three KIR/ligand combinations was observed in AML and NHL patients, but neither association remained significant after correction, suggesting there is likely limited improvement in protection when considering ligand over that offered by KIR alone.

Analysis of representative world populations revealed distinctively strong linkage disequilibrium between *Bw4*$^+$*HLA-B* and *C1*$^+$*HLA-C* in East Asians (**Figure 3A**). *HLA-B*46*, which is the only frequent *HLA-B* allele that expresses a C1 motif, is characteristic to East Asia, common in the Chinese Southern Han (∼15%), and is linked to *C1*$^+$*HLA-C* (21, 40). Also in the Chinese Southern Han, all *KIR A* haplotypes express KIR2DL3*001, which is an inhibitory receptor that interacts with C1$^+$HLA-C (26). KIR2DL3*001 also predominates in Japanese, another East Asian population (44). Similarly, the majority of East Asian *KIR A* haplotypes (>70%) express either KIR3DL1*001 or KIR3DL1*015, which are strongly-inhibitory KIR3DL1 allotypes specific for Bw4$^+$HLA (44). Of note, the non-expressed *KIR3DL1*004* allele that is common in Europeans (25) is absent from East Asians (44–46). Thus, in addition to the high frequency of *KIR A* haplotypes, a given Chinese Southern Han *KIR A* haplotype is more than twice as likely to express KIR3DL1*001 or *015 than a European *KIR A* haplotype (**Figure 3B**). Taken together, these observations indicate that Chinese Southern Han having the *KIR AA* genotype are more likely to have multiple interactions between strongly inhibitory KIR and their corresponding HLA ligands than are European individuals, who are more commonly studied. Thus, these strongly inhibitory KIR/HLA ligand interactions may be important factors in the NK cell control of ALL and to a lesser degree AML, in Chinese Southern Han.



B

	KIR A (%)	3DL1^{hi} (% of *KIR A*)
EUR	50.3	34.7
CSH	74.1	72.9
JPN	74.9	47.5

FIGURE 3 | Distinctive HLA and KIR haplotypes in Chinese Southern Han. **(A)** Mean frequencies of *Bw4*$^+$*HLA-B*/*C1*$^+$*HLA-C* haplotypes from multiple populations representing major world groups, Oce—Oceanian (3 populations), Am—Amerindian (4 populations), Eur—European (4 populations), SSA—sub-Saharan African (6 populations), EA—East Asian (8 populations). Data were obtained from Solberg et al. (42) and haplotype frequencies calculated using the Expectation Maximization algorithm (43). Vertical bars are S.D. **(B)** Frequency of *KIR A* haplotypes in European (EUR), Chinese Southern Han (CSH) and Japanese (JPN) populations, and the percentage of *KIR A* haplotypes that express either KIR3DL1*001 or KIR3DL1*015 (KIR3DL1^{hi}).

In contrast to ALL and AML, the strength of protection from developing CML offered by the *KIR AA* genotype was enhanced by the C2 ligand (OR = 0.34, 95% CI = 0.18–0.65, *Pc* = 0.004) to a much greater degree than by the C1/Bw4 ligands (**Figure 2C**). Although the sample size of the CML group is relatively small, the strength of the effect provides compelling evidence that Chinese Southern Han individuals having the *KIR AA* plus *C2*$^+$*HLA-C* genotype are less likely to develop CML than those who do not have this compound genotype. KIR2DL1, which is encoded by the *KIR A* haplotype, has high specificity for C2$^+$HLA-C, but the strength of inhibition resulting from the interaction varies across KIR2DL1 subtypes (47, 48). In East Asians, only two KIR2DL1 subtypes predominate: strongly inhibitory KIR2DL1*003 encoded by *KIR A* haplotypes, and weakly inhibitory KIR2DL1*004 encoded by *KIR B* haplotypes (44)

(and ZD, PJN unpublished). NK cells expressing KIR2DL1*003 are highly effective at killing cells that have reduced $C2^+$HLA-C expression, whereas those expressing KIR2DL1*004 are significantly weaker in this regard (47, 48). Thus, individuals who have the *KIR AA* genotype in this population likely have two genomic copies of the strongly inhibiting KIR2DL1 allotype, KIR2DL1*003, and should be best equipped at recognizing and eliminating tumor cells having aberrant $C2^+$HLA-C expression. This could take the form of reduction or loss of HLA expression (49, 50) or presentation of tumor neoantigen peptides by the $C2^+$HLA-C molecules, abrogating interaction with KIR2DL1 (5, 51). This result thus identifies a role for strongly functional KIR2DL1 allotypes in protection from CML in Chinese Han. This finding strikes strong parallel with several reproductive diseases including preeclampsia, where the *KIR AA* genotype in combination with C2 ligand impacts the risk of developing the disease (37), and highlights the need for study of populations in addition to Europeans for understanding mechanisms of protection from disease (52).

In summary, the *KIR AA* genotype confers differential protection against ALL, AML, CML and NHL; whereas the C2 ligand enhances *KIR AA* protection against CML, the C1 and Bw4 ligands enhance *KIR AA* protection against ALL, and the *KIR AA* genotype alone protects from AML and NHL.

NK Cells From *KIR A* Homozygous Individuals Are Strongly Cytotoxic to Leukemia Cells

To investigate whether individuals who are *KIR* AA^+ have stronger responses to leukemia than other individuals do, we measured the cytotoxicity against leukemia cells, of NK cells from healthy individuals with and without *KIR AA*. We used standard assays for NK cell activity (30, 31, 35, 36) to study six healthy Chinese Southern Han subjects having the *KIR AA* genotype, and nine without it. NK cells from individuals with the *KIR AA* genotype demonstrated significantly greater cytotoxicity against the HLA class I negative K562 leukemia cell line, a standard target used to measure NK cell cytotoxicity, than NK cells isolated from individuals without the *AA* genotype (mean killing 65 vs. 49%, $p = 0.002$; **Figure 4A**). These data suggest that NK cells from *KIR AA* individuals are better educated to respond to loss of HLA class I expression than are those from individuals carrying other *KIR* genotypes.

Next, the same NK cells were tested for their cytotoxicity against primary leukemic cells, which were isolated from five patients with AML and two with ALL (**Supplemental Figure 3**). Again, NK cells isolated from individuals who have the *KIR AA* genotype had a stronger cytotoxic response than those isolated from non-*AA* individuals (29.9 vs. 20.5%, $p = 0.001$; **Figure 4B**). In line with previous observations on European subjects (53), we observed variability in the baseline killing of leukemia cells across NK cell donors. This may be due to differential education by donor HLA class I allotypes. Although all the donors and patients studied possess the C1 ligand (**Supplemental Figure 3**), the very high number of subjects required precludes from completely controlling for HLA variation. Nevertheless, a higher degree of cytotoxicity was consistently observed for NK cells from *KIR* AA^+ donors as compared to *KIR* AA^- donors regardless of the source of leukemia cell targets ($p < 0.0001$ by paired *t*-test; **Figure 4C**). This result was further validated in an independent set of 18 healthy individuals and a complementary method, which measured the cell surface expression of CD107a that accompanies degranulation of NK cells (35). Here, a mean of 25.4 % NK cells from *KIRAA*$^+$ subjects expressed CD107a following contact with K562 cells, vs. 17.8% from non-*AA* subjects ($p = 0.03$: **Figure 4D**). In summary, these findings show that in Chinese Southern Han, NK cells from individuals with the *KIR AA* genotype exert greater cytotoxicity against leukemic cells than those from individuals having any other *KIR* genotype.

DISCUSSION

KIR are highly polymorphic and the distribution of *KIR* haplotypes differs markedly across human populations (11). Because multiple previous studies were consistent with, but had not identified, a role for the *KIR AA* genotype in protection from leukemia, we studied a cohort of Chinese Southern Han from East Asia, where the *KIR A* haplotype is at highest frequency. We showed that possession of the *KIR AA* genotype protects individuals from developing either of three types of leukemia and one lymphoma analyzed. These results were further refined by considering the HLA ligands for KIR in the analysis. Importantly, *in vitro* functional assays demonstrated that NK cells from healthy individuals with *KIR AA* genotype had strong reactivity against leukemic cells.

Because there have been conflicting reports on the role of KIR in protection from leukemia, we sought to study a large number of patients and controls that were matched by clearly defined age and ethnicity. We studied over 1,000 patients and 745 controls and observed no significant difference in the overall frequency distribution of *HLA class I* alleles between the two groups or compared with previous studies of Chinese Southern Han (40). Because allele frequency spectra of these highly polymorphic genes characterize and distinguish human populations (42), this finding validates the self-defined ancestry of the cohorts as well as their suitability for use in these comparisons. We studied subjects from China, where the mean age for leukemia diagnosis is younger than that seen for Europeans (54–56), and compared them with healthy blood donors having a similar age distribution. The high frequency of *KIR AA* homozygous individuals observed in our control group is consistent with multiple previous studies of healthy Chinese Southern Han (19, 57–59). We also observed a difference in *KIR AA* frequency between AML patients and controls of similar magnitude as that seen in a previous study of Chinese subjects from Singapore (19). One caveat is that we studied patients selected as potential transplantation recipients, but we consider it unlikely that *KIR/HLA* genotype affects this initial decision. Moreover, our finding that possession of the *KIR AA* genotype consistently protects from at least three types of leukemia and one lymphoma was further validated by *in vitro* functional assays of NK cell cytotoxicity and lytic granule release.

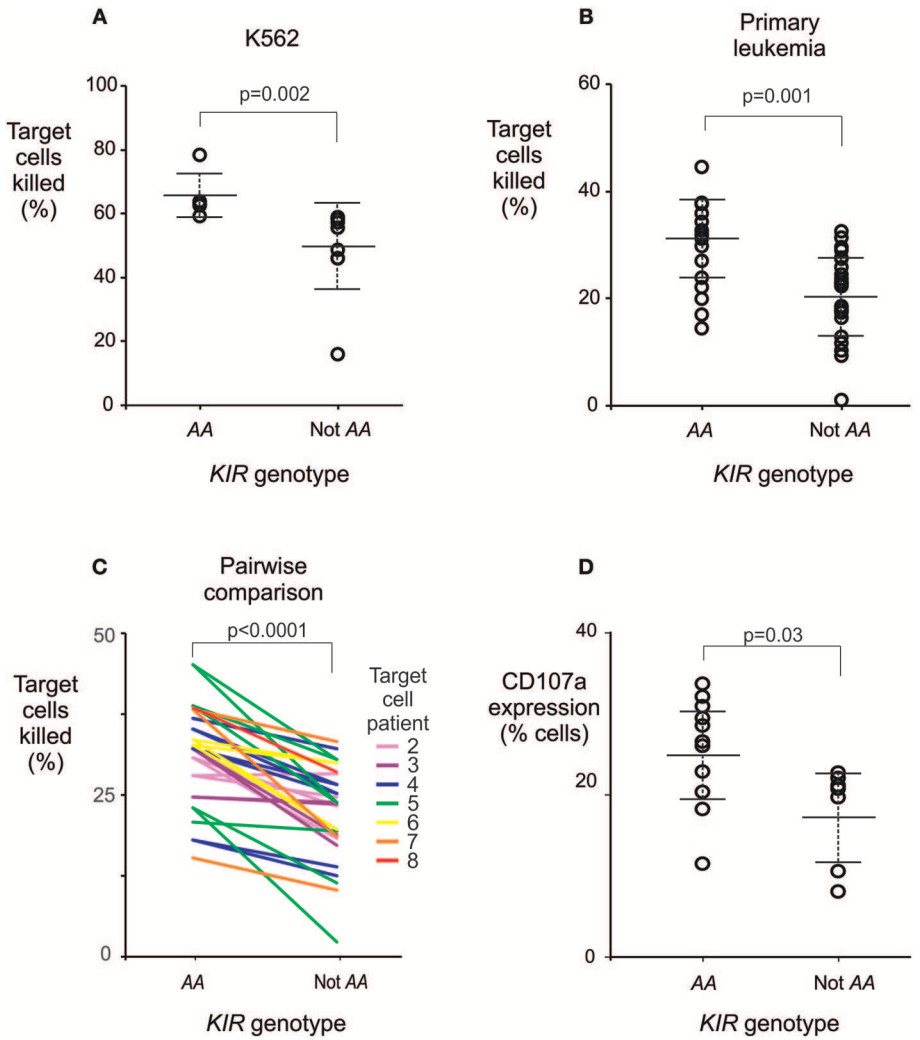


FIGURE 4 | The *KIR AA* genotype correlates with enhanced potential of NK cells to lyse leukemic cells. **(A)** Mean % killing of K562 target cells by NK cells isolated from six healthy individuals with (left) and nine without (right) the *KIR AA* genotype. Each dot represents the mean result from a duplicated assay (**Supplemental Figure 3E**). *P*-value is from a Mann-Whitney *U*-test. **(B)** Mean % killing of primary leukemia cells using the same NK cells as **(A)**. The primary leukemia targets were obtained from two ALL patients and five AML patients (**Supplemental Figure 3**). Each dot represents the mean result from a duplicated assay (**Supplemental Figure 3**). *P*-value is from a Mann-Whitney *U*-test. Six NK cell donors were tested against two targets, seven were tested against three targets, and two against four targets, as detailed in **Supplemental Figure 3**. **(C)** Pairwise comparisons of % killing by NK cells from *KIR AA* vs. non *AA* individuals against identical target leukemia cells. Each line represents one of the seven targets, incubated with NK cells from an AA^+ donor (left) or an AA^- donor (right), at the same time and conditions. Line colors indicate the seven individual targets as given in **Supplemental Figure 3** (Target 1 is K562; not shown for clarity). *P*-value is from a paired *t*-test. **(D)** Shows CD107a expression by NK cells following incubation with K562 targets. The NK cells were isolated from a further 11 healthy individuals with (left) and seven without (right) the *KIR AA* genotype. *P*-value is from a Mann-Whitney *U*-test. Error bars are mean ± sd. The NK killing assays are described in **Supplemental Figure 3**.

Our results suggest that any contradictory findings in the association of KIR/HLA and leukemia that have been reported are likely to be driven by the relative population frequencies of the *KIR A* haplotypes and the KIR allotypes they encode. Many previous studies are concordant with the results presented here (14, 15, 17–19, 60), while others that differ could be explained by cryptic differences in ancestries of patients and controls, resulting in differences in *KIR A* haplotype frequencies, and the alleles present on those haplotypes (11, 26, 61–65). We hypothesize that the strongly inhibitory KIR allotypes that characterize East Asian *KIR* haplotypes efficiently educate NK cells to detect changes in HLA expression that can occur in leukemic cells (20, 44). These changes may include loss or reduction of HLA expression, or alterations to the peptide repertoire that may be detected by NK cells through KIR binding (5, 51). That the same strongly-inhibiting KIR allotypes are present in other populations, albeit to lower frequencies (11), suggests their effect on leukemia control will be similar, but much larger sample sizes will be required to detect them. We studied a population with relatively low genetic diversity, such that possession of the *KIR A* haplotype is accompanied by a uniform set of alleles of the component genes. High resolution analyses will likely be required to account for the substantial allelic diversity of any further population groups analyzed (66).

Although the *KIR AA* genotype appears to protect against leukemia and NHL in Chinese Southern Hans, there were differences in the HLA ligand required to strengthen this effect. These differences may be due to the distinct maturation stages and the non-overlapping subsets of lineage markers and fusion proteins expressed by the leukemia subtypes (67, 68). The clearest ligand effect was observed for CML, which is likely due to the relative uniformity of this leukemia class (69). The least defined ligand effect was observed for AML, likely due to the highly heterogeneous nature of this disease (70). Another ligand for inhibitory NK cell receptors is HLA-E, which can present peptide fragments derived from the expression of HLA-A, -B or -C. CD94/NKG2A educates and modulates NK cells through binding HLA-E, complementing the role of KIR and extending the capacity of NK cells to monitor HLA class I expression (41, 71). Although CD94/NKG2A is conserved, diversity across individuals occurs because only some HLA-B alleles encode the −21M variant peptide that binds to HLA-E. Accordingly, a recent study showed −21M HLA-B associated with increased survival for AML patients receiving immune therapy (72). However, as we observed no significant differences in HLA allele frequencies (**Supplemental Figure 2**), there were no significant differences in frequency of the HLA-B −21M variant between the patients and controls of our study. This indicates that HLA-B −21M polymorphism is unlikely to affect leukemia susceptibility in Chinese Southern Han.

Although the endpoint of our analysis was disease diagnosis, a limitation is that we were not able to perform functional tests of NK cells extracted from leukemia patients, or phenotypic analyses of HLA expression by leukemic cells. The exception is the K562 target, which is a leukemia cell known to lack HLA class I expression. Another limitation is that the relatively low sample numbers we obtained for the functional tests did not allow for thorough analysis of KIR ligand mismatches between NK cell donor and leukemia target cells. It will be important in future work to examine how changes in HLA expression or neo-antigen peptide presentation may affect the NK cell response to cells that could be leukemogenic, and ultimately how they may influence disease course. These kinds of experiments require pre-genotyping of large numbers of individuals, and will be greatly facilitated by involving populations where the respective genotypes are at high frequency, including the Chinese Southern Han.

In conclusion, this study enhances our understanding of the immune response to leukemia in the world's largest population group, shows the benefit of including specific well-defined populations in study of diseases involving highly polymorphic genes, and identifies a role for NK cell education by inhibitory KIR in control of leukemia. In light of recent studies that promote expansion beyond the usual European populations analyzed (73), the benefits of studying well-defined population groups is becoming increasingly apparent.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

The study was approved by the ethics review board of Shenzhen Blood Center, Shenzhen, Guangdong, China. Written informed consent was obtained from all study participants.

AUTHOR CONTRIBUTIONS

ZD, SC, JZha, WH, MC, and PN: experiment and analysis design. ZD, YQ, RC, JZhu, JZhe, MZ, GZ, LH, HZ, LL, and WZ: data generation and interpretation. ZD, YQ, QY, MM, XG, and PN: Data analysis. ZD, JZha, XG, MM, MC, and PN: Manuscript preparation. All authors: manuscript review and approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01646/full#supplementary-material

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REVIEW
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ILC2s: New Actors in Tumor Immunity

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

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

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INTRODUCTION

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

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

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

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

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

ILC2s IN HEMATOLOGICAL MALIGNANCIES

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

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

ILC2s IN UROGENITAL TRACT CANCERS

ILC2s in Prostate Cancer

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

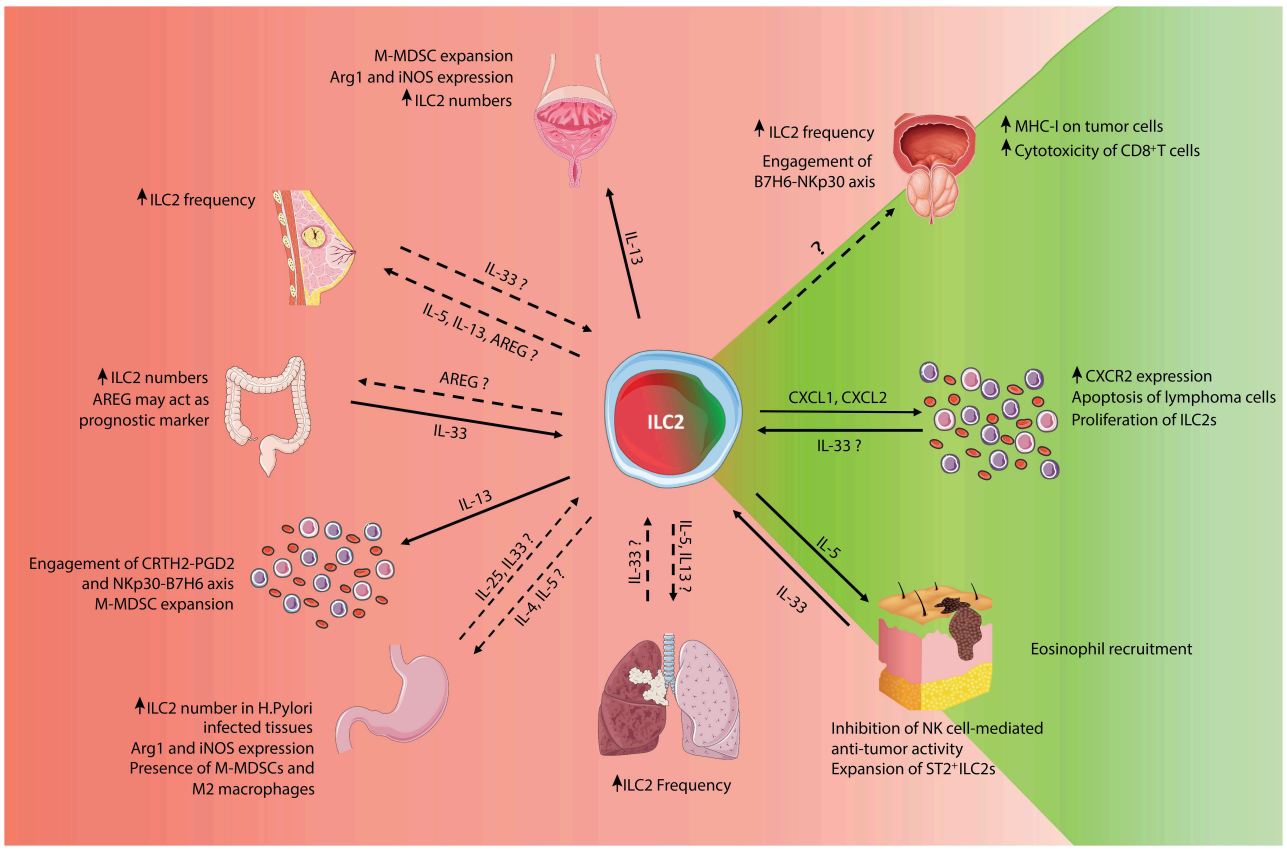

FIGURE 1 | Schematic representation of pro- and anti-tumor roles of ILC2s. Summary of the known pro- and anti-tumor roles of ILC2s, classified by tumor types. For bibliographic details refer to the work cited in the main manuscript.

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

ILC2s in Bladder Cancer

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

ILC2s IN CANCERS OF THE GASTROINTESTINAL SYSTEM

ILC2s in Colorectal Cancer

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

ILC2s in Gastric Cancer

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

ILC2s IN BREAST CANCER

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

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

ILC2s IN MELANOMA

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

ILC2s IN LUNG CANCER

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

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

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

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

AUTHOR CONTRIBUTIONS

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

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REVIEW
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The Interplay Between Innate Lymphoid Cells and the Tumor Microenvironment

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The multifaceted roles of Innate Lymphoid Cells (ILC) have been widely interrogated in tumor immunity. Whereas, Natural Killer (NK) cells possess undisputable tumor-suppressive properties across multiple types of cancer, the other ILC family members can either promote or inhibit tumor growth depending on the environmental conditions. The differential effects of ILCs on tumor outcome have been attributed to the high degree of heterogeneity and plasticity within the ILC family members. However, it is now becoming clear that ILCs responses are shaped by their dynamic crosstalk with the different components of the tumor microenvironment (TME). In this review, we will give insights into the molecular and cellular players of the ILCs-TME interactions and we will discuss how we can use this knowledge to successfully harness the activity of ILCs for anticancer therapies.

Keywords: innate lymphoid cells, tumor microenvironment, crosstalk, immune evasion, immune modulation

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INTRODUCTION

In the past years, Innate Lymphoid Cells (ILCs) have emerged as crucial players in cancer growth and therapy. The ILC family members are classified into five groups namely Natural Killer (NK) cells, group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), group 3 ILCs (ILC3s), and lymphoid-tissue inducer cells (LTis) (1, 2). Initially described in the 1970s, NK cells are the founding members of the ILCs family (3). They develop in the bone marrow from common lymphoid progenitors and follow a sequential maturation and differentiation process, which is regulated by a variety of transcription factors (4). The T-box protein 21 (T-BET) and Eomesodermin (EOMES), for instance, undertake non-redundant roles in this process through stabilizing distinct NK cell subsets during maturation (5). Once in tissues, NK cells are potentially capable of eliminating infected or transformed cells via several mechanisms including degranulation, death receptor ligation, or the production of inflammatory cytokines (e.g., IFNγ, TNFα) (6). The latter feature is shared with another member of the family, the ILC1s, which are phenotypically very similar to NK cells (7, 8). However, the two lineages have been shown to diverge early in ontogeny and to differ in terms of cytotoxic machinery and tropic properties (9). Thus, ILC1s are typically defined as tissue resident since they are not found in the blood or lymphoid tissues, but rather in organs such as the gut, the liver, the salivary glands or the reproductive tract (9). In the liver, for example, T-bet dependent ILC1s have been shown to contribute to immune responses against haptens and viral antigens (10). In contrast, the intestinal ILC1 subset rather controls microbial pathogens and contributes to chronic inflammation (11).

ILC2s are characterized by their ability to produce Th2 cytokines (IL-4, IL-5, IL-9, or IL-13) and therefore contribute to type 2 inflammation promoting pathological responses associated to asthma

or allergy, but also conferring protection against helminths (12). ILC2s require the transcription factor GATA-3 for their development (13). In the mucosal tissues, where they typically reside, ILC2s can be activated by epithelial-derived alarmins (IL-33, IL-25, or TLSP), whose contribution to ILC2 activation depends on the tissue type as well as on the nature and magnitude of the pathological insult (14). Thus, whereas IL-33 is believed to play a crucial role in the context of allergic airway inflammation, IL-25 is particularly relevant for the amplification of type 2 immune responses in the gut (15, 16).

ILC3s comprise a heterogenous and plastic population. They are divided into two subsets based on the expression of the natural cytotoxicity receptor (NCR) NKp46 in mice and Nkp44 in humans namely NCR^+ILC3s and NCR^-ILC3s (17). Both subsets require the transcription factor RORγt for their development and represent a major source of the cytokine IL-22 (18), which regulates interactions with the commensal flora and controls mucosal infections at barrier sites (19). ILC3s can also take proinflammatory roles through the release of IL-17 or IFNγ, contributing to the progression of psoriasis and colitis, respectively (20, 21). The production of abundant amounts of IL-17 and IL-22 is also a defining feature of LTis, which functionally resemble the population of NCR^-ILC3s (22). LTis, however, are derived from a developmental pathway starting in the embryo (23), where they engage in the formation of lymphoid tissues through the production of lymphotoxins (24).

The properties of ILCs have been widely investigated in the context of tumorigenesis. Due to their high cytotoxic capacity, NK cells are particularly suitable to eliminate tumor cells. Indeed, several preclinical studies have revealed a central role for NK cells in tumor control, especially in metastatic disease (25–30). ILC2s and ILC3s can also modulate antitumor responses, but their role rather depends on the environmental cues they encounter in their resident tissue. Thus, whereas IL-12-stimulated NCR^+ILC3s were found to control primary melanoma growth (31), the growth of this tumor type in the lungs is modulated by an IL-5-producing subset of ILC2s (32). This contrasts with the protumorigenic role of ILCs described for other tumor models. For example, IL-13-producing ILC2s promote tumor growth in leukemia and breast cancer (33, 34), and IL-22-producing ILC3s do likewise in the gut (35, 36). Finally, the recruitment of RORγt$^+$ILC3s to tumors mediated by CCL21 was able to promote lymph node metastasis by modulating the local chemokine milieu in the TME (37).

With the growing interest in harnessing ILC responses for immunotherapeutic strategies against cancer, it is important to better understand the multifaceted roles of ILCs in tumor development. Here, we will first discuss how ILCs migrate and expand in the tumor site. Further, we will review current knowledge on how ILCs communicate with the environment, including the interactions they establish with the tumor cells and the different components of the TME. Finally, we will discuss whether these interactions are beneficial or deleterious to tumor growth and invasion.

ILCs IN THE TUMOR SITE: MIGRATION vs. LOCAL EXPANSION

Parabiosis studies have shown that NK cells are a highly mobile subset that constantly circulate throughout the bloodstream and the lymphatic system, whereas the rest of the ILC family members are defined as tissue resident cells (38–40). Confirming the maintenance by local self-renewal within tissues, only very small numbers of ILCs can be found in healthy human and mouse circulation (41, 42). It has been shown that some ILC progenitors express the integrin α4β7 and the chemokine receptors CCR7, CCR9, or CXCR6, which may enable them to migrate to peripheral and lymphoid tissues (42–44). Also mature ILCs express several tissue homing receptors such as CXCR6, which promotes the accumulation of ILC3s in the gut (45) and provides survival signals to maintain ILC1s within the hepatic niche (46). Other markers of tissue residency for ILCs are the integrin CD49a and the early activation marker CD69, which are upregulated during ILC activation (47–49).

Within the TME, NK cells represent by far the most abundant innate lymphocyte subset identified (48). However, despite correlating with a better prognosis, NK cell homing is highly inefficient in most solid tumors (50, 51). There are a few exceptions including clear cell renal carcinoma, which harbors unusually high numbers of intratumoral NK cells (52, 53). The mechanisms leading to NK cell paucity in the TME are not well-studied, but what it is by now clear is that the majority of NK cells infiltrating tumor tissue belong to the mouse $CD27^{high}$ and the human $CD56^{bright}$ subsets, which are recruited to the tumor in a CXCR3-dependent manner (54, 55). Even though the immature $CD56^{bright}$ NK cell population has been traditionally considered as a "cytokine producer," whether it can control tumors as efficiently as the mature $CD56^{dim}$ population is still a matter of debate. Due to their high motility, NK cells can also be recruited in strategic locations in order to prevent further cancer spread. As such, highly cytotoxic populations of NK cells from both $CD56^{dim}$ and $CD56^{bright}$ subsets have been found in tumor-draining lymph nodes of melanoma patients (56, 57). On the other hand, immunosuppressive mediators such as TGFβ might favor the retention of NK cells in the bone marrow through the upregulation of CXCR4 and the downregulation of CX3CR1 (58).

Despite their residency properties, a few ILCs have been reported to circulate in human blood. Thus, increased frequencies of ILC1s and ILC2s were found in patients with colorectal cancer (59) and with gastric cancer, respectively (60, 61). RORγt$^+$ILC3s were also reported to migrate via the bloodstream toward the tumor site in response to CCL21 in mouse models of breast cancer (37) and in melanoma (62). Within the TME, ILC subsets other than NK cells are only found at extremely scarce numbers. In human lung cancer, a NCR^+ population of ILC3s was found to accumulate at the edge of lymphoid structures in the tumor tissue (37, 62, 63). An enrichment of ILCs in tumors compared to healthy tissue has also been observed for ILC1s in gastrointestinal tumors (49) or ILC2s in gastric, breast and prostate cancer (34, 49, 60). Despite the presence of ILCs in the above-mentioned types of cancer, whether

they contribute to the underlying pathology in humans is still a matter of debate. Also, whether the enrichment of ILCs results from newly recruited cells or from local *in situ* proliferation has not been thoroughly investigated. The latter phenomenon was however observed for ILC2s in IL-33-treated breast cancer (33), and for ILC1s in mouse mammary pre-cancerous lesions (64).

THE BIDIRECTIONAL CROSSTALK BETWEEN ILCs AND TUMOR CELLS: RECOGNITION vs. IMMUNE EVASION

From all the ILC family members, NK cells show the highest cytolytic activity, while the primary role of other ILCs is to produce cytokines in response to different stimuli. In order to eliminate transformed cells, NK cells are equipped with a plethora of activating and inhibitory receptors, which need to be tightly regulated to determine whether a target cell will be killed or spared (65). Once activated, NK cells eliminate target cells via death receptors pathways (e.g., Fas/FasL) or through the release of cytotoxic granules at the immunological synapse (66). The usage of these two cytotoxic pathways appears to be tightly regulated. As such, whereas NK cells use the fast granule-mediated pathways for their first killing events, they switch to death receptors-mediated killing during the last encounters with the tumor cells (67). Despite possessing such an efficient cytotoxic machinery, NK cells from tumor-bearing mice or cancer patients are often functionally impaired and display low amounts of effector molecules such as granzyme B, IFNγ, or FasL (68). This is mostly due to the signals these cells receive from the TME, and especially from the surrounding tumor cells.

Within the TME, tumor cells are constantly exposed to stress conditions, which induce the upregulation of ligands for NK cell activating receptors (69). Although *a priori* this would favor NK cell-mediated immune surveillance, cancer cells have developed several mechanisms that allow them to evade immune recognition. Among those, we highlight the dysregulation of ligands that bind NKG2D, a major NK cell activating receptor critical for antitumor immunity (70). A commonly proposed mechanism for evading NK cell surveillance has to do with the shedding of the NKG2D ligands MICA and MICB from the cell membrane, leading to soluble forms that promote the internalization and posterior degradation of the receptor (71–73). This was however challenged in a study performed in murine tumor models, which reported that the soluble high affinity NKG2D ligand MULT-1 actually caused NK cell activation and tumor rejection (74). Irrespective of whether NKG2D ligands are soluble or membrane-bound, what is clear by now is that it is their chronic engagement which causes the desensitization of the NK cell receptor as well as related signaling pathways (75). Moreover, although tumor cells represent the main source of ligands for activating receptors, the induction of NKG2D ligands on myeloid cells and endothelial cells has also been shown to contribute to impaired NK antitumor responses (76, 77). Finally, other ILC family members such as intestinal ILC1s and ILC3s can also express NKG2D on the cell surface (78). Whether this receptor is able to modulate the activity of these cells in the TME is however not known.

Besides desensitizing NKG2D, tumor cells use additional mechanisms to evade NK cell surveillance including the secretion of immunosuppressive molecules such as TGFβ, IL-10, prostaglandin E2 (PGE2) or indoleamine 2,3-dioxygense (IDO) (79, 80). The production of these factors is not restricted to cancer cells, and a variety of cell types populating the TME can also contribute to the immunosuppressive pool leading to impaired NK cell function. Nevertheless, TGFβ and PGE2 are able to shape NK cell activity directly via the inhibition of activating receptors (79–81), or indirectly through the recruitment of immunosuppressive cells types such as myeloid-derived suppressor cells (MDSCs) or regulatory T cells (Tregs) (82, 83).

ILCs have a remarkable plasticity allowing them to acquire features of another ILC population in order to adapt to changes in the tissue microenvironment. In tumors, ILC plasticity was suggested as a mechanism by Gao et al., who reported a TGFβ-dependent conversion of NK cells into "ILC1-like" cells in a mouse model of chemically induced sarcoma (84). This conversion, which is characterized by the upregulation of the integrin CD49a and the downregulation of Eomes, appears to be detrimental for tumor control (84). A similar CD49a^{high} ILC1-derived subset with a tissue-residency phenotype was however found to exert cytotoxicity in oncogene-induced murine tumor models (64). Given the overlapping phenotypes between NK cells and ILC1s (85), it is difficult to postulate whether one subset really converts into the other or if cells rather evolve on a continuum. A complete transition seems unlikely, since ILC1s and NK cells lineages are believed to separate early during the differentiation process (78, 86).

The dependence of NK cell into ILC1 conversion on TGFβ supports increasing evidence that this cytokine does not only induce NK cell dysfunction, but also plays a crucial role in regulating ILC plasticity. Interestingly, TGFβ-imprinting is essential for the differentiation of the ILC1s residing in murine salivary glands via suppression of Eomes and the upregulation of CD49a (87). In humans, TGFβ was also described to enable the transition between mature CD16^{+} peripheral blood NK cells into a CD16^{-}CD9^{+} phenotype that resembles a population of decidual NK cells (88). But TGFβ is not the sole factor reported to induce ILC plasticity. In fact, the proinflammatory cytokine IL-12 was shown to induce the differentiation of ILC2s into IFNγ-producing ILC1s, a process that was reversed by IL-4 (89, 90). Further, IL-12 mediates the conversion of ILC3s into type 1-like ILCs in a variety of pathological conditions (31, 91–93). The so-called "ex-ILC3s" were found to display cytotoxic activity in humans (93) and to effectively suppress tumor growth in a mouse model of melanoma (31). In the context of intestinal inflammation, the ILC3 to ILC1 plasticity was reversible in the presence of IL-23 (92). Together, these results reinforce the notion that ILCs are highly plastic cells which fine-tune immune responses to adapt to the changing environment.

The wide number of events that take place in the TME to evade ILC surveillance have been summarized in **Figure 1**.

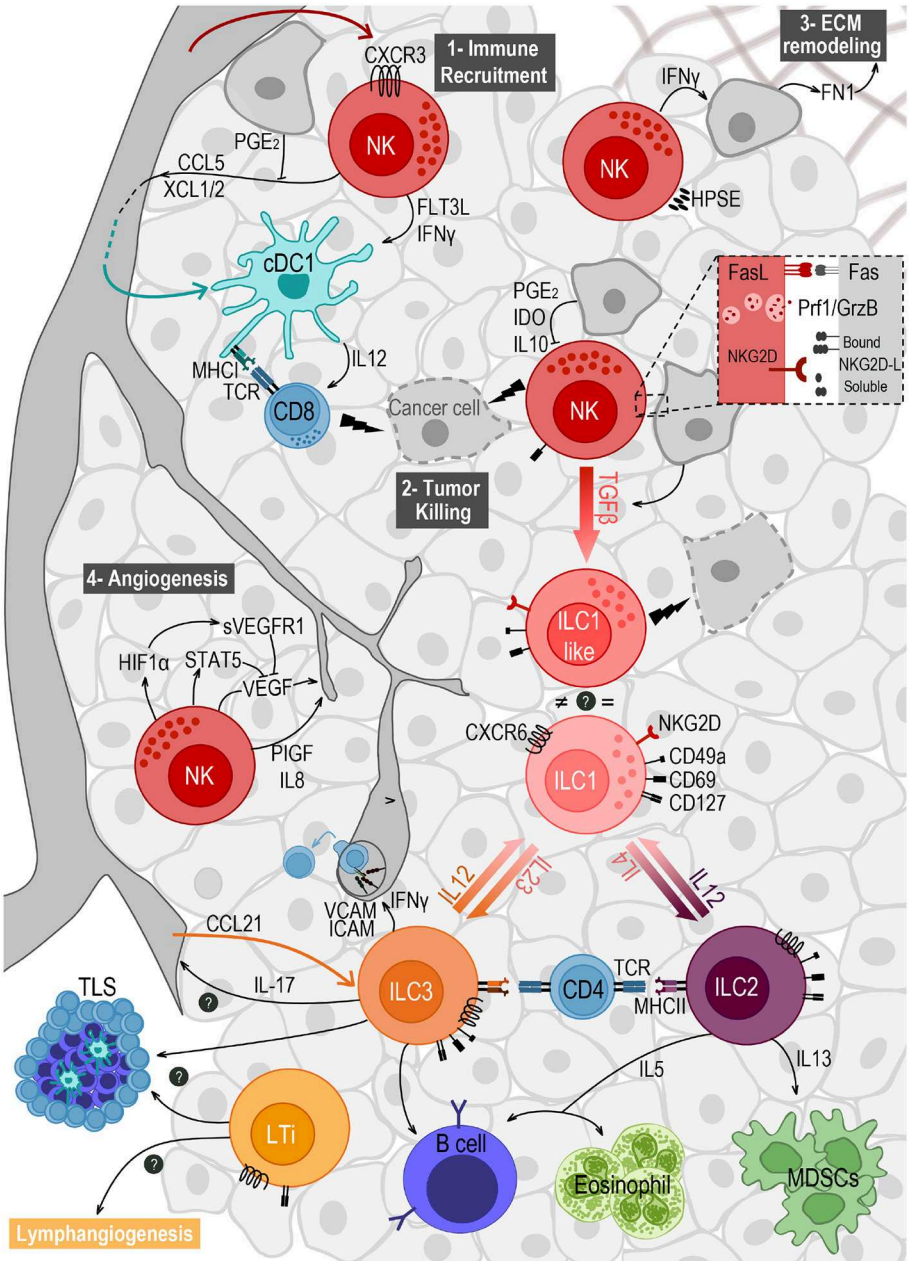

FIGURE 1 | Crosstalk of ILCs and the different components of the tumor microenvironment. NK cells are the main ILC subset found in the TME, after migrating from the blood in a CXCR3-dependent way. They play important roles in (1) immune recruitment, (2) tumor cell killing, (3) extracellular matrix (ECM) remodeling, and (4) angiogenesis. (1) NK cells recruit cDC1s to the TME by secreting CCL5 and XCL1/2, and promote their survival and maturation through FLT3L and IFNγ. (2) NK cell mediated killing is mostly achieved by the engagement of death receptors (e.g., Fas/FasL) and by the release of cytotoxic granules containing perforin (Prf1) and granzymes (GrzB). This response can be triggered by the expression of stress markers on tumor cells, recognized by the receptor NKG2D. Ligands to this receptor (NKG2D-L) are membrane-bound but can also be shed and released in soluble form. (3) NK cells express the enzyme heparanase (HPSE) to degrade heparin sulfate proteoglycans, allowing them to migrate in the tumor tissue. NK cells' secretion of IFNγ induces the production of fibronectin 1 (FN1) by tumor cells, further remodeling the ECM. (4) NK cells modulate angiogenesis by releasing VEGF, PIGF and IL-8. VEGF secretion can be repressed by STAT5 or by soluble VEGF receptor (sVEGFR1) induced as a response to hypoxia. Tumor cells' secretion of TGFβ induces the conversion of NK cells into ILC1-like cells expressing CD49a and CD69, and exhibiting anti-tumor cytotoxic activity. Plasticity within the ILC family also includes the conversion from ILC2 to ILC1, and from ILC3 to ILC1, both induced by IL-12 and reversed by IL-4 and IL-23, respectively. ILC2s have a pro-tumorigenic role via secretion of IL-13 driving the expansion of myeloid-derived suppressor cells (MDSCs), and an anti-tumorigenic role through IL-5 mediated recruitment of eosinophils and activation of B cells. ILC3 can also stimulate antibody production by B cells, and can favor leukocyte recruitment to the TME when sensing IL-12, through IFNγ-mediated upregulation of ICAM-1 and VCAM-1. They may also play a role in angiogenesis through the production of IL-17. NCR^{+}ILC3s and LTis accumulate in tertiary lymphoid structures (TLS) and may also promote lymphangiogenesis. Finally, both ILC2 and ILC3 express MHC class II and were able to prime CD4 T cells *in vitro*.

ILCs AS MODULATORS OF VASCULAR REMODELING

Angiogenesis, the formation of new blood vessels from pre-existing ones, is needed to satisfy the increasing demand of oxygen and nutrients of the growing tumor. This process is supported by several immune cell types via the production of pro-angiogenic growth factors (94, 95). NK cells, for instance, were the first ILC subset reported to modulate tumor vascularization. Hence, a population of $CD56^{bright}CD16^{-}$ NK cells was shown to produce high amounts of the proangiogenic molecules VEGF, PlGF, or IL-8, leading to the formation of capillary-like structure in patients with NSCLC, melanoma, breast and colon carcinoma (96, 97). Interestingly, this population is reminiscent of a $CD56^{bright}CD16^{-}$ subset of decidual NK cells, which may be involved in the tissue remodeling process associated with angiogenesis during embryonic development (98).

NK cells are able to regulate the production of the proangiogenic factor VEGF through various mechanisms. Firstly, the expression of VEGF can be repressed by the transcription STAT-5, leading to inhibition of angiogenesis and tumor growth (99). Since STAT-5 is required for NK cell cytotoxicity, it was proposed that cytokines that signal through this transcription factor (e.g., IL-2 and IL-15) may regulate tumor growth by promoting the conversion from angiogenic to cytotoxic NK cells (99). NK cells can also regulate their own production of VEGF when adapting to hypoxia (100). Thus, the induction of HIF-1α on NK cells induces the upregulation of the soluble receptor VEGFR-1 (sVEGFR-1), which sequesters VEGF leading to the formation of more functional vessels that induce tumor growth (100). Further, ILCs can induce changes in the tumor vasculature through the modulation of adhesion molecules (101, 102). A tumor-suppressive subset of IL-12-driven $NKp46^{+}$ ILC3s promoted leukocyte recruitment through the induction of the adhesion molecules ICAM and VCAM on the tumor vessels (102), similarly to what was observed by tumor-infiltrating NCR^{+}ILC3s in NSCLC tissues (63). ILC3s producing IL-17 may also play a role in regulating the tumor vasculature. IL-17 induces blood vessel permeability in pulmonary endothelial cells, thus leading to metastatic growth (103). Further, IL-17 signals through stromal cells to induce a variety of proangiogenic factors (e.g., VEGF, TGFβ, or IL-8) (104, 105). Finally LTis may also play a role in promoting lymphatic vessel growth, which actively participates in metastatic tumor dissemination (106). LTis interact with Mesenchymal Stem Cells (MSCs), which produce pro-lymphoangiogenic factors such as VEGF-C (107). Although the LTi-MSC crosstalk has been proposed to mediate lymph node metastasis in breast cancer (37), the involvement of the lymphatic vasculature in this setting remains unknown at this time.

Not only the vasculature, but also the extracellular matrix (ECM) is modified during the course of cancer progression. The ECM is a complex network of proteoglycans and fibrous proteins that support the surrounding cells and provide molecular cues for cell migration and differentiation (108). During cancer progression, the deregulation of the ECM promotes invasion, angiogenesis and facilitates immune cell infiltration (109). It has been shown that NK cells can modulate the ECM through the secretion of fibronectin 1 (FN1), leading to structural changes in the primary tumor and decreased metastasis (110). In addition, NK cells can facilitate their own migration through the ECM thanks to the expression of heparanase, an enzyme known to degrade heparin sulfate proteoglycans (HSPGs) (111). This raises concerns about the use of EMC inhibitors to block tumor cell invasion, since it may be detrimental for a proper migration of NK cells and possibly other subsets of immune cells.

Taken together, these reports highlight the importance of ILCs in modulating the tumor vasculature and the remodeling of the ECM (**Figure 1**), which could be exploited for immunotherapeutic purposes. Further work will have to address specific contributions of the distinct ILC subsets to this process. For instance, whether and how ILC2-signature cytokines regulate the angiogenic process has yet to be studied.

ILCs INTERACT WITH A BROAD SPECTRUM OF IMMUNE CELLS WITHIN THE TME

ILCs establish continuous interactions with a wide variety of cells within the TME. As such, understanding the nature of this crosstalk is crucial to unleash the full potential of ILC responses against developing tumors. Defined NK cell interactions in the cancer context include the interplay with DCs, the main sentinels of the innate immune system (112, 113). DCs can support NK cell responses through the secretion of several proinflammatory cytokines (IL-12, IL-15, IL-18, and Type I Interferon) (114). NK cells can in turn trigger DC maturation via the production of IFNs and Tumor Necrosis Factor (TNF) (115, 116). Within the TME, NK cells promote the recruitment of cDC1s, the DC subset capable of priming tumor-specific CD8 T cells (117). This is mediated through the secretion of CCL5 and XCL1/2 by intratumoral NK cells, and antagonized by PGE2 produced by the tumor cells (113). Apart from promoting the recruitment of DCs, NK cells can also prime and ensure their expansion. Thus, NK cells activated by MHC Class I low tumor cells can prime DCs to produce IL-12 and to induce protective CD8 T cell responses (118). Further, they appear to be the main source of the cytokine fms-like tyrosine kinase 3 ligand (FLT3L), a survival factor for DCs (112, 119). In contrast, the use of less immunogenic tumor cells led to the inhibition of DC activation by NK cells, which was mediated by the (TNF)-related apoptosis-inducing ligand (TRAIL) (120). This controversy was not observed in the human disease, where a high expression of NK cell and cDC1 signatures correlated with better prognosis and response to immunotherapy in a wide array of cancers (112, 113).

NK cells are not the only ILC subset that is able to shape myeloid cell responses. ILC2s, for instance, have been shown to either limit anti-tumor responses by triggering the expansion of MDSCs via secretion of IL-13 (34), or to enhance anti-cancer immunity by cooperating with DCs or eosinophils in the lung in a IL-5-dependent manner (32, 121). Also the

crosstalk between ILC1s, ILC3s and myeloid cells has been shown to promote chronic inflammation leading to tumor initiation. First, ILC1s and "ex-ILC3s" have been found to accumulate in chronically inflamed guts in response to myeloid-derived cytokines such as IL-12 or IL-15 (47, 91). In this scenario, these two ILC subsets secrete high amounts of IFNγ, which engages neutrophils and macrophages to cause tissue injury (47, 122). Further, group 3 ILCs are particularly responsive to IL-23, a key pathogenic inducer of chronic intestinal inflammation (123). IL-23, which is primarily produced by cells of the myeloid lineage, induce the production of GM-CSF, IL-17 or IL-22 by ILC3s (20, 124, 125). Whereas GM-CSF feeds back on the myeloid cells to promote tissue damage and colitis (20, 125), IL-17 and IL-22 limit inflammation by maintaining the integrity of the epithelial barrier (126, 127). This contrasts with the protumorigenic role that both IL-22 and IL-17 exert in colorectal cancer (105, 128), where they were shown to have pro-proliferative and pro-angiogenic functions, respectively (105, 128).

In humans, the levels of IL-17 appear to be upregulated in colorectal cancer patients, where they associate to poor prognosis (35, 129, 130). Further studies will be required to determine the contribution of ILC3s to the total pool of IL-17 or IL-22-producing cells. Nevertheless, blocking the ILC-myeloid axis in tumors of intestinal origin arises as a promising approach and may represent a promising anti-cancer therapeutic strategy.

ILCs may also directly modulate the quality of T cell responses without prior DC crosstalk. NK cell-secreted IFNγ, for example, was shown to promote Th1 polarization in the lymph nodes in mouse models of infection (131, 132). A close cooperation between NK cells and T cells has also been shown in established lung carcinoma, where the stimulation of NK cells induced the recruitment of highly active T cells, leading to a more efficient tumor control (133). Also the engagement of NK cells with the TNF superfamily member LIGHT was found to trigger CD8 T responses at the tumor site (134). ILC2s can also modulate adaptive immune responses. The IL-33/ST2 signaling pathway, which drive ILC2 activation, shape an immunosuppressive microenvironment during intestinal tumorigenesis dominated by regulatory T cells (Tregs) (135). A possible mechanisms by which ILC2s might shape the Treg phenotype is through production of AREG, an EGF-like growth factor that enhances regulatory T cell functions (136), or via the production of Arginase 1 (Arg1), which inhibits T cell activation (137).

Upon activation, both ILC2s and ILC3s were able to upregulate the expression of MHCII molecules (138–142). In the case of ILC3s, this was accompanied by high levels of costimulatory molecules and the capacity to process antigens, thus promoting *in vitro* CD4 T cell priming (141). Whether this priming can also take place in the tumor setting is currently now known. Interestingly enough, the population of NCR^+ILC3s described by Carrega et al. in human tumors was found to be located at the edge of tertiary lymphoid structures, an ectopic hub for acquired immune responses (63). Not only T cells, but also B cell responses can be regulated by ILCs. ILC2s, for instance, can modulate B cell function and antibody production through the production of IL-5 and expression of CD40 ligands (143, 144). Also, a population of splenic $ROR\gamma t^+$ ILC3s located in the marginal zone was shown to help B cells for antibody production (145).

Collectively, the above-mentioned studies demonstrate that ILCs are poised to interact with other immune cells within the TME, and thereby modulate both innate and adaptive immune responses against tumors (summarized in **Figure 1**).

CONCLUDING REMARKS

Manipulating ILC responses has emerged as an attractive therapeutic strategy against cancer. In principle, NK cells are the best-suited members of the ILC family to exploit for therapeutic purposes, due to their indisputable cytotoxic properties. In the past years, however, other ILC subsets endowed with either pro- or anti-tumor activity have gained increasing attention as important modulators of the TME. Irrespective of the subset, ILC therapies face a major obstacle, namely the poor capacity to infiltrate and to survive in the hostile tumor bed (50, 51). To date, we still miss a complete picture of the mechanisms regulating ILC migration into tumors. This would be of high interest not only to enhance the number of endogenous ILCs that reach the tumor, but also to increase the efficacy of cell transfer therapies. The latter includes, for instance, the utilization of NK cells engineered to express chimeric antigen receptor (CAR) specific for tumor antigens, which arises as a safe off-the-shelf therapy against refractory malignancies (146).

Once ILCs reach the tumor site, they encounter a hostile microenvironment, which imposes several limitations to dampen the activity of ILCs. Although tumor cells are the key drivers of NK cell dysfunction, other immunosuppressive cells populating the TME can significantly contribute to this process. The TME is characterized by a high degree of intra- and inter-tumor heterogeneity, which challenges the identification of targetable factors aimed at restoring tumor surveillance by ILCs. Nevertheless, a better understanding of the mechanisms that ILCs utilize to communicate with the TME will be key to effectively manipulate these cells for site-specific anticancer therapies. This can be achieved using multi-omics approaches that allow for the integration of data from diverse platforms, including single-cell transcriptomics, cytometry by time-of-flight (CyTOF) or multiplexed tissue imaging. A detailed and personalized multi-omics profile of the TME will be crucial for the design of novel approaches for cancer immunotherapy in the era of precision medicine.

It is important to point out that most of the studies directed to investigate ILCs-TME interactions relies on the use of non-specific strategies to deplete ILC subsets (e.g., blocking antibodies against asialo GM1, CD25, or CD90.2). Although this is being gradually substituted by genetic tools that allow for selective ablation and mapping of ILCs (147), the high plasticity observed within the different ILC family members complicates our ability

to track and target these cells in the TME. The matter is further complicated for ILC1s, due to the lack of ILC1-knockout mice or antibodies that specifically deplete this ILC population. This calls for caution when interpreting the effects of the ILC1 population on both physiological and pathological conditions.

Clearly, as we only now start to understand the complex biology of the ILC family members, it is time to study the power of these cells not only from the direct effects they exert on cancer cells, but also from their ability to communicate with the TME. This will provide valuable insights into how to effectively manipulate ILCs for immune-mediated anticancer therapies.

AUTHOR CONTRIBUTIONS

All authors participated in the intellectual conception of the review, drafts, and final approval of the manuscript.

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MINI REVIEW
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Innate Lymphoid Cells in Colorectal Cancers: A Double-Edged Sword

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The immune system plays a fundamental role at mucosal barriers in maintaining tissue homeostasis. This is particularly true for the gut where cells are flooded with microbial-derived signals and antigens, which constantly challenge the integrity of the intestinal barrier. Multiple immune cell populations equipped with both pro- and anti-inflammatory functions reside in the gut tissue and these cells tightly regulate intestinal health and functions. Dysregulation of this finely tuned system can progressively lead to autoimmune disease and inflammation-driven carcinogenesis. Over the last decade, the contribution of the adaptive immune system in controlling colorectal cancer has been studied in detail, but the role of the innate system, particularly innate lymphoid cells (ILCs), have been largely overlooked. By sensing their microenvironment, ILCs are essential in supporting gut epithelium repair and controling bacterial- and helminth-mediated intestinal infections, highlighting their important role in maintaining tissue integrity. Accumulating evidence also suggests that they may play an important role in carcinogenesis including intestinal cancers. In this review, we will explore the current knowledge about the pro- and anti-tumor functions of ILCs in colorectal cancer.

Keywords: tumor immunology, adaptive immunity, innate immunity, tumor immunosurveillance, immunotherapy

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INTRODUCTION

Colorectal Cancer—Epidemiology, Pathophysiology, and Risk Factors

Colorectal cancer (CRC) is the third most common cancer worldwide with more than 1.8 million patients diagnosed each year (1). Despite intensified screening programs, most patients are diagnosed at the later stages of disease (2). This results in CRC emerging as the second leading cause of death with more than 900,000 deaths every year (1). CRC is a multifactorial and heterogeneous disease (3) with most cases being sporadic and fewer than 5% of patients with CRC have a positive familial history with inherited germline mutations that increase the risk of tumor development (3, 4).

Tumors are widely distributed from the proximal region of the colon to the rectum and each location is associated with different tumor-intrinsic characteristics (5). Most of these tumors arise from benign lesions called polyps, which progressively acquire genetic and epigenetic modifications to form malignant tumors and metastases (3, 4, 6, 7). These include deletions, amplifications, mutations or methylation of the promoter of certain genes such as *APC*, *SMAD4*, *TP53*, *BRAF*, *MYC*, *KRAS*, *PTEN*, or *MLH1* (3, 4, 8). The risk of developing CRC increases with age (4, 5)

and is higher in men than in women (9). Other environmental factors such as exposure to smoking, alcoholic beverages, the presence of visceral fat and poor dietary patterns are all features associated with a higher risk of developing CRC (3, 5). In addition, inflammatory bowel disease (IBD) and chronic colitis have also been associated with an increased risk of CRC development (10, 11). However, improved anti-inflammatory treatments and increased surveillance have been effective in reducing CRC incidence for these patients (12).

Treatment

The most effective first line treatment for patients diagnosed with localized CRC is surgery. In certain cases, neoadjuvant chemotherapy allows early reduction of the tumor burden to increase the chances of complete tumor resection (3, 4). For patients with metastatic CRC, there are increased treatment options available which include chemotherapies and targeted therapies.

Chemotherapy

Tumor recurrence occurs in 15–50% of patients who have undergone complete resection of loco-regional tumor lesions (4). To reduce this risk, patients often receive adjuvant chemotherapy treatment as a first line approach increasing both patient progression free and overall survival (13, 14). More recently, chemotherapy has been combined with targeted therapy to further improve patient overall response rates.

Targeted Therapies

Multiple treatment approaches have been developed in an endeavor to provide curative outcomes for patients. These include targeting oncogenic drivers (RAS, BRAF), receptors of growth factors (EGFR) or pathways involved in angiogenesis such as VEGFR (15). As an example, Bevacizumab, a human IgG1 antibody directed against VEGF-A, increased patient overall and progression-free survival when combined with chemotherapies (14, 16, 17).

Recent breakthroughs in tumor immunology have revolved around the clinical efficacy of therapeutic antibodies that are designed to block critical checkpoint molecules expressed at the membrane of circulating and tumor-infiltrating immune cells (18). Programmed death-1 (PD-1) blocking antibodies have been shown to induce remarkably durable clinical responses in many different cancer types, including CRC (19, 20). These anti-PD-1 sensitive CRC tumors harbor defective mismatch DNA repair mechanism (called microsatellite instability high or MSI high tumors) and is associated with enhanced mutational load, neoantigen formation, T cell infiltration of the tumor, and immune checkpoint expression (4, 8, 20, 21). However, only half of these CRC patients experience durable clinical responses (22) and many others develop resistance during treatment. To understand the underlying mechanisms, Grasso and colleagues performed large scale genomic analyses of more than 1,200 CRC tumors (23). This revealed that MSI high tumors have a higher rate of mutated genes in critical immune-modulating pathways such as antigen presentation that then drive resistance to treatment. In addition, upregulation of the WNT pathway in both MSI high and microsatellite stable CRC leads to a "cold" tumor microenvironment that is poorly infiltrated by anti-tumor T cells (23). Combined infusion of anti-CTLA-4 and anti-PD-1 blocking antibodies increased patient survival in metastatic CRC compared to anti-PD-1 treatment alone. However, it also induced higher levels of toxicity (24).

Currently, the use of immunotherapy and its integration into strategies for CRC care are growing (25). However, the quality of the immune response has not yet been fully factored into treatment decisions despite significantly influencing CRC patient outcomes. A better understanding of the tumor immune infiltrate would greatly inform treatment decisions and allow more strategic design of future combination therapies.

THE TUMOR IMMUNE CONTEXTURE IN COLORECTAL CANCER—THE SUCCESS OF THE ADAPTIVE IMMUNE SYSTEM

Most colorectal tumors develop from glandular epithelial cells of the colon or rectum (26) and evolve through close interaction with their microenvironment and diverse immune cell types. A critical balance between pro- and anti-tumor immune responses determines either the eradication of nascent lesions or the development and progression of transformed cells that form malignant tumors (27). It has been demonstrated that in patients where initial failure to eradicate emerging tumors occurs, immune cells still play a critical role in dictating the patient response to treatment and outcome (28–32). To account for the quality of the immune infiltrate, a scoring system called Immunoscore™ (33) has been developed by tracking adaptive immune cells such as $CD3^+CD8^+$ and memory ($CD45RO^+$) T cells (**Table 1**). This allows the immune infiltrate and its location within the tumor sample to be integrated to stratify patients and accurately predict dissemination to distant metastasis (34), disease-free and overall survival (30, 31, 35) (**Tables 1**, **2**). However, the impact of innate cells has not been considered. In this review, we will summarize the latest evidence implicating a role for innate lymphoid cells in CRC development and their impact on disease outcome.

TABLE 1 | Parameters examined in Immunoscore™.

Cell type	Cytotoxic lymphocytes ($CD3^+CD8^+$) Memory T cells ($CD45RO^+$)
Location	Tumor center Invasive margin
Density	0—low density of both cell populations in both regions 4—high density of both cell populations in both regions
Prognosis-Cox analysis	Disease free survival Overall survival Disease specific survival

TABLE 2 | Immunoscore™ and its association with the risk of relapse.

Immunoscore	Risk of relapse
0—low density of both cell populations in both regions	High
1—one cell population in one region	
2—one or both cell population(s) in one or both region(s)	
3—one or both cell population(s) in one or both region(s)	
4—high density of both cell populations in both regions	Low

ROLE OF THE INNATE LYMPHOID CELLS IN COLORECTAL CANCER

Innate lymphoid cells (ILCs) are the innate counterpart of the T lymphocytes, mirroring key aspects of their phenotype and function. ILCs are divided into five subsets, classified based on their development, transcription factor expression, cytokine production and functions. These are NK cells, ILC1, ILC2, ILC3, and Lymphoid Tissue inducers (LTi) (36). They are enriched in mucosal tissues, including the intestinal tract (37) and respond to signals derived from their micro-environment such as cytokines, alarmins, and other inflammatory and non-inflammatory stimuli (38, 39) to drive appropriate immune responses and maintain tissue homeostasis. In addition, ILCs express particular receptors and ligands at their surface regulating further their function (40, 41). NK cells and ILC1 are mainly involved in the early protection against viruses (42), bacteria (43, 44), and cancer (45) through the secretion of interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (46). ILC2 are essential in host protection against helminth and parasites throughout interleukin (IL)-4, IL-5, and IL-13 production (47). ILC3 express mainly IL-17 and IL-22, both cytokines critical to mucosal immune responses and epithelium regeneration (48). Finally, LTi are critical for lymphoid organogenesis (49). Beyond this, significant plasticity occurs between ILC subsets depending on environmental stimuli they receive, thus directly impacting on their effector functions and immune responses (50–55). While ILCs are essential to lymphoid organogenesis and tissue homeostasis, they also participate to the development of autoimmune diseases (56, 57) or inflammation-driven carcinogenesis (58) (**Figure 1**).

Type 1 Innate Lymphoid Cells

High-dimensional cytometric analyses have revealed that human colorectal tumors are highly infiltrated by NK cells and intraepithelial-like ILC1 (59, 60). They secrete cytotoxic molecules such as granzymes and perforin (59, 60) and cytokines such as IFN-γ following stimulation (59), making them key potential players in cytotoxic anti-tumor responses (**Figure 1A**). In contrast, a separate study found impaired NK cell infiltration despite elevated levels of chemokines found in CRC tumors (61) (**Table 3**).

Studies tracking intraepithelial ILC1s have shown that they accumulate in the intestine of patients with Crohn's Disease and in CRC (51, 59, 62). These intraepithelial ILC1s can be activated by chemokines and cytokines such as IL-18 produced by intestinal epithelial cells (63) or IL-12 from dendritic cells and monocytes (62). This induces IFN-γ production in ILC1 which, in contrast to previously mentioned studies (59, 60), drives the pathogenesis of anti-CD40-induced colitis in *Rag1*$^{-/-}$ mice (62) (**Table 3** and **Figure 1A**). Given these divergent outcomes, further studies are required to analyze the exact role of intraepithelial ILC1, their receptor expression and cytokine production using genetically-modified conditionally-deficient mice and human samples. How intraepithelial ILC1 influence patient outcomes and responses to therapy, is, to date, poorly understood.

NK cells are known to mediate anti-tumourigenic activity (45). In CRC, NK cells effectively eliminate CRC stem cells and cancer-initiating cells, which express lower levels of class I major histocompatibility complex (MHC) but high levels of ligands that bind the natural cytotoxicity activating receptors (NCR) NKp30 and NKp44. Consequently, these cells are "marked" for NK cell mediated lysis (64) providing a mechanism to potentially prevent tumor cell re-emergence or CRC metastasis. However, tumor cells can circumvent this process by differentiating and upregulating MHC class I expression to suppress NK cell activation or by reducing their expression of NCR ligands to escape NK cell-targeted killing (64). Thus, circulating NK cells in CRC patients are often phenotypically altered reflecting these induced changes. These patients exhibit reduced NKp44 expression on their CD56^{dim} cells (65), a key subset that normally exhibit potent effector function. In addition, high NKp30 expression on circulating CD56^{bright} cells has been associated with a shortened disease-free survival period in CRC patients (65). Other receptors and ligands expressed on NK cells of CRC patients such as activating and inhibitory KIRs (66) or TIGIT (67) have also been suggested to play distinct roles in the development, progression, and metastasis of CRC tumors (**Table 3** and **Figure 1A**). In mice, neutralizing TIGIT markedly prolongs the survival of tumor bearing animals. However, when NK cells are depleted, this effect was completely abrogated highlighting the critical role of NK cells in this context. In addition, tumor infiltrating NK cells can transdifferentiate into ILC1-like cells in the presence of transforming growth factor-β (TGF-β) which is particularly abundant in the tumor microenvironment and associated with tumor progression (54). Does TGF-β accumulate in CRC tumors? If so, similar NK cell—ILC1 transdifferentiation could occur in CRC tumors and this would potentially with worsen prognosis. Collectively, it is clear that NK cells are involved in the control of CRC progression and metastasis but are also susceptible to tumor-directed dysregulation (41). To circumvent such events, current NK cell-based therapies are designed to boost NK cell functions in an effort to strategically further improve patient outcomes (68–71).

Type 2 Innate Lymphoid Cells

The role of ILC2 has been examined in a number of different cancers including lung and gastric cancer (72, 73). Nevertheless, their role in colorectal cancer is, to date, only poorly understood. In fact, they are found to a relative lower frequency compared to other ILC subsets that reside in the intestine. Nevertheless, ILC2 frequency is increased in mucosal samples from IBD patients

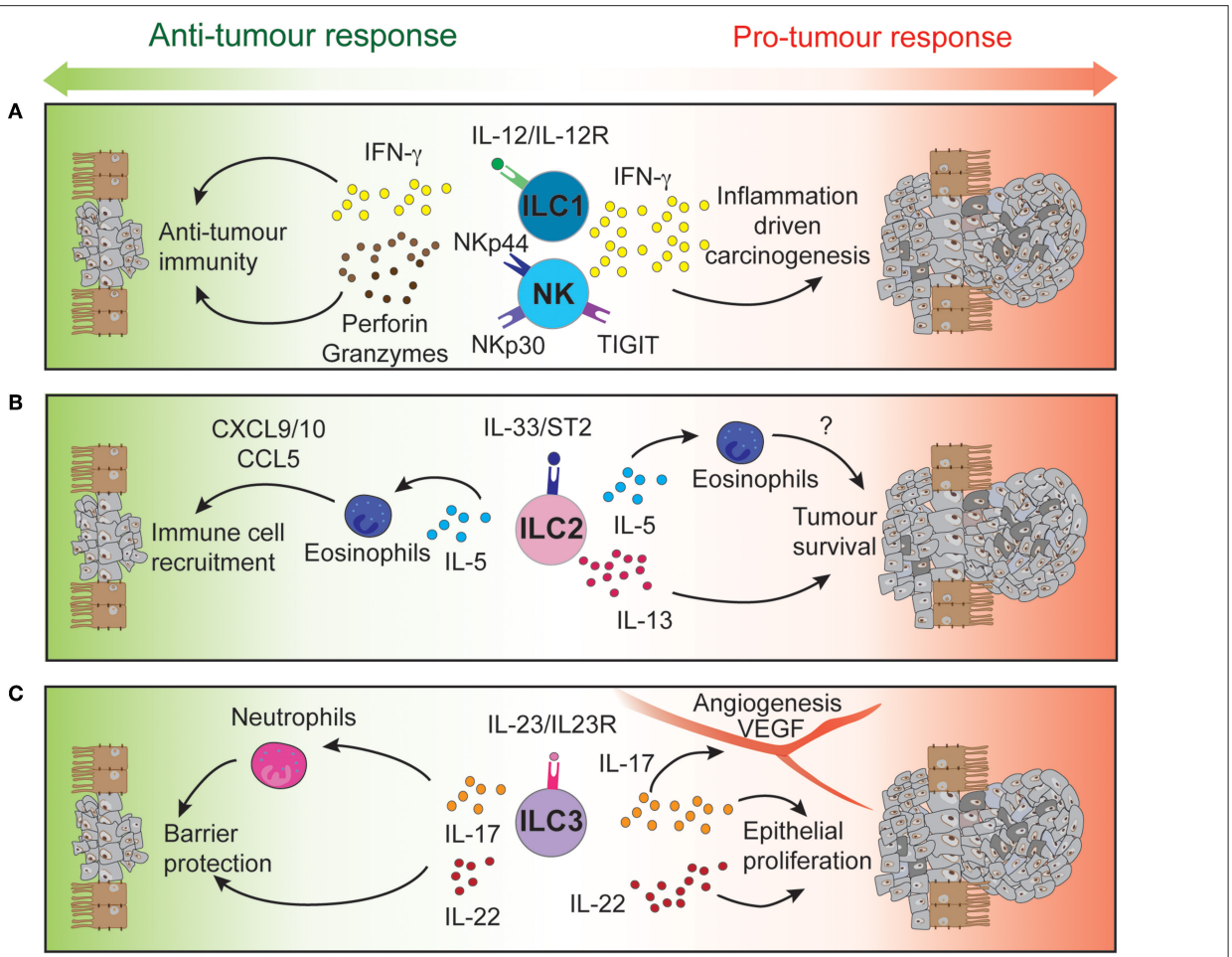

FIGURE 1 | Intestinal ILCs drive both pro- and anti-tumor functions tipping the balance for tumor development. Signaling of NK cells and intraepithelial ILC1 **(A)**, ILC2 **(B)**, and ILC3 **(C)** through their activatory or inhibitory receptors regulates the function of these immune cells. Through the secretion of cytokines and cytotoxic molecules, ILCs can modulate the tumor microenvironment to either control or promote CRC development and progression.

compared with healthy individuals (74). In CRC tumors, ILC2s express higher levels of ICOS and CD69 compared with ILC2 found in normal colon tissue (59, 60). These phenotypically distinct ILC2s could be a potential source of IL-4, IL-5, and IL-13 as both IL-4 and IL-13 are found at elevated levels in the serum of CRC bearing mice (75).

IL-13 can act directly on tumor epithelial cells in both an autocrine or paracrine manner to promote their survival and tumorigenesis (**Figure 1B**). In CRC, high expression of the IL-13Rα2 receptor is associated with more advanced disease stages, lymph node involvement, the presence of metastases and reduced survival (76). In contrast, Saigusa et al. found that low preoperative serum IL-13 was a predictive marker for poorer prognosis of colorectal cancer patients (77). These discrepancies indicate that IL-13 expression and its predictive value may be context dependent. The role of IL-5 has not been explicitly studied in the context of CRC. Nevertheless, it is important for eosinophil recruitment, expansion and activation (78, 79). In turn, these cells secrete chemo-attractants such as CXCL9, CXCL10, and CCL5 which promote the recruitment of $CD8^+$ T cells to the tumor site (80) (**Table 3** and **Figure 1B**). The presence of peritumoral eosinophils is an independent prognostic factor associated with favorable progression free survival (81). In contrast, patients suffering from ulcerative colitis, have a higher risk of CRC development and were found to have elevated IL-5 transcript levels (82). Neutralizing IL-5 in mice reduced eosinophil numbers and diminished the inflammation associated with DSS-induced colitis (83).

The role of ILC2s, their activation pathways and subsequent downstream signaling in CRC are highly contentious. This is because the same biomarkers or effector molecules (e.g., IL-5 and IL-13) have been found to exhibit opposing roles in CRC outcome, and thus, differentially impact patient prognosis. A similar effect is seen for the cytokine IL-33, a molecule released by damaged epithelial cells and regulator of ILC2 activation. Several studies have found both the cytokine IL-33 and its receptor ST2, to be elevated in mouse and human CRC tissues (84–86), suggesting a potential role of this IL-33/ST2 pathway

TABLE 3 | Role of ILC in promoting or inhibiting CRC tumorigenesis.

Immune cell type	Prognosis		References
	Anti-tumor	Pro-tumor	
NK cells	• High cytotoxicity • Recognition of cancer initiating cells	• Low number of infiltrating NK cells • Reduced level of activating receptors (e.g., NKp44, NKp30) • Increased expression of inhibitory receptors (e.g., TIGIT) • Low cytotoxicity	(61, 64, 65, 67)
ILC1	• Produce IFN-γ and cytotoxic molecules associated with anti-tumor immunity*	• Accumulate in inflamed tissue • IFN-γ promotes inflammation*	(58, 62)
ILC2	• High pre-operative serum IL-13 • IL-5 recruits eosinophils	• Local IL-13 promote tumor epithelial survival and proliferation. • Elevated IL-5 in ulcerative colitis, anti-IL-5 reduced eosinophils and colitis*	(76, 77, 81–83)
ILC3	• IL-17 recruits neutrophils to protect tissue barrier* • IL-22 promotes wound healing* and protects intestinal epithelial cells from genotoxic stress-induced DNA damage	• Overproduction of IL-17 promote inflammation and angiogenesis, and disrupt the intestinal epithelial barrier • Overproduction of IL-22 dysregulates epithelial proliferation	(96, 102, 103, 106, 108, 109, 112, 113)

**Indirect evidence of involvement in CRC development.*

in tumor development and progression. Mechanistically, IL-33 was found in adenomatous polyposis coli $(APC)^{min/+}$ mice to promote intestinal polyp formation by activating stromal cells and modulating angiogenesis (85, 87), and by promoting cell growth and proliferation of primary CRC cells (88). Serum soluble ST2 levels were inversely correlated with the tumor stage despite being elevated in CRC patients. Indeed, soluble ST2 was shown to act as a decoy receptor suppressing IL-33-driven angiogenesis (89) and inhibiting CRC growth, while loss of ST2 expression conferred protection in AOM/DSS-induced CRC (90). Conversely, reduced levels of serum IL-33 is observed in CRC patients compared with healthy individuals and decreased expression of ST2L, the transmembrane isoform of the ST2 receptor, in CRC tumors is associated with higher tumor grade and worse prognosis (91). These conflicting results indicate that the IL-33/ST2 axis may playing a dual role in CRC pathogenesis and is capable of both promoting or protecting against CRC tumor development and progression depending on the environmental context. IL-33 has multiple downstream targets as ST2 is not only expressed on ILC2 but is also found on other intestinal cell types such as epithelial cells or regulatory $CD4^+$ T cells (86). Therefore, the role of the IL-33/ST2/ILC2 pathway in CRC warrants further investigation using conditional mouse models, which would allow cell-specific responses to be disected.

Type 3 Innate Lymphoid Cells

Inflammation is a key driver of cancer progression and the link between IBD and the development of CRC is well-established (92). This inflammation can be exacerbated by the loss of barrier protection occurring early during CRC development (93), allowing for the infiltration of microbes and their associated products into the tumor microenvironment. This activates dendritic cells and macrophages to produce IL-23 (94), a key cytokine regulating ILC3 function and plasticity (53, 95). Additional stimuli such as IL-12, IL-18, TGF-β, or Notch ligand can profoundly influence ILC3 plasticity (50–52). In the presence of IL-12 or TGF-β, IL-22-expressing ILC3 can acquire ILC1 features and transdifferentiate into IFN-γ producing cells (52). At the molecular level, the transcription factors T-BET, AIOLOS, and Signal Transducer and Activator of Transcription (STAT) 4 induce an ILC1 transcriptional program, repressing in parallel the ILC3 lineage genes (52, 53). Transitional ILC3/ILC1 cells were found in human intestinal epithelium (52) and accumulate in inflamed lesions of patients suffering from Crohn's disease (51) suggesting that such plasticity may also occur in inflamed CRC lesions.

IL-23 has been found to be overexpressed in both mouse and human CRC tumors (93, 96, 97) and IL-23 deficiency has been shown to protect against tumor development in a number of mouse models of cancer (97). Conversely, chronic IL-23 injection induces ILC3-dependent duodenal adenomas development in mice (98). Furthermore, local IL-23 expression drives ILC3 accumulation and activation within the colonic lamina propria (99, 100). These intestinal ILC3 express higher levels of the activating receptors ICOS and CD69 (59, 60), regulators of tumor responses CD244 and CD39 (59), and exhibit enhanced production of the downstream signaling cytokines IL-17 and IL-22 that contribute to the pathogenesis of CRC (96) (**Table 3** and **Figure 1C**).

Several studies have reported high IL-17 expression in both mouse and human CRC compared with normal adjacent tissues (96, 101–103). ILC3s produce high levels of IL-17 but other immune and non-immune intestinal cell types also express this cytokine. These include Th17 cells, γδ T cells, and Paneth cells (104). Importantly, IL-17 transcripts are increased during progression of tumors from the adenoma to carcinoma stages in CRC patients (105). Furthermore, IL-17 can alleviate cell cycle inhibition, thereby promoting epithelial proliferation and intestinal inflammation (106) and increase angiogenesis by inducing VEGF expression (103). Administration of anti-IL-17 antibodies have been shown to ameliorate inflammation severity (96) while genetic ablation of IL-17 was able to reduce tumor burden (93, 100, 106, 107). Although, these studies show that IL-17 is a pro-inflammatory cytokine involved in CRC pathogenesis, IL-17 may also play a protective role. Lin and colleagues (102) reported better patient overall survival associated with high

IL-17 levels (102) and neutralizing IL-17 exacerbated Crohn's disease and DSS-mediated colitis by increasing gut permeability (108, 109) (**Table 1**).

In a similar pattern to IL-17, the ILC3-driven IL-22 pathway plays both protective and pathogenic roles during colitis and tumorigenesis (**Table 3**). Under normal conditions, IL-22 is required for homeostatic epithelial cell proliferation through STAT3 activation (110, 111). Furthermore, IL-22 production by ILC3 and γδ T cells protects the intestine from genotoxic stress-induced DNA damage through effective initiation of the DNA damage response program (112). Loss of IL-22 or IL-22 receptor on epithelial cells results in delayed tissue repair, exacerbated inflammation (113) and tumor development when intestinal cells are exposed to carcinogens (112). Conversely, sustained IL-22-dependent epithelial proliferation leads to tumor progression (**Figure 1C**) and neutralizing IL-22 reduced CRC development (96). In addition to this, loss of IL-22 binding protein, a soluble decoy receptor neutralizing IL-22-driven cell activation, has been shown to increase epithelial cell proliferation and was associated with a higher tumor burden (114).

These studies unanimously suggest that IL-23 signaling in ILC3 is central in driving CRC development. Indeed, the absence of ILC3 despite chronic IL-23 injections protects mice from tumor development (98). However, the downstream cytokine IL-17 and IL-22 signaling pathways seem to play a dual role in CRC. These findings suggest that initial activation of the IL-23/ILC3 pathway is sufficient to trigger tumor development but additional mechanisms influence CRC progression and outcomes. Collectively, further investigations are warranted to shed light on the role of these cytokines in CRC pathogenesis.

CONCLUDING REMARKS

Despite increasing evidence for the crucial role of the immune system in dictating patient's prognosis, the evaluation of the tumor immune cell infiltration in primary CRC is not yet fully integrated into assessments in routine clinical practice (115). It is, however, clear that adaptive immune cells, particularly $CD8^+$ memory T cells, play an important role to limit tumor recurrence and prolong patient's survival (30). How adaptive immune cells are influenced by resident innate immune cell populations, the first line of immune defense, is still poorly understood (116). Direct and indirect evidence tend to suggest that ILCs could play a dual role in colorectal cancer. However, most of the aforementioned findings resulted from studying knockout mice or mice lacking adaptive immune cells. Thus, further studies are warranted to ascertain the role of ILCs in immunocompetent animals using genetically-modified conditionally-deficient mouse models. Collectively, given their function and strategic location, harnessing ILC responses would open up new possibilities through the development of combination therapies to further constrain CRC progression.

AUTHOR CONTRIBUTIONS

QH and NJ wrote the initial draft. WC designed the figure. All authors provided critical insight, edited, and approved the final version.

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MINI REVIEW
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Tumor Microenvironment-Associated Extracellular Matrix Components Regulate NK Cell Function

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The tumor microenvironment (TME) is composed of multiple infiltrating host cells (e.g., endothelial cells, fibroblasts, lymphocytes, and myeloid cells), extracellular matrix, and various secreted or cell membrane-presented molecules. Group 1 innate lymphoid cells (ILCs), which includes natural killer (NK) cells and ILC1, contribute to protecting the host against cancer and infection. Both subsets are able to quickly produce cytokines such as interferon gamma (IFN-γ), chemokines, and other growth factors in response to activating signals. However, the TME provides many molecules that can prevent the potential effector function of these cells, thereby protecting the tumor. For example, TME-derived tumor growth factor (TGF)-β and associated members of the superfamily downregulate NK cell cytotoxicity, cytokine secretion, metabolism, proliferation, and induce effector NK cells to upregulate ILC1-like characteristics. In concert, a family of carbohydrate-binding proteins called galectins, which can be produced by different cells composing the TME, can downregulate NK cell function. Matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAM) are also enzymes that can remodel the extracellular matrix and shred receptors from the tumor cell surface, impairing the activation of NK cells and leading to less effective effector functions. Gaining a better understanding of the characteristics of the TME and its associated factors, such as infiltrating cells and extracellular matrix, could lead to tailoring of new personalized immunotherapy approaches. This review provides an overview of our current knowledge on the impact of the TME and extracellular matrix-associated components on differentiation, impairment, and function of NK cells.

Keywords: NK cells, tumor microenvironment, galectin, MMP, ADAM

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INTRODUCTION

Innate lymphoid cells (ILCs) are lymphocytes derived from a common lymphoid precursor. Unlike T and B lymphocytes, ILCs do not express adaptive antigen receptors, instead being activated through cytokine receptors (1). Natural killer (NK) cells are classified as group 1 ILCs together with ILC1 (2), previously known as tissue resident NK cells (3), due to their shared dependence on the transcription factor T-bet, production of specific cytokines (e.g., interferon-gamma, IFN-γ), and surface receptor expression (e.g., NK1.1, NKp46 in mice, and NKp30 in humans) (1, 4). During development in the bone marrow, both mouse and human NK cells appear dependent on

the transcription factor Eomesdermin (Eomes) (5), then during maturation they repress Eomes and increase T-bet production (4). However, in humans, mature liver ILC1s can express Eomes (6). Although ILC1 are tissue resident and unlikely to migrate to other tissues (7–9), their function in cancer is poorly understood. Recent studies have revealed that transforming growth factor-beta (TGF-β) signaling (either by TGF-β itself or indirectly by Activin-A) can suppress cellular metabolism and effector functions (10–12). This suppressive signaling drives the upregulation of ILC1-related markers in circulating mouse or human NK cells, suggesting the possibility of intercellular plasticity which could be important within the tumor microenvironment (TME) (11, 13, 14). NK cell cytotoxicity can be controlled by many stimulatory (NKp30, NKp44, NKp46, CD16) and inhibitory (PD1, TIM3, TIGIT, KLRG1) surface receptors (15). Even with their ability to kill transformed cells, NK cell immunosurveillance can be evaded by tumor cells due to their ability to manipulate the TME in favor of immune equilibrium and escape, allowing tumor survival and the possibility of further metastatic spread (16–18). Once established, the TME is composed of different immune and non-immune cell subsets recruited by the tumor (e.g., fibroblasts, pericytes, endothelial cells, macrophages, lymphocytes, etc.) (19), bioactive products, such as extracellular matrix (ECM) proteins, cytokines, and growth factors (20), and specific glycosylation pattern (21, 22). In this review we will discuss some of the molecules present in the TME (summarized on **Table 1**), with a focus on their potential impact on NK cell functions.

TABLE 1 | TME molecules and the effect on NK cells.

TME molecule	Effect on NK cells	Cancer type	References
Hyaluronan	Impair access to tumor and ADCC	Breast and ovarian caner	(23)
Heparanase	Decrease recognition of target cells	Breast cancer	(24)
Galectin-1	Impair cytotoxicity	Glioma	(25)
Galectin-3	Galectin-3$^{-/-}$ mice have more effective cytotoxic CD27^{high}CD11b^{high} NK cells	Melanoma	(26)
	Increase of galectin-3 impair NK cells cytotoxicity	Adenocarcinoma, cervix cancer	(27)
Galectin-9	Increase of NK cells infiltration	Melanoma	(28)
	Downregulation of stimulatory genes (LTB, KLRF1, FCGR3A) and impair cytotoxicity against K562 cells	Leukemia	(29)
	Galectin-9 binds to TIM-3 leading to NK cells exhaustion	Gastrointestinal tumors	(30)
Sialic acid	Low sialylation of tumor cells increases NK cell cytotoxicity	Melanoma	(31)
Siglec-7/9	Membrane inhibitory receptor on NK cells that recognize sialic acid	Melanoma, basal cell carcinoma, squamous cell carcinoma, and cutaneous T cell lymphoma	(32)
MMP-9	Cleaves MIC-A, MIC-B and ULBP-2 from tumor cells membrane avoiding killing by NK cells	Human gastric cancer, lung adenocarcinoma and osteosarcoma	(33–35)
ADAM-10/17	Cleave MIC-B, ULBP-2 and B7-H6 from tumor cells membrane avoiding killing by NK cells	Human pancreatic adenocarcinoma, melanoma, cervical, breast, hepatocellular carcinomas and glioblastoma	(36–38)

GLYCOSAMINOGLYCANS AND PROTEOGLYCANS

Glycosaminoglycans (GAGs) are a family of linear polysaccharides composed of repeating disaccharide units. Depending on the disaccharide composition, GAGs can be classified as: keratan, chondroitin, dermatan or heparan sulfate (HS), heparin, or hyaluronan (39). Except for hyaluronan, all GAGs can be linked to proteins, forming proteoglycans (PGs) (40).

Hyaluronan is the only non-sulfated GAG, first isolated and characterized from bovine vitreous humor in 1934 (41). It is produced and secreted to the ECM by the transmembrane hyaluronan synthase (42), which is encoded by three conserved genes in both mice (*Has1, Has2,* and *Has3*) and humans (*HAS1, HAS2,* and *HAS3*) (43, 44). In cancer, hyaluronan is associated with tumor cell proliferation, angiogenesis, and evasion of immune responses and apoptosis (45–50). The presence of hyaluronan in the TME appears to be detrimental to NK cell function against cancer cells; hyaluronan rich tumors can inhibit both NK cell access to tumor cells and antibody-dependent cell-mediated cytotoxicity (ADCC) (23). Although hyaluronan does not form PGs, it can bind to PGs by linking proteins (51). Our group recently identified a poor prognostic association between the *HAPLN3* gene (Hyaluronan and Proteoglycan Link Protein 3) and a low NK cell infiltration in malignant melanoma patients, suggesting a potential inhibition of anti-tumor immune functions by *HAPLN3* and identifying this gene as a potential target for immunotherapy (52).

Heparan sulfate proteoglycans (HSPGs) can be found on the cell surface (glypicans and syndecans families) or in the ECM (perlecan, agrin, collagen XVIII) (53). Many types of tumors overexpress HSPGs, which is associated with increased angiogenesis in hepatocellular and colon carcinomas, breast and pancreatic cancers, and melanoma (54–58). HSPGs are also associated with invasion and metastasis in melanoma and breast cancer (59–61). Some reports have suggested that HS chains can be ligands for NKp30 (62, 63), NKp44 (63, 64), NKp46 (62, 63, 65), and for the NKG2D and CD94 complex (66). This tumor production of HSPG is not sufficient to stimulate NK cell cytotoxicity, and there are two potential hypotheses for this observation:

i) Tumor cells present altered expression of many enzymes related to the HSPG modifications, such as sulfatase 2 and heparan sulfate 6-O- sulfotransferase 2 (67–69), leading to production of PGs containing distinctly sulfated HS chains (70, 71). Differences in sulfation pattern could impair the recognition of HS chains by NKp30, NKp44, and NKp46 (62, 63, 65).
ii) Melanomas, multiple myeloma, bladder, prostate, breast, colon and liver cancers overexpress heparanase (72–76), which is an endo β-D-glucuronidase that cleaves specific regions of HS into small fragments (77, 78), decreasing NK cells ability to recognize target cells (24). However, a previous study showed that heparanase produced by NK cells is also unexpectedly important for the host tumor surveillance by allowing NK cell navigation through the ECM (79).

GALECTINS

Galectins are a group of proteins with two main features: β-galactoside binding sites and conserved carbohydrate recognition domains (CRDs) (80). The first galectin was isolated in 1975 from an electric fish (*Electrophorus electricus*) and named electrolectin (81). Just in 1994, the name galectin was given to this family of lectins and all members were numbered in order of discovery (80). Galectins are divided into three groups: prototype have one CRD domain (galectins 1, 2, 7, 10, 11, 13, 14, and 15); tandem-repeat type have two CDR domains (galectins 4, 8, 9, and 12); chimera-type have a single CRD domain and an amino-terminal polypeptide rich in proline, glycine, and tyrosine residues (galectin-3) (82).

Galectins are expressed in many different mammalian tissues (83, 84) and are involved in early development, tissue regeneration, immune homeostasis, and some pathologies (e.g., cancer, obesity, type II diabetes) (85). In some types of cancer, galectins may be associated with angiogenesis, cancer cell survival, invasion, metastasis, and avoiding immunosurveillance (86). Here we will discuss and revisit the potential contribution of different galectins for the TME, NK cell function, and anti-cancer responses.

Galectin-1 is important for maturation of B cells in the bone marrow (87, 88) and T cells homeostasis (89–91). It is overexpressed in some types of cancer such as ovarian, breast, myeloma, and melanoma (92–95), and can contribute to tumor survival by inhibition of NK cells (25). Glioma cells deficient for galectin-1 showed reduced tumor growth, increased intra-tumor NK cell infiltration, and elevated expression of granzyme B when implanted into the striatum of *Rag1*$^{-/-}$ mice (which develop NK, but not T or B cells) when compared to *Rag1*$^{-/-}$ mice injected with wild-type cells (25). In the same study, galectin-1 deficient glioma cells were injected into NGS (T, B, and NK cells deficient) or C57BL/6 immunocompetent mice treated with anti-asialo GM1, which depletes NK cells. Enhanced tumor growth was observed in both models, proving the inhibitory effect galectin-1 has on NK cell anti-tumor function (25).

Galectin-3 was initially discovered in macrophages and named Mac-2 (96). It starts to be expressed in many normal tissues during embryogenesis (in both mice and humans) (97) and is involved in angiogenesis (98) and migration of monocytes and macrophages (99). In cancer, galectin-3 overexpression in the TME is associated with angiogenesis (98), tumor progression (97), and immune escape by inducing T cell apoptosis (100, 101). Some reports have also shown the impact of galectin-3 on NK cells. For example, galetin-3-deficient mice are resistant to lung metastasis development by B16-F1 melanoma cells, potentially due to an increase of CD27^{high} CD11b^{high} NK cells in their spleen compared with the wild type (26), suggesting an inhibitory effect of galectin-3 on NK cell immunosurveillance. Additionally, HeLa cells overexpressing galectin-3 are more resistant to human NK cell-mediated death; yet when galectin-3 is knocked out the killing capacity of NK cells is restored in a mechanism mainly mediated by NKp30 (27). Considering the potential for galectins as cancer treatment targets, clinical trials using galectin inhibitors have already started for both galectins 1 (ClinicalTrials.gov identifier: NCT01724320—for advanced solid tumors; NCT00054977—for advanced solid tumors in combination or not with 5-Fluorouracil) and 3 (NCT02575404—for advanced melanoma, non-small cell lung cancer, and head and neck squamous cell cancer in combination with Pembrolizumab; NCT02117362—for advanced melanoma in combination with Ipilimumab).

Galectin-9 was first described in mouse embryos and later discovered during homeostasis in many adult organs such as liver, kidney, spleen, and lungs (102). In some cancers, galectin-9 is related with a good prognosis (103). In breast, pancreatic cancer and melanoma, expression of galectin-9 correlates with good prognosis for those patients (28, 104, 105). Galectin-9 appears to promote patient survival in part through NK cell modulation (106). C57BL/6 mice that had B16-F10 melanoma injected into their peritoneal cavity followed by galectin-9 treatment showed prolonged survival compared with untreated controls, which also correlated with increased NK cell infiltration into the peritoneal cavity; however, when NK cells were depleted by anti-asialo GM1, those positive effects were lost, suggesting a stimulatory effect of galectin-9 on NK cells (106). Despite these findings, the role of galectin-9 may be ambiguous, as inhibitory effects over NK cells have also been demonstrated (29). Human NK cells exposed to galectin-9 downregulate many NK cell stimulatory genes (e.g., LTB, KLRF1, FCGR3A), resulting in less efficient killing of target leukemia K562 cells (29). A possible explanation for galectin-9 mediated inhibition of NK cells could be its interaction with and activation of TIM-3 (T cell immunoglobulin and mucin domain 3) (107), which is a transmembrane receptor associated with NK cell exhaustion (108, 109). Additionally, a positive correlation was found between galectin-9 expression on human gastrointestinal stromal tumor and TIM-3^{+} expression on infiltrating NK cells (30). This study suggests that targeting galectin-9 or preventing its interaction with TIM-3 could potentially act as a novel immunotherapy approach to enhance NK cell functions against cancer (30).

SIALIC ACID AND MUCINS

Sialic acids (Sia) are a family of carbohydrates composed of *N*-acetylneuraminic acids (110) linked to many proteins, lipids,

and other polysaccharides on the cell surface. The most common Sia are *N*-acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic (Neu5Gc) acids (111). Humans only express Neu5Ac, due to the lack of an enzyme called cytidine monophospho-*N*-acetylneuraminic acid hydroxylase, which converts Neu5Ac to Neu5Gc (112). Sia are associated with many biological processes, but an important function is recognizing self and non-self (113). Many types of cancer, including breast cancer and cervix squamous cell carcinoma, are hypersialyated (114, 115) due to the overexpression of Sia synthesis enzymes (116, 117). This hypersialytion is associated with increased metastasis (117) and immune system evasion (118). A study using "Sia low" B16-F10 cells demonstrated that after their subcutaneous injection into C57BL/6 mice, tumors grew more slowly and exhibited increased NK cell infiltration when compared with standard B16-F10 cells (31). Additionally, after NK cell depletion (using anti-NK1.1) "low Sia" tumors grew at a similar rate to the control group, highlighting the importance of NK cells during the defense against sialyated tumors (31).

The interactions between cells and Sia are mediated by transmembrane proteins called Siglecs (sialic acid-binding immunoglobulin-type lectins) (119). Siglecs are expressed in all immune cells and are divided into two broad groups: CD33 and CD33-related Siglecs, which have high homology with CD33 in their extracellular domains, and CD33-unrelated Siglecs which have high homology between human, rodents and other vertebrates (120). Both groups consist of both activating and inhibitory receptors, where the inhibitory Siglecs contain the intracellular immune receptor tyrosine-based inhibition motifs (ITIM), leading to tyrosine phosphorylation and tyrosine phosphatases SHP-1 and SHP-2 (121) (and as exemplified in **Figure 1A**). ITIMs are associated with NK cell inhibition and

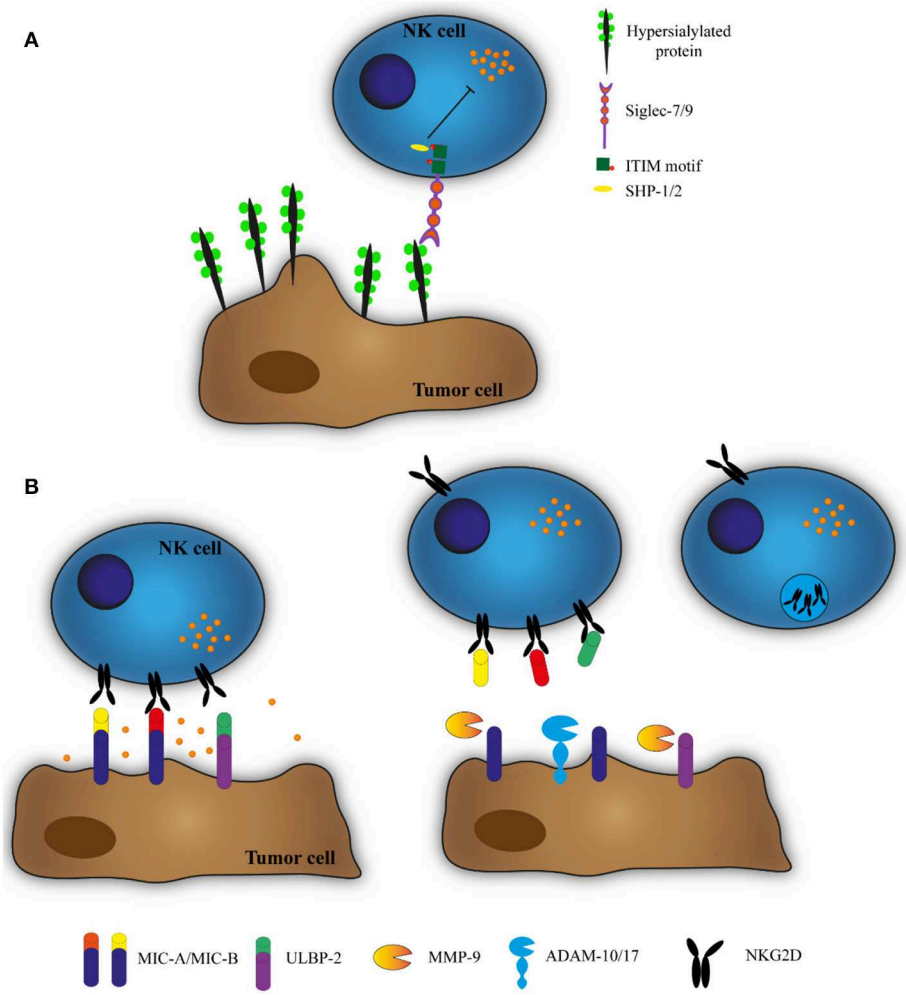

FIGURE 1 | (A) Hypersialysation of tumor cells inhibits NK cell cytotoxicity. To impair recognition by NK cells, tumor cells change their glycosylation pattern, expressing more sialic acid on the cell membrane. NK cells express membrane receptors that recognize this sialic acid (Siglecs). Siglecs have an intracellular immune receptor tyrosine-based inhibition motif (ITIM) that recruits tyrosine phosphatases SHP-1 and SHP-2 and inhibits NK cell cytotoxicity. **(B)** ADAMs and MMPs cleave MIC-A, MIC-B, and ULBP-2 and downregulate NKG2D expression. NK cells can recognize and kill target cells by the interaction between the stimulatory receptor NKG2D and the ligands MIC-A, MIC-B, and ULBP-2. However, the TME contains ADAMs and MMPs that cleave these ligands, allowing the soluble proteins to bind to NKG2D and stimulate its degradation.

are related to other inhibitory receptors (e.g., Ly-49 and NKG2-A) (122, 123). Human NK cells express Siglec-7 (also named as p75/AIRM1) (124, 125) and Siglec-9 (126) on the cell surface. Siglec-7 is expressed in all human NK cells (124, 125) whereas Siglec-9 is expressed selectively in a subset of $CD56^{dim}$ NK cells (32, 127). Jandus and colleagues demonstrated that various human tumor samples (melanoma, basal cell carcinoma, squamous cell carcinoma, and cutaneous T cell lymphoma) and tumor cell lines (e.g., A375, HeLa, SW1116, and K562) have ligands for both Siglec-7 and 9. They also found that NK cells displayed increased cytotoxicity against HeLa and K562 after enzymatic treatment to remove the Sia from the target cell surface (32). Cell lines of multiple myeloma (e.g., RPMI 8226 and H929) pre-treated with a sialyltransferase inhibitor were also more susceptible to NK cell-mediated killing (128). In a separate study, Balb/c mice injected with desialylated MCA-induced fibrosarcoma cells developed less lung metastasis, an effect which could be abolished when NK cells were depleted by antibodies (129). Besides inhibitors of sialytranferase and enzymes that cleave Sia, other strategies can be applied to avoid Sia-mediated inhibition of NK cells, and antagonists for Siglecs-7 and 9 could be an option (130). This has been demonstrated by Prescher and collaborators, who described a small molecule inhibitor of Siglec-7 which increased cytotoxicity of human NK cells toward Mel1106 melanoma target cells (130).

Siglec-9 can also interact with mucin-1 and 16 (127, 131), which are rich in Sia (132, 133). Mucins are proteins that have tandem repeat structures which are highly glycosylated and rich in proline, threonine, and serine (PTS domains) (134). They are normally expressed by epithelial cells, but are overexpressed in some types of cancer, particularly ovarian (135). Some reports have shown that murine ovarian cancer cells knocked down for mucin-16 are more susceptible for NK cell killing, showing that mucin-16 has an impact on NK cells (136, 137). While mucin-1 is also a ligand for Siglec-9, it has only been demonstrated to have a direct inhibition on macrophages (138). However, mucin-1 may have other effects on NK cells (139). In human metastatic bladder cancer, tumor cells overexpress the enzyme 2β-1,6-N-acetylglucosaminyltransferase (C2GnT) that adds a poly-N-acetyllactosamine on Mucin-1. The increased glycosylation of Mucin-1 raises its affinity for galectin-3 binding. Consequently, this Mucin-1/galectin-3 complex is suggested to generate a shield around tumor cells, which impairs recognition by NK cells (139).

MATRIX METALLOPROTEINASES (MMPs) AND A DISINTEGRIN AND METALLOPROTEINASES (ADAMs)

Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) belong to a superfamily of zinc-dependent metalloproteinases known as metzincins, which process or degrade virtually all structural ECM proteins, growth factor–binding proteins, cell-cell adhesion molecules, and cell surface receptors (138, 139). MMPs are found either on the cell surface or soluble, and are involved in tissue remodeling and wound healing (140). ADAMs are single-pass membrane proteins that are important in shedding proteins and embryogenesis (141). In many types of cancer, MMPs and ADAMs are associated with tumor progression through angiogenesis, invasion, metastasis, and regulation of the immune response (142, 143).

MMPs and ADAMs can cleave NKG2D ligands from the tumor cell surface, including MHC class I chain-related A (MIC-A), MHC class I chain-related B (MIC-B), and UL16-binding protein (ULBP) (144, 145). The soluble forms of cleaved proteins from tumor cell membrane bind to NKG2D, inducing endocytosis and degradation of this receptor, resulting in the tumor evasion from the surveillance of this receptor (144, 146) (**Figure 1B**). This effect has been observed in multiple studies using different tumor cell lines, and in all of them the NK cell function returns to normal after using inhibitors for MMPs or ADAMs (33–37). *Ferrari de Andrade* and collaborators developed an antibody that binds to the MIC-A α3 domain, the site of proteolytic shedding, to avoid MIC-A cleavage, and demonstrated this could increase NK cell cytotoxicity toward human melanoma cells (147).

MMPs can also shed intercellular-adhesion molecule 1 (ICAM-1) from the tumor cell surface, a protein that is important for the adhesion of cytotoxic T lymphocytes and NK cells to target cells (148, 149). Interaction of NK cells with target cells expressing ICAM-1 leads to an expression of IFN-γ (150). Many types of cancers express ICAM-1 (151), however it is thought to be shed from the surface of tumor cells to avoid an immune response (152, 153). Indeed, when comparing the human breast cancer cell line MDA-MB435 ($ICAM\text{-}1^{+}$ and $MMP\text{-}9^{-}$) to transfected MDA-MB435 ($ICAM\text{-}1^{+}$ and $MMP\text{-}9^{+}$), the transfected cells had a higher concentration of soluble ICAM-1 in the supernatant and were more resistant to NK cells. This resistance was reversed when those cells were co-cultured in the presence of MMP-9 inhibitors (154).

ADAM-10 and 17 can also catalyze the cleavage of B7-H6, one of the ligands for NKp30 (both only expressed in human) (38). Using many different human tumor cell lines (pancreatic adenocarcinoma, melanoma, cervical, breast, and hepatocellular carcinomas), Schlecker and colleagues observed that these cells produced B7-H6 at the mRNA level; however they had a low abundance of this protein on the cell membrane compared to what was detectable in the culture supernatant, showing ADAM-10 and 17 cleaving activity (38). The high levels of soluble B7-H6 decreased the expression of NKp30 on the NK cell membrane, leading to a decrease of degranulation. However, in the presence of inhibitors or siRNA for ADAM-10 or 17, the levels of soluble B7-H6 decreased and the degranulation of NK cells was restored (38). Curiously, several reports have also described the effects of ADAM-17 in cleaving CD16 (FcgRIIIA), one of the most important activating receptors responsible for recognition of antibody-coated target cells and NK cell-mediated ADCC, suggesting the potential for inhibitors of ADAM17 as a novel therapeutic approach to increase NK cell anti-tumor potency during immunotherapy (155). As an alternative to prevent ADAM-17-mediated shedding of CD16, Jing and

colleagues showed that replacing the serine at position 197 of the cleavage site of CD16 with proline completely prevented ADAM-17-mediated cleavage of both CD16a and b, enhancing NK cell function to antibody-opsonized tumor cells (156). More recently, the same group provided evidence that amino acid replacement to generate uncleavable CD16 can be feasibly employed in induced human pluripotent stem cells (hiPSC), as a renewable and gene-editable source of off-the-shelf NK cell products with enhanced functionality (157).

Peng and collaborators showed that MMPs can also have a direct effect on NK cells, leading to their dysfunction. NK cells were co-cultured with a pancreatic cancer cell line (SW1990), and an increase of MMP-9 production was observed compared with NK cells co-cultured with a normal pancreatic cell line (hTERT-HPNE) (158). It was also observed that NK cells after been co-cultured with SW1990 presented a reduction in the percentage of cells positive for NKG2D, NKp30, NKp44, NKp46, DNAM-1, perforin, and granzyme B, and those cells were less cytotoxic against K563 (158). However, after incubation with an inhibitor for MMP-9 (TIMP-1) the levels of NKG2D, NKp30 and perforin were partially restored and the killing capacity was recovered (158). Additionally, in concert with our previous observations in murine NK cells (13), Bruno and colleagues described that infiltrating NK cells in human colorectal tumors display a "decidual" behavior by expression of CD49a (among other tissue resident-related markers) and MMP-9 (159). The same study also revealed MMP-9-expressing NK cells as important contributors of tumor angiogenesis, and that inhibition of MMP-9 with immunotherapy could help repolarize NK from pro-angiogenesis to anti-tumor effector cells (159). These recent findings reveal that MMPs might not only play a role in NK cell migration and *in vivo* positioning as previously believed (160), but also directly impact their anti-tumorigenic function and potentially be considered as novel inhibitory checkpoints in NK cell biology.

CONCLUSION

Many components of the TME can impair the cytotoxic activity of NK cells by changing or cleaving ligands that could lead the activation of NK cells, or by an increasing the availability of factors that can downregulate NK cells effector functions. There is an arising interest for identifying novel immune checkpoints for NK cells. Studies around the composition of the TME, such as ECM proteins, enzymes, and glycosylation patterns, are now a field of interest to understand how to overcome tumor inhibitory signals and discover new therapeutic targets.

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GR and FS-F-G wrote the manuscript. ET and FS-F-G reviewed the manuscript and provided critical input.

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Helper Innate Lymphoid Cells in Human Tumors: A Double-Edged Sword?

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Innate lymphoid cells (ILCs) were found to be developmentally related to natural killer (NK) cells. In humans, they are mostly located in "barrier" tissues where they contribute to innate defenses against different pathogens. ILCs are heterogeneous and characterized by a high degree of plasticity. ILC1s are Tbet$^+$, produce interferon gamma and tumor necrosis factor alpha, but, unlike NK cells, are non-cytolytic and are Eomes independent. ILC2 (GATA-3+) secrete type-2 cytokines, while ILC3s secrete interleukin-22 and interleukin-17. The cytokine signatures of ILC subsets mirror those of corresponding helper T-cell subsets. The ILC role in defenses against pathogens is well-documented, while their involvement in tumor defenses is still controversial. Different ILCs have been detected in tumors. In general, the conflicting data reported in different tumors on the role of ILC may reflect the heterogeneity and/or differences in tumor microenvironment. The remarkable plasticity of ILCs suggests new therapeutic approaches to induce differentiation/switch toward ILC subsets more favorable in tumor control.

Keywords: innate lymphoid cells, antitumor immune response, checkpoint inhibitors, natural killer cells, immunotherapy

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INTRODUCTION

Innate lymphoid cells (ILCs) belong to a family of immune cells involved in innate host defenses against pathogens and tumors. In addition, they play an important role in lymphoid organogenesis and, after birth, in the remodeling and tissue repair of mucosal epithelial cells. Five major groups of ILCs have been defined on the basis of the pattern of cytokine production and developmental transcription factor (TF) requirements, namely, natural killer (NK) cells and the non-cytolytic "helper-ILC," including ILC1s, ILC2s, ILC3s, and lymphoid tissue inducers (LTi) (1). Recently, Crome et al. described a new subset of ILC with regulatory function (regulatory ILC) (2). ILCs derive from a common CD34$^+$ haemopoietic precursor expressing the ID2 TF (3, 4). Remarkably, the ratio between different ILC subsets generated from precursors varies depending on the source of CD34$^+$ cells (e.g., umbilical cord blood vs. peripheral blood). In addition, the use of granulocyte-colony stimulating factor employed for CD34$^+$ cell mobilization in hemopoietic stem cell transplantation adds a further degree of complexity in the generation of different ILC, a finding that should be taken into account for therapeutic use (5). In this context, it should be underlined that ILCs display a high degree of plasticity, not only during their differentiation but also in their mature compartments. Thus, a shift from one to another ILC subset has been well-documented and implies that their functional capability may change substantially in given microenvironments,

particularly at tumor sites under the influence of soluble factors or cellular interactions (6). In addition to these polarizing signals in tissues, ILC plasticity depends on specific chromatin regions to be accessible to TFs, whereas regulatory regions controlling the expression of signature cytokine genes in CD4 T-helper cells become accessible only after activation by cytokine signals or infection. ILC regulatory elements are already poised or active before stimulation. This distinctive feature of ILCs suggests that ILC regulomes are highly prone to dynamic changes in response to the microenvironment (7).

ILCs are particularly enriched in barrier tissues such as gut, uterus, lung, and skin, suggesting that their main biological function is to respond rapidly to infections and to environmental or inflammatory signals (8–11). During the early phases of infections and/or tissue damage, ILC represent a suitable and rapid source of cytokines that play a critical role in the activation of immune responses, induction of neo-angiogenesis, and promotion of tissue repair and barrier integrity. Considering the particular set of cytokines produced, ILC subsets could play a role also in shaping the tumor microenvironment (TM) (12). Moreover, given their ability to produce large amounts of cytokines, ILCs may be important players in tumor-associated inflammation (13). While the role of NK cells in controlling tumor growth and metastasis is now well-established, the involvement of non-cytotoxic "helper" ILCs in tumors remains controversial and poorly understood. Studies in both murine experimental models and human patients are clearly needed to clarify whether ILCs may represent a reliable tool or a target for immunotherapy (14). NK cells are cytotoxic cells not only responsible for the innate cytolytic defenses against tumors and viruses, but they are also able to secrete proinflammatory cytokines, primarily interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). The antitumor effect of NK cells is well-established and extensively reviewed in recent publications (15–17). The main NK-mediated mechanisms of defense are related to their cytolytic activity and to cytokine production. However, even their activity may be sharply inhibited in the TM due to inhibitory factors or PD-1/PD-L1 interactions (18–21). In addition, particular conditions, such as low NK/tumor cell ratios, may favor tumor escape (IFN-γ-induced HLA-I upregulation in tumor cells or induction of epithelial–mesenchymal transition) (22). Overall, however, NK cells play a positive role against tumors, primarily by controlling the tumor growth and metastatic spread.

Helper ILC classification into ILC1, ILC2, and ILC3 is made on the basis of their TF profile and the set of cytokine produced (1). Recently, the presence of helper ILC in different tumor types has been documented (13). However, the actual involvement of ILC in antitumor defenses or in tumor progression through their ability to regulate inflammatory processes, neo-angiogenesis, tumor proliferation, and formation of tertiary lymphoid organs is far from being elucidated.

ILC1

ILC1s are Tbet^{+}, secrete IFN-γ and TNF-α, and are distinguished from NK cells because they are not dependent on Eomes and lack cytolytic activity (23). Although the hallmark of ILC1 is their ability to produce IFN-γ, a CD49a^{hi} ILC1-like population, capable of lysing tumor cells, has been described by Dadi et al. in mouse mammary tumors expressing granzyme B and TNF-related apoptosis-inducing ligand and intermediate levels of Eomes (24). Analogously, another group identified two distinct subsets of ILC1s in murine tumors. These cells are CD49a^{+}CD49b^{-}Eomes^{int} or CD49a^{+}CD49b^{+} Eomes^{+} ILC1-like cells, respectively. However, despite the presence of a lytic machinery, such ILC1s were found to be ineffective in controlling carcinogenesis and even potentially able to promote metastasis in a transforming growth factor beta (TGF-β)-rich TM. The authors suggested that these ILC1s could derive from NK cells and that TGF-β not only is responsible for such plasticity but can also suppress ILC1s antitumor function while promoting their proangiogenic effect (25).

In humans, CD56^{+}CD16^{-} cells were found in solid tumors as well as in peritoneal and pleural fluids of cancer patients. Because these cells also expressed CD9 and CXCR3, they were thought to derive from NK cells acquiring an ILC1-like phenotype. They produced VEGF suggesting that, analogous to ILC1-like subsets present in mice, such NK-derived infiltrating ILC1s might favor tumor growth (26). An analogy between this ILC subset and decidual NK cells has also been proposed. Indeed, both in TM and in decidua the presence of factors, such as TGF-β, could induce the conversion of conventional NK cells into cells with proangiogenic and immune-tolerant features (27).

In Crohn's disease, interleukin (IL)-12 signaling has been shown to convert RORγt^{+}ILC3s into RORγt^{-}Tbet^{high} ILC1s and induce IFN-γ production. While the IL-12Rβ1 is expressed at comparable levels by all ILC subsets, ILC1s were found to be the only ILC subset expressing the IL-12 receptor B2 subunit. Thus, IL-12 may be a key driver for the production of effector cytokines by ILC1. In this context, it is noteworthy that intestinal barrier defects are usually associated with higher levels of IL-12 production by mononuclear cells of the *lamina propria* (23). The ILC1s production of proinflammatory cytokines such as IFN-γ and TNF-α supports the hypothesis that they mainly contribute to the progress and chronicity of inflammation thus favoring the malignant transformation (17). However, the role of ILC1s in the development of tumors or in the control of their growth remains ambiguous. Thus, it has been reported that IFN-γ, a key cytokine produced by ILC1, may display either a pro- or antitumorigenic effect. In particular, the antitumor effects of IFN-γ include its ability to recruit and activate effector immune cells (by the upregulation of costimulatory molecules, cytotoxicity, and cytokine production) and to inhibit tumor growth (induction of apoptosis). On the other hand, the protumorigenic effect of IFN-γ consists in the induction of tumor escape mechanisms through the upregulation of ligands for the major inhibitory checkpoints (i.e., PD-L1) and HLA class I molecules as well as induction of epithelial–mesenchymal transition. On the other hand, ILC1-derived cytokines may also be involved in antitumor immunity, suggesting that the ILC1 function may depend on the microenvironment context. In general, the effect of IFN-γ is related to the tumor type/microenvironment and to the intensity of IFN-γ signal (28, 29).

ILC2

ILC2s express high levels of the TF GATA3 and are defined by their capacity to produce the type 2 cytokines IL-4, IL-5, and IL-13. ILC2s were shown to play a predominant detrimental role in various tumor settings (30). One of the first observations related to ILC2 and tumors was reported in 2014. In these studies, elevated ILC2 numbers and high levels of transcripts of ILC2-related genes including RORα, GATA3, and CRTH2 were found in peripheral blood of patients with gastric cancer (31). In addition, in acute promyelocytic leukemia, high numbers of ILC2 have been reported, which became activated upon sustained interaction of CRTH2 and NKp30 with their tumor-associated ligands (32). In acute promyelocytic leukemia, ILC2 could enhance the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs) through IL-13 production (32). In line with these findings, an ILC2–MDSC immunoregulatory axis was described in human bladder cancer and in murine prostate tumors. In bladder cancer patients who underwent the standard intravescical *Bacillus* Calmette–Guerin (BCG) immunotherapy, high numbers of tumor-infiltrating ILC2 were detected and found to correlate with low T cell/MDSC ratios, and unfavorable prognosis (33). *In vitro*, BCG was shown to stimulate the production of IL-13 by ILC2, which, in turn, supported both the recruitment and the immunosuppressive activity of MDSC. This suggested that the ILC2/IL-13 axis could counteract the effectiveness of the therapeutic activity of BCG in this tumor (33). On the other hand, ILC2 might also be involved in antitumor immune responses. Indeed, Kim et al. reported that growth of lymphoma cells injected subcutaneously in mice could be inhibited by local overexpression of IL-33, a major cytokine promoting differentiation and function of ILC2s. In this model, it was shown that ILC2s mediate antitumor activity either by producing cytokines relevant in tumor control (IL-5, IL-13, and GM-CSF) or by producing CXCR2 ligands (CXCL1 and CXCL2), which could induce the apoptosis of $CXCR2^+$ tumor cells (34). Although this study suggests an important antitumor role for ILC2s dependent on IL-33 activation, this role remains to be confirmed in a more physiological tumor setting, where IL-33 is not overexpressed.

A recent study investigated in murine models the role of ILC2s in both primary and metastatic lung tumors. It was reported that mice genetically lacking ILC2s displayed a significantly increased tumor growth rate and a higher frequency of circulating tumor cells resulting in increased metastatic spread to distal organs. In addition, the same authors demonstrated that *in vitro* ILC2 coculture with tumor cells, T cells, and peptide-pulsed dendritic cells correlated with increased MHCI expression on tumor cells and elevated level of Granzyme B expression, resulting in T-cell-mediated enhanced killing activity of tumor cells (35). Thus, although type 2 responses, in general, and ILC2s, in particular, have been associated to tissue remodeling and establishment of a tumor-promoting environment, these findings suggest that, at least in particular instances, they may play a favorable role against tumors.

ILC3

Among helper ILCs, ILC3s are $ROR\gamma t^+$ and secrete IL-22, IL-17, IL-8, and TNF-α. They are critical for maintaining mucosal tissue homeostasis, and their dysregulation has been associated to chronic intestinal inflammation and cancer. An association between IL-23-driven gut inflammation and tumorigenesis has been reported (36). Since IL-23 plays an important role in ILC3 development, it is not surprising that, as suggested by some studies (34, 35), the ILC3-derived IL-17 and IL-22 may contribute to the development of colorectal cancers. In this context, transgenic overexpression of IL-23 in wild-type mice was shown to be sufficient to induce adenoma formation in an ILC3-dependent manner, partly through IL-17 production (37). In addition, the production of IL-22 by $NKp46^-$ILC3 was shown to play a role in tumor maintenance in a bacteria-induced colon cancer model (38). A similar subset of $NKp46^-$IL-22-producing ILCs has been described to be able to regulate the activity and the expansion of T cells present in the TM (2). In this context, IL-17 and IL-22 produced by ILC3 may counteract tumor growth by favoring the recruitment of CD8 T cells, NK cells, and neutrophils. On the other hand, they may favor tumor proliferation/metastasis by inducing macrophage polarization toward M2 and Treg recruitment (38–40).

ILC3s are also involved in promoting tissue remodeling/repair and in maintaining tissues homeostasis. In particular, LTi-like cells are a subset of ILC3s that have been associated to improvements of antitumor immunity, by facilitating the infiltration of leukocytes in the tumor. In a skin metastasis mouse model, IL-12 has been shown to initiate local antitumor immunity by stimulating $NKp46^+$ LTi cells, which, in turn, induced upregulation of adhesion molecules in the tumor vasculature resulting in increases in leukocyte invasion (41). In non-small cell lung cancer, $NKp46^+$ILC3s with LTi-like properties were consistently found at the edge of intratumor tertiary lymphoid structures (that they contribute to organize through the production of TGF-β) and correlated with the density of such lymphoid aggregates within the tumor. Importantly, their presence was associated with a better clinical outcome. Also in non-small cell lung cancer, ILC3s induced the expression of adhesion molecules by newly formed endothelial cells in the tumor (9). Since the presence of tumor-associated ectopic lymphoid-like structures appears to correlate with a better prognosis in different tumors and considering that ILC3 may induce ectopic lymphoid-like structures formation and promote lymphoid organogenesis, the analysis of TI-ILC numbers and function may acquire an important prognostic value (42). Also in breast cancer, the presence of $ROR\gamma t^+$ ILC3s able to facilitate tumor invasion into lymphatic system has been reported, thanks to their ability to modulate the production of soluble factors present in the TM (43).

Similarly to what occurs for Th17 cells, also the involvement of ILC3s in cancer immunosurveillance is pleiotropic. Conflicting available data could reflect, at least in part, the heterogeneity of ILC3 subsets, the tissue of origin, and the type of microenvironment of different tumors.

INHIBITORY CHECKPOINTS

While NK cell activation and function is under the control of inhibitory receptors (checkpoints) such as the HLA-I-specific KIR and CD94/NKG2A (expressed constitutively) and PD-1, limited information exists on checkpoints possibly expressed by ILC3. In this context, PD-1, a well-known checkpoint controlling T-cell activation, has recently been shown to control also NK cell function (19, 21, 44). Checkpoint inhibitors, such as anti-PD-1 blocking antibodies, are currently used in the treatment of different advanced solid tumors. The unexpected positive clinical outcome has changed substantially the prognosis of tumors considered otherwise incurable (45). Notably, the selection of patients suitable for treatment is mostly based on percentages of PD-L1+ tumor cells in the tumor biopsy. However, the evaluations is made on heterogeneous tumor samples, such as surgical specimens or biopsies (in some instances inadequate in numbers), or using different anti PD-L1 clones, which may substantially vary in their reactivity, thus leading to possible discordances and incorrect clinical decisions (46, 47). The physiological role of PD-1 is important since it is involved in the induction and maintenance of peripheral immune tolerance. In this context, PD-1 expression at the feto-maternal interface during the early phases of normal human pregnancy seems to be crucial for a successful pregnancy. Peculiar NK cells and ILC3 are present in human decidua where they appear to play a relevant role in controlling the balance between inflammation and tolerance, as well as in inducing neoangiogenesis, tissue remodeling, and placentation (48). Indeed, during the first trimester, decidual ILC3 expresses high levels of PD-1. Specifically, high percentages of PD-1 were detected on both LTi-like cells and NCR^+ILC3. In addition, these cells were found to express/coexpress TIM-3, another immune checkpoint. Importantly, the PD-1 expression undergoes progressive reduction during pregnancy reaching significantly lower levels at the third trimester. In particular, the negative correlation between proportions of $PD\text{-}1^+$ILC3 and the stage of pregnancy suggests a role for $PD\text{-}1^+$ ILC3 in the control of early stages of implantation. Of note, $PD\text{-}1^+$ ILC3 displays a reduced capacity to release cytokines as compared to the $PD\text{-}1^-$ ILC3 population. Moreover, mAb-mediated PD-1 cross-linking results in inhibition of the production of IL-22, IL-8, and TNF-α by $PD\text{-}1^+$ ILC3 (20).

The immune checkpoint-mediated inhibitory mechanisms on T-cell (and, in part, NK cell) function has been studied primarily in tumors. Along this line, we have recently shown that also different ILC subsets are present in pleural effusions (PEs) from primary (mesothelioma) or metastatic tumors (adenocarcinoma). Thus, besides NK cells, helper ILC including ILC1, ILC2, and ILC3 were also detected in PEs in cancer. Upon specific stimulation, ILCs produce their typical cytokines suggesting that they could mediate an antitumor effect. Notably, both PE-NK cells and PE-ILC3 may express functional PD-1, while tumor cells may express PD-L1, suggesting a PD-1-mediated inhibitory effect on cells with potential antitumor activity (19).

Besides pregnancy and tumor settings, PD-1 expression was reported in mice on ILC-committed progenitors, capable of generating ILC1s, ILC2s, and ILC3s and a small number of circulating NK cells (49, 50). PD-1 expression is lost upon differentiation but expressed on effector tissue resident ILC2s upon IL-33 stimulation, resulting in reduction in their ability to release cytokines (49, 51). In addition to PD-1, it was reported that ILC2s express PD-L1 during murine pulmonary infection, and their interaction with PD1-expressing $CD4^+$ T cells favors Th2 polarization (52). While the role of PD-1 expression on ILC2s seems to be relevant mainly in infections, these findings suggest that blocking PD-1/PD-L1 axis in the context of cancer could also affect type 2 responses. The possible favorable or unfavorable contribution of this ILC2-mediated response to therapy with checkpoint inhibitors should be further explored to improve the efficacy of cancer treatment.

Another inhibitory checkpoint expressed by ILCs is OX40L, which could interact with its natural receptor (OX40) on Th1 or Th2 cells. In particular, $OX40L^+$-ILC2 induce an expansion of Th2 cells and Treg, suggesting a dual role of ILC2 in the induction or regulation of immune response (53). It is known that inflammatory stimuli (i.e., TNF-like ligand 1A) upregulate OX40L on ILC3 that, in turn, induce Th1-type inflammatory responses *in vivo*. It is possible to speculate that, in the absence of inflammatory stimuli or in TM, ILC3 could not induce T-cell responses against tumor cells (54).

CONCLUSIONS

A number of experimental evidences have highlighted the relevant role of cells of the innate immunity in the positive or negative regulation of tumor growth and metastasis. Thus, polarization of different innate cell types may determine their effect on tumor control (6). While the first seminal evidences of the consequence of cell polarization at the tumor site were provided by Mantovani and colleagues for tumor-associated macrophages (55), other innate cell types present in the TM were subsequently shown to favor tumor escape from antitumor immune responses. Among ILCs, ILC2 may shape downstream adaptive immunity and favor "negative" type 2 responses. Notably, mechanisms similar to those exploited by tumors to subvert immune responses are known to play a physiological role in certain tissue environments. This is evident in decidua where NK cells, ILC3, macrophages, and other myeloid cells are polarized toward suppressive functional activities. The recent finding that PD-1 may be expressed also by NK cells provided an important information for the usefulness of therapy with mAbs disrupting the PD-1/PD-L1 axis in tumors that have lost (partially or completely) HLA-Cl-I molecules, thus becoming undetectable by cytolytic T cells. Indeed, in HLA-Cl-I-defective tumors, the function of $PD\text{-}1^+$ NK cells can be unleashed, thus restoring their antitumor activity. As shown recently, a further potentiation of NK cell function can be achieved by blocking KIRs and/or NKG2A (56, 57) or using a monoclonal antibody (Ipilimumab) able to block the interaction between CTLA4 and its ligand B7 (58, 59). Of note, in the haploidentical hemopoietic stem cell transplantation setting, this blocking may render "alloreactive" all donor NK cells. In addition, the plasticity of ILC could be better exploited to favor the generation of cell subsets useful for their antitumor activity. This may be achieved *in vitro* not only by

inducing (with appropriate cytokines) differentiation of $CD34^+$ progenitors toward given ILC but also by acting on mature ILC or their precursors.

In conclusion, although the recent years, thanks to immunotherapy, witnessed unprecedented progresses toward the cure of solid tumors and high-risk leukemia, it is evident that further progresses are required, which may be based on better knowledge of the pathophysiology of different cell types, including ILCs and on new combined therapies, to minimize the effect of tumor escape mechanisms (60).

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All authors discussed together the general outline of the article and contributed to the elaboration of the final version of the manuscript. NT, PV, LQ, and LM wrote the first draft that was subsequently reviewed by EM, FMo, AP, and FMa.

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REVIEW
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Pleiotropic Role and Bidirectional Immunomodulation of Innate Lymphoid Cells in Cancer

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Innate lymphoid cells (ILCs) are largely tissue resident and respond rapidly toward the environmental signals from surrounding tissues and other immune cells. The pleiotropic function of ILCs in diverse contexts underpins its importance in the innate arm of immune system in human health and disease. ILCs derive from common lymphoid progenitors but lack adaptive antigen receptors and functionally act as the innate counterpart to T-cell subsets. The classification of different subtypes is based on their distinct transcription factor requirement for development as well as signature cytokines that they produce. The discovery and subsequent characterization of ILCs over the past decade have mainly focused on the regulation of inflammation, tissue remodeling, and homeostasis, whereas the understanding of the multiple roles and mechanisms of ILCs in cancer is still limited. Emerging evidence of the potent immunomodulatory properties of ILCs in early host defense signifies a major advance in the use of ILCs as promising targets in cancer immunotherapy. In this review, we will decipher the non-exclusive roles of ILCs associated with both protumor and antitumor activities. We will also dissect the heterogeneity, plasticity, genetic evidence, and dysregulation in different cancer contexts, providing a comprehensive understanding of the complexity and diversity. These will have implications for the therapeutic targeting in cancer.

Keywords: ILCs, cancer, tumor immune microenvironment, immunosurveillance, heterogenity, plasticicity, immunothearpy

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INTRODUCTION

Innate lymphoid cells (ILCs) derive from common lymphoid progenitors (CLPs) (1, 2) and are regulated by multiple endogenous signals including neuropeptides, hormones, cytokines and other alarmins (3). Tissue-resident ILCs represent a heterogeneous group of cells that interact with a wide variety of hematopoietic and non-hematopoietic cells through direct or indirect communication. These ILCs act as functional effectors contributing to the integration of innate and adaptive immune responses and the orchestration of physiological and pathological processes throughout the body (1). ILCs are considered to be counterparts of T-cell subsets resembling both their phenotypical and functional characteristics. Five subgroups of ILCs have been classified as natural killer (NK) cells and helper-like ILCs (ILC1s, ILC2s, and ILC3s) and lymphoid tissue inducer (LTi) cells in terms of their lineage-determining transcription factors and cytokine secretion profiles according to a redefined nomenclature (4) (**Figure 1**). NK cells derive from NK-cell precursors (NKP), which directly differentiate from common innate lymphoid progenitors (CILPs),

whereas other ILCs derive from common helper innate lymphoid progenitors (CHILPs) (2, 5–7). CHILP can differentiate to ILC precursors (ILCPs) and LTi precursors (LTiPs) that give rise to all ILC subsets (ILC1, ILC2, and ILC3) and LTis, respectively (4, 8). The transcriptional repressor, inhibitor of DNA binding 2 (Id2), is sequentially expressed in the ILC lineage framework, and Id2-dependent precursors can further differentiate with lineage-specific transcription factors (9). Data from both humans and mice demonstrate that LTi cells and conventional NK (cNK) cells are developmentally related yet represent distinct lineages (10–12). Recent studies reveal the previously unappreciated ILCP heterogeneity by using polychromic reporter mice to identify the ILCP lineage. The results suggest that a fraction of CHILP highly expresses PLZF (Zbtb16) and PD-1 and retains potential for all CD127^{hi}ILCs but not LTis. In addition, these cells can also generate NK cells that express Eomes and perforin, indicating additional lineage potential (13, 14). Likewise, an analysis of Id2 and PLZF reporter mice reveals that Id2^{+}Zbtb16^{+}ILCPs define multipotent NK and/or ILCPs and are associated with loss of LTi potential at a clonal level, suggesting a revised model for ILC differentiation that redefines the cell-fate potential of helper-ILC-restricted Zbtb16^{+}ILCPs (15) (**Figure 1**).

NK cells and ILC1s share the same transcription factor T-bet and produce type 1 cytokines, including interferon-gamma (IFNγ) and the tumor necrosis factor-alpha (TNFα), in response to interleukin (IL)-12 and IL-18, whereas NK cells also require the transcription factor eomesodermin (Eomes) and produce cytotoxic proteins, perforin, and granzymes in mice and granulysin in humans (16). NK cells and ILC1s are functionally analogous to cytotoxic CD8^{+} T cells and CD4^{+} T helper1 (Th1) cells, respectively. ILC2s depend on GATA3 and produce IL-4, IL-5, IL-9, IL-13 (17, 18), and amphiregulin (AREG) (19) in response to IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which resembles the Th2 cells response (4). ILC3s are heterogeneous but consistently require transcription factor RORγt for their development and function. LTis are related to ILC3s with distinct functions in the secondary lymphoid organ formation during both embryonic and adult stages of development (8, 11, 20). In general, these cells found in adult mice are termed LTi-like cells, which have a similar phenotype to LTis (21). Both ILC3s and LTis are capable of producing IL-17, IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in response to IL-23, IL-1β, or natural cytotoxicity receptor ligands (NCR-L) that reflect Th17 response (3, 22). Regardless of the functional association with ILC3s, LTis are considered a separate ILC lineage. Fetal liver-derived LTiPs express α4β7 integrin and C-C Motif Chemokine Receptor 6 (CCR6), whereas adult LTis derived from bone marrow precursors display upregulated RORγt in a Notch-dependent manner (9, 20). Adult mice also have T-bet-expressing CCR6^{-}NKp46^{+}ILC3s derived from CCR6^{-}NKp46^{-}ILC3s (23) (**Figure 1**).

In contrast to the polarized T-cell subsets that take over several days for activation and clonal expansion, most ILCs can produce significant amounts of cytokines upon stimulation without further differentiation. Hence, ILCs are considered to be the first line of defense to confront and sense the changes in the local environment and then react rapidly as the host response in peripheral tissue (24). Dysregulation of ILCs manifested by the changes in cell numbers or subset proportions is associated with diverse inflammatory diseases and cancer (25–28). The role of ILCs in cancer is ambivalent based on both protumor and antitumorigenic activities depending on their phenotype, the variety of cancer, and the tumor microenvironment (TME) context. The remarkable heterogeneity, distinct signature cytokines they produce, the various surface markers they express, and the plasticity among all the different subsets make ILC functions divergent and less comprehensible.

Given that the role of NK cells in cancer has been extensively reviewed (29, 30), this review will mainly focus on helper-like ILCs (ILC1s, ILC2s, and ILC3s) and LTis in the context of cancer. The plasticity of ILCs and their interactions with the extracellular matrix (ECM) and adaptive immune cells shape the TME. In return, changes in the secretion of cytokines by these cells also polarize ILC functions and influence plasticity under pathological conditions in the TME. These bidirectional interactions are crucial for controlling tumor growth and metastasis. Untangling the pleotropic roles and bidirectional regulations of ILCs in cancer will ultimately help to provide a rationale for the design of therapeutic strategies for cancer treatment.

INNATE LYMPHOID CELLS AND TUMOR IMMUNE MICROENVIRONMENTS

Tumor-infiltrating leukocytes were first discovered in the 1800s, suggesting a functional relationship between immune cells and cancer (31). These infiltrating immune cells had been considered to have antitumor properties only until the last three decades, when the adverse role of promoting tumor progression has come into light. Tumor development and progression are profoundly influenced by a variety of resident host cells, stromal cells, the ECM, the blood and lymphatic vascular networks, infiltrating immune cells, and signaling molecules including growth factors, cytokines, and chemokines, collectively known as the TME (32–35). Recognizing the importance of the TME in cancer has revolutionized cancer treatment from targeting tumor cells to deciphering the tumor ecosystem complexity. This fundamentally determines the fate of the primary tumors whether it is eradicated or a premetastatic niche is established that favors metastatic dissemination (36, 37). Substantial immune cell infiltration varying in size, composition and distribution is a distinct characteristic present in almost all types of malignant tumors. These infiltrating immune cells, including tumor-associated macrophages (TAMs), mast cells, T cells and ILCs within the TME, comprise the tumor immune microenvironment (TIME) (26, 38–43).

Each of these immune components is believed to be involved in tumorigenesis, tumor invasion, and metastasis (44, 45). From immunosurveillance to tumor escape, infiltrating immune cells mutually interact with other cells within the TME niche and play a dual role that can either promote or attenuate malignant outgrowths. Given that tumor-induced immunological changes affect metastatic progression even before disseminated tumor cells reach secondary organs (38), targeting the premetastatic

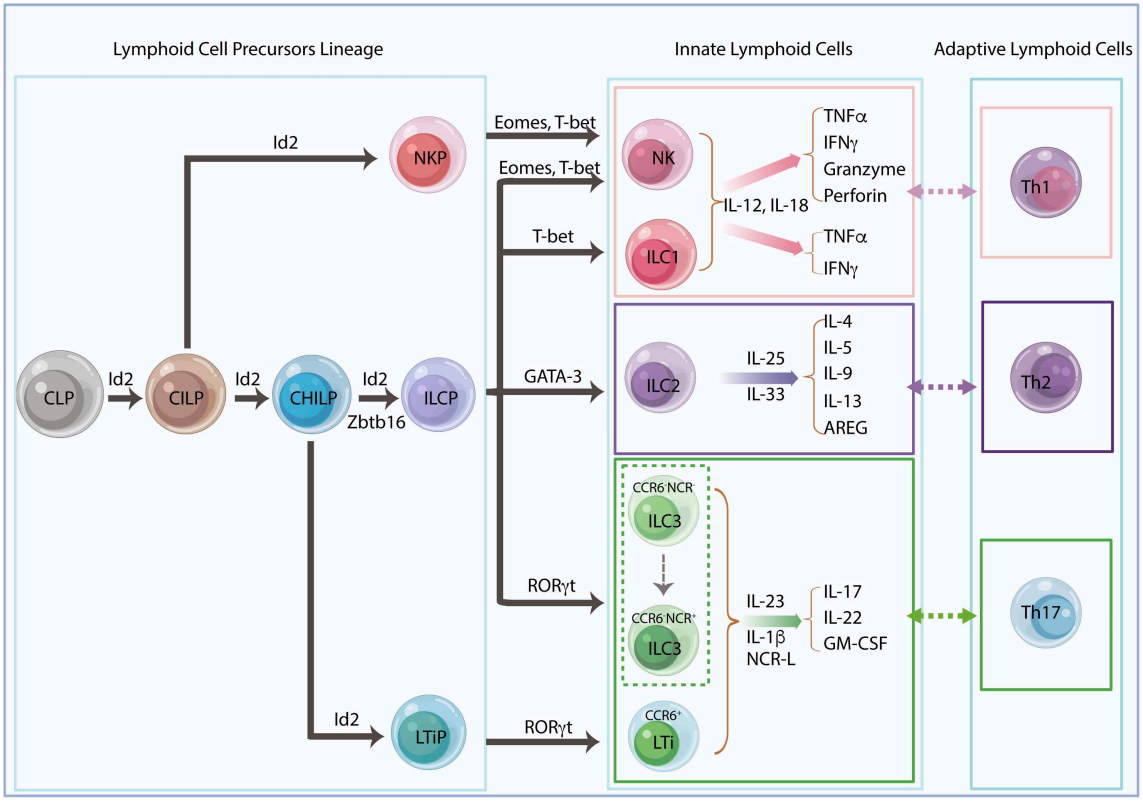

FIGURE 1 | Innate lymphoid cell lineage and classification. Innate lymphoid cells (ILCs) derive from common lymphoid progenitors (CLPs) and give rise to common innate lymphoid progenitors (CILPs) and common helper innate lymphoid progenitors (CHILPs). CILPs differentiate into natural killer (NK) cell progenitors (NKPs) and then terminally differentiate into NK cells. CHILPs divide into innate lymphoid cell progenitors (ILCPs) and lymphoid tissue inducer progenitors (LTiPs), which generate all helper-like ILC subsets, ILC1s, ILC2s, ILC3s, and LTis, respectively. The transcriptional repressor Id2 is developmentally required and sequentially expressed in the ILC precursors. Id2-dependent precursors can further differentiate with lineage-specific transcription factors. Recent studies suggest that Zbtb16^{+}ILCPs harbored extensive NK and/or ILC precursors potential, indicating a revised model for ILC differentiation. The different ILC subsets need their unique transcription factors for development and secretion of their signature cytokines in response to different stimulators, which mirror the phenotype and the function of adaptive helper T cells, Th1, Th2, and Th17, respectively.

TIME is believed to be critical for the success of therapeutic strategies (40, 42, 46).

ILCs are tissue-resident cells in lymphoid and non-lymphoid organs and functionally act as sentinels to maintain and shape tissue microenvironment. They respond rapidly to environmental changes by secreting significant amounts of cytokines and expanding locally under physiologic and pathological conditions, suggesting a role for these cells in the early phases of tumorigenesis and in shaping the TME (46).

ILCs in Extracellular Matrix Remodeling

ECM is a major component of the local TME, and its remodeling plays a prominent role in tumor development. ECM dysregulation promotes cancer cell invasion, induces angiogenesis and facilitates immune cell infiltration (47). Activated immune cells including ILCs closely interact with ECM, which may decisively affect the outcome of tumor progression (42).

Among the ILC family members, NK cells were first identified as being involved in the remodeling of the tumor ECM. NKp46-mediated IFNγ secretion increases the expression of fibronectin 1 (FN1) by tumor cells leading to ECM structural changes in the primary tumors and decreased metastasis (48). Reciprocally, FN1 stabilizes NK cell survival and facilitates NK cell migration by inducing anti-apoptotic protein B-cell leukemia 2 (Bcl-2) (49). These data suggest a bidirectional regulation between NK cells and ECM remodeling. IL-12-responding ILC1s induce a signature TME characterized by strong upregulation of IFNγ and type I-associated chemokine receptors (CXCR6, CCR5, CXCR3, and CCR1) to recruit tumor invading *Rorc*$^{fm+}$ILCs in a mouse model of melanoma (50). Adipose ILC1s were also found to contribute to adipose tissue fibrogenesis in humans (51). Other studies of ILC2s show that activation by IL-33 induces IL-13 secretion, which in turn stimulates hepatic stellate cells to produce ECM proteins leading to pathologic tissue remodeling in mouse liver (52) (**Figure 2**). Depletion of RORγt-dependent LTis and RORγt-independent ILCs impairs lung function and tissue repair, whereas IL-33/IL-33R signaling is critical for lung

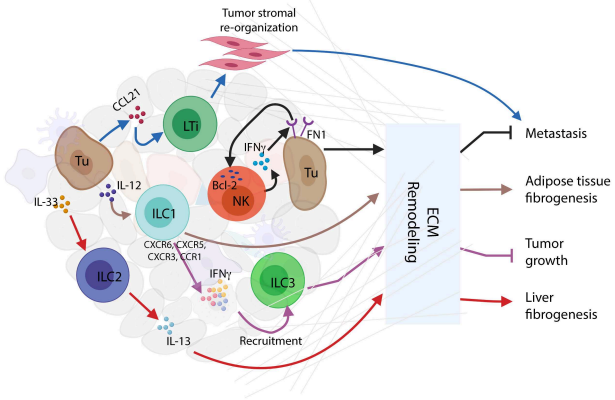

FIGURE 2 | Innate lymphoid cells (ILCs) and extracellular matrix (ECM) remodeling. Activated ILC interactions with ECM in tumor immune microenvironment (TIME) conclusively affect the consequences of tumor progression. Interferon-gamma (IFNγ) secreted by natural killer (NK) cells stimulate tumor cells to express FN1 that causes ECM remodeling resulting in the inhibition of tumor metastasis. Mutually, FN1 stabilizes NK cell survival and facilitates NK cell migration through the induction of B-cell leukemia 2 (Bcl-2) expression. In response to IL-12, ILC1s upregulate several cytokines and chemokines, such as IFNγ, CXCR6, CCR5, CXCR3, and CCR1, to recruit ILC3s to the tumor site, thus causing ECM remodeling and tumor growth repression. Through ECM remodeling, adipose ILC1s contribute to adipose tissue fibrogenesis. Similarly, in mouse liver, IL-33-activated ILC2s release IL-13 to stimulate hepatic stellate cells to produce ECM proteins, thus inducing liver fibrogenesis. Moreover, tumor cells recruit CCL21 to activate LTis, whereby causing tumor stromal reorganization and facilitation of lymph node metastasis.

ILC response through the production of AREG via IL-13/ IL-22-independent mechanisms (53).

As described above, the remodeling of ECM causes organ dysfunction and contributes to chronic disease mechanisms including cancer. Innate immune cytokines derived from ILCs subsets directly regulate fibroblast functions that are independent of adaptive immunity (54). Taken together, these studies demonstrate a critical role for ILCs as important regulators of cytokine networks involved in ECM remodeling and the consequences for health and disease. This also suggests the clinical relevance of controlling pathologic remodeling to prevent metastasis.

ILCs in Tumor Angiogenesis and Lymphatic Vascular Networks

Tumoral angiogenesis is a characteristic feature of tumor survival and progression, which differs considerably from the vascular structure generated for the regular blood vessels (55, 56). The formation of new blood vessels in tumors is driven by hypoxic tumor cells, tumor-associated stromal cells (TASCs), and the ECM in which they are embedded, producing vascular endothelial growth factor A (VEGFA) to initiate tumor angiogenesis (57). Tumor-infiltrating immune cells, such as ILCs, are also important pro-angiogenic mediators to increase VEGFA bio-availability and signaling during the angiogenic switch (58). ILCs stimulate endothelial cell proliferation and upregulation of adhesion molecules by releasing pro-angiogenic factors, contributing to immune cell recruitment and perpetuation of inflammation (42, 55, 56, 59).

Tumor-infiltrating NK cells, transformed from $CD16^{+}$NK to $CD16^{-}$NK subset upon transforming growth factor-beta (TGF-β) stimulation, predominate in non-small-cell lung carcinoma (NSCLC) and produce elevated levels of pro-angiogenic factors such as VEGF and placenta growth factor (PIGF) to sustain tumor progression (55, 60, 61). This pro-angiogenic effect can be reversed by transcriptional factor STAT5 via repressing VEGFA transcription in NK cells, in both mice and humans (62). The secretion of VEGF by NK cells is associated with angiogenesis in human melanoma, and breast and colon carcinoma (42). These studies suggest that the TIME is able to functionally affect NK cells by inhibiting their cytotoxic ability or promoting pro-angiogenic phenotypes by a wide array of cytokines and soluble factors such as TGF-β, PGE2, VEGF, and adenosine (55). Likewise, tumor-infiltrating ILC1s in mouse fibrosarcoma also showed upregulation of angiogenesis gene sets that also expressed in NK cells (63). ILC1s impaired tumor neovascularization mediated by IL-12, which required the presence of NK cells to induce endothelial cell cytotoxicity in lymphomas (64). ILC1s produce two signature cytokines, IFNγ and TNFα, which are associated with cell proliferation and angiogenesis. IFNγ activates the transcription factor STAT1 to inhibit tumor cell proliferation and angiogenesis (65, 66). TNFα can either destroy tumor vasculature and induce apoptosis as an antitumor effector or stimulate the expression of angiogenic and growth factors to promote tumor formation and growth (38). ILC2s in response to IL-33, the cytokine that induces angiogenesis and vascular permeability through ST2 receptor binding (67), can enhance the re-epithelialization and promote the restoration of skin integrity after injury (68). ILC3s contribute to preserving epithelial integrity and maintaining tissue homeostasis by the release of IL-22 (59). Secretion of IL-17 by ILC3s promotes angiogenesis via stimulation of vascular endothelial cell migration and cord formation, resembling those indirect angiogenic stimulators such as TGF-β and platelet-derived growth factor B subunit homodimer (PDGF BB) *in vivo* (69). The indirect role of ILC3s in tumor angiogenesis is also manifested by their recruitment of myeloid-derived suppressor cells (MDSCs) and regulatory T cell (Treg) cells, which in turn promote M2-like macrophages in the TIME (70, 71). Apart from IL-17 and IL-22, the LTi-like neuropilin (NRP)1^{+}ILC3 subset was also found to release CSF2, TNFα, B-cell-activating factor, and CXCL8, in association with VEGF production that might contribute to angiogenesis (59) (**Figure 3**).

The other prominent feature of tumor angiogenesis is the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM), which conveys the apparent tumor-immune privilege. In a subcutaneous melanoma mouse model, $NKp46^{+}$LTi cells alter the tumor microvasculature upon IL-12 stimulation, which leads to upregulation of VCAM and tumor suppression (72). Indeed, LTis modulate not only blood vasculature but also

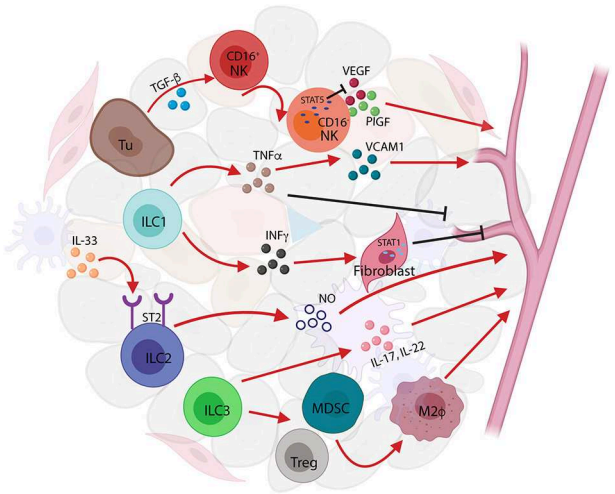

FIGURE 3 | Innate lymphoid cells (ILCs) in tumor angiogenesis. ILCs act as tumor angiogenesis modulators by releasing pro-angiogenic factors and by inducing the recruitment and infiltration of immune cells to affect tumor-related inflammation. Transforming growth factor-beta (TGF-β) secreted by tumor cells activate natural killer (NK) cell to produce vascular endothelial growth factor (VEGF) and placenta growth factor (PIG) to induce tumor angiogenesis; conversely, the transcription factor STAT5 represses the expression of VEGF resulting in the inhibition of angiogenesis and tumor growth. ILC1s produce two signature cytokines, interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα), that are associated with cell proliferation and angiogenesis. TNFα secreted by ILC1s increases vascular cell adhesion molecule (VCAM)1 expression causing tumor vascular formation, whereas in a different context, TNFα-producing ILC1s can either destroy tumor vasculature or induce apoptosis acting as antitumor effectors. Furthermore, IFNγ released from ILC1s causes STAT1 activation, thereby inhibiting angiogenesis formation. ILC2s respond to IL-33 and induce angiogenesis and vascular permeability through ST2 receptor binding. IL-17 and IL-22 released by ILC3s promote angiogenesis via stimulation of vascular endothelia cell migration and cord formation. The indirect role of ILC3s in tumor angiogenesis is also shown in the recruitment of myeloid-derived suppressor cells (MDSCs), regulatory T cell (Treg) cells, and the promotion of M2-like macrophages in the tumor immune microenvironment (TIME).

the lymphatic vascular system. LTis induce mesenchymal stem cells (MSCs) to produce chemokines, CCL19, CCL21, or CXCL13, which promote lymphocyte recruitment and spatial compartmentalization (73). This cross talk also plays a role in promoting lymph node metastasis in breast cancer. In the 4T1.2 triple-negative breast cancer (TNBC) mouse model, ILC3s are recruited to the primary tumors by CCL21 and stimulate tumor stromal cells to release CXCL13, leading to enhanced tumor cell motility, lymphangiogenesis, and lymph node invasion by tumor cells (74). These data suggest that the number of infiltrating ILCs within the primary breast tumors could be used as a predictor of metastatic and malignancy potential (74).

Tumor angiogenesis and lymphatic vascular formation prompt tumor invasion and metastasis, the landmark events that transform a locally growing tumor into a systemic metastatic and life-threatening disease. As tumor-infiltrating ILCs can polarize the TME to either protumor or antitumor effects by the modulation of angiogenic activities and lymphatic vascular networks, these cells represent valid targets for antitumor immunotherapy and cancer preventive strategies (55).

Interplay Between ILCs and Cytokines, Chemokines and Growth Factors in Tumor Immune Microenvironment

Initiation of ILC response relies on sensing the cytokines, alarmins, and inflammatory mediators that are derived from tissue sentinels such as myeloid cells, dendritic cells (DCs) and macrophages, or epithelial cells to translate environmental signals into a specific cytokine profile (75). The complex, diverse and dynamic interplay with surrounding environments amplifies ILC signaling and determines their function. Tumor-infiltrating immune cells engage in an extensive and dynamic interaction with TIME and shape the TME, whereas tumor cells also induce an immunosuppressive microenvironment by the secretion of the cytokines and other soluble factors (33).

In a model of subcutaneous melanoma, ILC1s respond to IL-12, produced by tissue sentinels such as DCs and macrophages, and alter the TME at an early stage of tumor development to facilitate tumor suppression by infiltrating immune cells (72). The production of TGF-β in the TME drives ILC fate outcomes between the ILC subsets from NCR^{-}ILC3s to NCR^{+}ILC3s (76). In human ovarian cancer, IL-22-producing ILC3s prevent tumor-infiltrating lymphocyte activation and proliferation (27), whereas the release of IL-22 and IL-17 by ILC3s modulates intestinal immune pathology (77). In a preclinical mouse model of human breast cancer, $ROR\gamma t^{+}$ILC3 promoted lymphatic metastasis by modulating the local chemokine milieu in the TME (74). Genetic mapping of the fate of ILC3 revealed that different subsets of ILC3 are phenotypically adapted to the local tissue environment they invade, indicating that the function of ILCs is shaped not only during their lineage commitment but also by the TME (50). Although ILCs are characterized by the distinct signature cytokine they express, tissue-derived cytokines can extrinsically shape ILC function (50), and this cross talk can drive both positive and negative consequences in tumor progression (78).

Interestingly, exosomes (extracellular vesicles, 50–150 nm in size released from the endocytic compartment) (79) derived from both tumors and stromal cells have been implicated as signaling mediators between cancer cells and surrounding cells that comprise the TME and contribute to therapy resistance. Several studies have identified the role of exosomes in premetastatic niche establishment that is favorable for future dissemination and metastatic seeding through the communication with immune cells like NK cells, macrophages, B cells, and T cells. Recent studies also revealed that chemotherapy-elicited extracellular vesicles in breast cancer cells can promote premetastatic niche formation in lung by delivering annexin 6 to induce CCL2, which promotes monocyte activation (80). The direct correlation of exosomes with ILCs has not been explored yet, although AREG, which is produced by ILC2s, can be found in distinct nanoparticles (DNPs), a subset of extracellular vesicles that are smaller than 50 nm with a dot-shaped morphology (81). Further investigation of ILCs and exosome communication

would potentially allow the development of effective clinical applications that may overcome the therapeutic resistance.

Interplay Between ILCs and Adaptive Immunity

Emerging evidence indicates that ILCs play an important role in coordinating tissue-specific adaptive immunity and act as master regulators in homeostasis and diseases. Substantial studies have demonstrated the direct communication between ILC2s and ILC3s with the adaptive immune system, whereas with ILC1s, it remains less well-investigated (21).

ILCs share several developmental and transcriptional signatures with T-cell subsets but have additional and/or unique, non-redundant functions in T-cell polarization and effector functions by secretion of regulatory cytokines, antigen presentation, or direct cellular interactions within complex tissue microenvironments. ILC2s functionally resemble Th2 cells and play a critical role in the type II immune response. ILC2-derived cytokine IL-13 stimulates tissue DC recruitment to the draining lymph node for eliciting production of Th2 cell-attracting chemokine CCL17, causing the Th2 response (82). The protumor activity of IL-13 is also involved in the activation and differentiation of MDSCs by induction of Treg cell expansion to establish an immune-suppressive TME (83). These studies may indicate that the role of ILC2/IL-13 axis in the initiation of cancers is linked to extensive tissue remodeling. During intestinal or lung inflammation, tissue-specific IL-33 secretion upregulates OX40L expression on ILC2s resulting in protective Th2 immunity and Treg cell expansion, which could be related to the induction of a tolerant microenvironment and inhibition of antitumor responses (84). In addition, ILC2-derived AREG limits antitumor immunity by stimulating Treg cell expansion, supporting the establishment of an immune-suppressive TME (85). ILC2s have been suggested as a key source of Th2 master regulator IL-4 (86), and the previously unrecognized role of ILC2-derived IL-4 in Th2 differentiation may indicate the potential involvement of ILC2/T-cell interactions in promoting tumor growth and metastasis (38). Notwithstanding the protumor role of ILC2s in cancer, ILC2-derived cytokines such as IL-5 are involved in an antitumor function. In a melanoma model, increased IL-5 production by ILC2 resulted in the recruitment and activation of eosinophils, which was correlated with lung tumor metastasis. Moreover, ILC2-derived IL-9 has recently gained attention in the context of tumor biology and has functional importance as growth factor for ILC2 and survival factor for other cell types (83). In a melanoma model, IL-9 deficiency promoted tumor growth, whereas IL-9 treatment decreased metastasis (87). It is possible that the role of IL-9 in cancer is associated with the promotion of infiltrating DC antigen presentation (88). Conversely, high expression of IL-9 was associated with T-cell transformation in humans, indicating that the anti-apoptotic properties on transformed cells promote tumorigenesis (89) (**Figure 4**).

Increasing evidence over the past decade also indicated the role of ILC3s in bridging innate and adaptive immune responses. In a bone marrow chimeric mouse model, RORγt$^+$ILCs (LTis) express OX40L and CD30L to regulate memory CD4$^+$ T-cell survival but not that of CD8$^+$ T cells in the homeostatic state (90), whereas in normal intestines ILC3s limit T-cell activation but decrease the regulatory effects under an inflammatory microenvironment through MCH-II presentation (91), indicating that the function of ILCs can be altered by mutual interaction with the microenvironment. ILC3s localize at the boundary between B-cell follicles and T-cell zone within intestinal draining lymph nodes to regulate T follicular helper cells (Tfh) responses and B-cell class switching via antigen presentation (92). In contrast to peripheral lymph nodes, resident ILC3s at mucosal draining lymph nodes express MHC-II and contribute to a distinct microenvironment constraining CD4$^+$ T-cell response to commensal bacteria in a contact-dependent manner dependent on CCR7 (93). Commensal microbiota and IL-1β stimulate the release of GM-CSF and IL-22 by ILC3 through MHC-II-dependent interactions to promote intestinal Treg cell expansion (94, 95). In addition, IL-1β stimulation leads to activation of peripheral ILC3s and consequently primes the CD4$^+$ T-cell immune response, whereas T cell-derived effector cytokines also cross-regulate the ILC response. The cognate interaction of ILC3 and CD4$^+$ T cells is bidirectional, indicating an unappreciated role of ILC3s in T cell-mediated immunity (96). ILC3 also activates local and systemic inflammatory T cells by increasing TNF-like ligand 1A (TL1A) and IL-1β (82, 95). In the peritoneal B-cell compartment, RORγt$^+$ILCs produce soluble lymphotoxin α3 (sLTα3) and membrane-bound lymphotoxin β (LTβ), both trimeric cytokines of the TNF superfamily, resulting in T cell-dependent and T cell-independent IgA production, respectively (97). Moreover, the adherent microbiota stimulates ILC3 to secrete IL-22 that triggers epithelial serum amyloid A protein (SAA) production to promote local Th17 responses in a STAT3-dependent manner (98) (**Figure 4**). The proliferative and anti-apoptotic capacities of ILC3-derived IL-22 may support malignant transformation in chronic inflammatory diseases. Those studies revealed the potential function of ILC3s as antigen-presenting cells and directly modulate adaptive immune responses. Even though ILCs show striking similarities to T-cell subsets, the non-redundant functions go beyond those shared with T cells through indirect and direct interaction with adaptive immune cells. In turn, adaptative immunity reciprocally regulates ILCs, which indicates that these bidirectional regulations are a crucial determinant of immune response within tissues. Given that the abundant evidence is mostly established under inflammatory conditions, a functional analysis between ILC3s and adaptive immunity in chronic inflammatory diseases may also imply the role of these interactions in the malignant transformations.

Collectively, these studies demonstrate key innate mechanisms in the regulation of the adaptive immune response and shed light on the tissue ILC-mediated control of adaptive immunity in orchestrating inflammatory and restorative tissue responses (99). Delineating the complex network of ILCs with other immune cells interactions will allow the manipulation of their specific function and ultimately open a way to innovative therapeutic strategies (41).

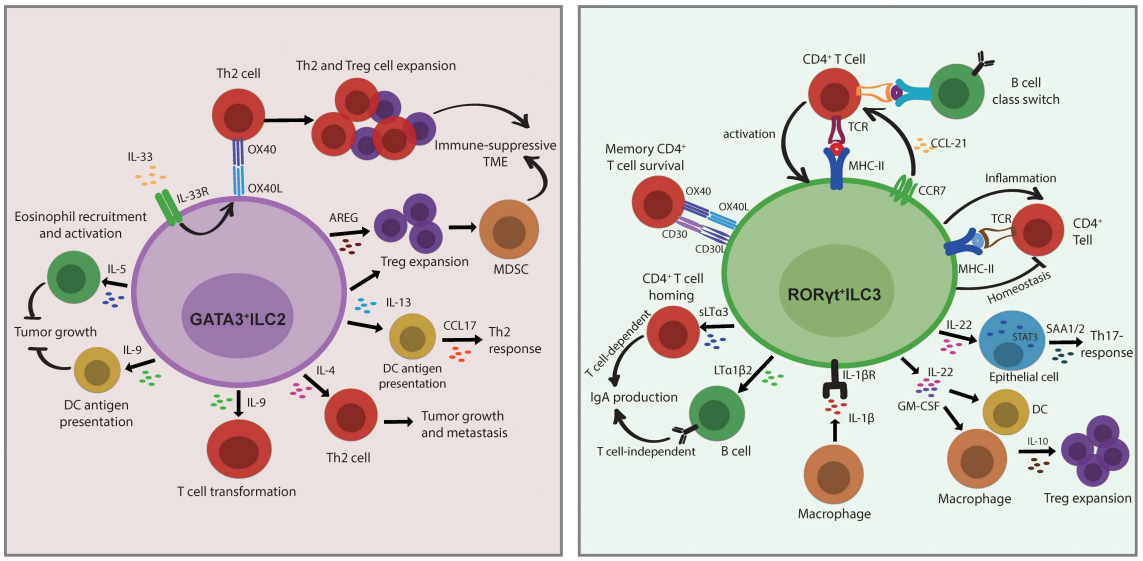

FIGURE 4 | Innate lymphoid cells (ILCs) modulate adaptive immunity by direct or indirect interactions with other cells in the tissue. ILC2s functionally resemble Th2 cells and play a critical role in the type 2 immune response. The role of ILC2s in the establishment of an by direct interactions with Th2 cells through OX40L/OX40 under IL-33 stimulation or through indirect interactions by the section of IL-13 or amphiregulin (AREG), which then causes regulatory T cell (Treg) cell expansion and myeloid-derived suppressor cell (MDSC) activation. ILC2-derived IL-13 also stimulates dendritic cell (DC) recruitment and CCL17 production to provoke a Th2 response. ILC2s produce IL-4 to regulate Th2 differentiation, which may suggest a potential role in promoting tumor growth and metastasis. In terms of antitumor function, the release of IL-5 and IL-9 by ILC2s restrains tumor growth through eosinophils recruitment and activation and DC antigen presentation. Conversely, high expression of IL-9 is associated with T-cell transformation in humans, indicating that the anti-apoptotic properties on transformed cells promote tumorigenesis. ILC3s are important in the Th17 immune response. The direct interactions with $CD4^+$T cells are either through MHC II/TCR or through OX40/OX40 and CD30L/CD30 to regulate T-cell survival or B-cell class switch, respectively. ILC3 migrate to the draining mesenteric lymph node to initiate an adaptive response that requires CCR7 expression. ILC3s promote IgA production in a T cell-dependent manner via the regulation of $CD4^+$T-cell homing by sLTα3 secretion and also in a T cell-independent manner via control of B-cell homing by the release of LTα1β2. ILC3-derived IL-22 triggers epithelial serum amyloid A protein (SAA) production to induce local Th17 response in a STAT3-dependent manner. The secretion of IL-22 and GM-CSF by ILC3 stimulates macrophages and DCs to release IL-10, and this consequently results in regulatory T cell (Treg) cell expansion. In addition, IL-1β produced by macrophages activates ILC3 and leads to $CD4^+$T-cell priming, whereas T cell-derived cytokines also cross-regulate ILC response, indicating the bidirectional interactions between ILC3 and T cell-mediated immunity.

INNATE LYMPHOID CELLS AND CANCER-RELATED INFLAMMATION

Inflammation is a well-known hallmark of cancer that sustains cancer progression by providing growth factors, proangiogenic factors, and ECM modifying enzymes. During the different stages of tumor development, the inflammatory response plays decisive roles in tumor initiation, promotion, malignant conversion, invasion, and metastasis (100). Inflammatory conditions can be present before or after a malignant change occurs, which induces an inflammatory microenvironment to promote tumor development (101). Cancer-related inflammation in the presence of inflammatory cells and their mediators, such as cytokines, chemokines and prostaglandins, in tumor tissue is similar to that observed in a chronic inflammatory response (101). The character of the inflammatory response and the composition of the immune cell infiltration are indispensable to shape the TIME (33).

ILCs are believed to be resident, whereas NK cells recirculate. Given their distribution throughout the body, ILCs play a critical role in coordinating inflammation and cancer (75). ILC1s are highly abundant in the small intestine, liver, uterus, and salivary glands; ILC2s are enriched in the skin and lung; and ILC3s are abundant in the colon and tonsils (27, 102). Mucosal ILC1s are important in barrier defense through escalating Th1-like responses against pathogenic infections (99). During inflammation, a higher frequency of IFNγ-producing ILC1 subsets in mucosal tissue is positively correlated with patients having Crohn's disease compared with the normal tissue, indicating a role for ILC1 in the pathogenesis of gut mucosal inflammation (103). ILC2s secrete the type 2 cytokines IL-5 and IL-13 to control helminths, restrain parasitic infections (99), and drive airway hyper-reactivity in mouse models of allergic asthma (103, 104). Tissue-derived cytokines can also shape ILC function in inflammation, and this is largely achieved by the IL-22 pathway (78). In response to IL-23 stimulation, IL-22-producing ILC3s facilitate tissue protection from the acute phase of *Citrobacter rodentium*-induced colitis (105). ILC3-dependent protection also occurs at other mucosal sites, including the lung and oropharyngeal barrier through IL-17 and IL-22 (106–108). ILCs are important in tissue repair and remodeling after infection. $IL\text{-}22^+$ILC3s limit inflammatory colitis, and their frequency correlates with mucosal repair in inflammatory bowel disease (IBD) (99). LTis are involved in tissue repair after clearance of lymphocytic choriomeningitis virus infection (109), and ILC2s assist lung tissue repair after influenza virus infection (53).

Indeed, ILCs act as a two-edged sword in inflammation. The same factors that inhibit acute inflammation and promote tissue repair can have pathogenic effects during chronic inflammation activation. IL-5-producing ILC2s boost lung inflammation by recruiting eosinophils that are the main allergic effectors, whereas maintenance of IL-13 production stimulates collagen deposition leading to chronic fibrotic tissue damage and pulmonary fibrosis in hyper-reactive lung airways (110–112). In the colon, chronic infection-induced IL-23 triggers RORγt$^+$ILC accumulation and increases IL-17 and IFNγ production that results in the bacteria-driven colitis (77). Furthermore, the protumor activities of ILC3 are mainly associated with chronic inflammation by IL-23-induced secretion of IL-17 and IL-22 (113). Antigen-presenting ILC3s act as a checkpoint to control Tfh and B cells response toward mucosal-dwelling microbiota (92). Interestingly, regulatory ILCs (ILCregs) are found in both human and mouse to protect intestines from inflammation through inhibition of ILC1 and ILC3 activation regulated by IL-10 secretion (114).

ILCs as mediators of inflammation in various organs play a key role in tissue remodeling and repair after recruitment and activation, which are controlled by cytokines and growth factors that are selective for each ILC member. Hence, targeting the regulation of the tissue-specific response of ILCs in inflammation may be beneficial in the setting of inflammation-driven cancer.

INNATE LYMPHOID CELLS IN PROTUMOR AND ANTITUMOR EFFECTS

The role of ILCs in cancer is ambivalent as a double-edged sword by engaging in cancer immune surveillance or enhancing the unique tumor-promoting milieu, depending on the conceptual framework. We review here the different subsets of ILCs in both antitumor and protumor activities in the diverse contexts of cancer (**Table 1**).

ILC1s

ILC1s are defined by the expression of T-bet and the production of the signature cytokine IFNγ. The prototypical member of ILC1s is the NK cell. NK cells recirculate and show strong cytotoxicity, whereas ILC1s are tissue resident and only exhibit weak cytotoxicity but produce more IFNγ and GM-CSF (131) than NK cells do (26). ILC1s in response to IL-12, a potent antitumor cytokine (115), produce the effector cytokines, IFNγ and TNFα, to limit tumor growth in a melanoma mouse model (132). The antitumor properties of ILC1s also depend on IL-15. The expanded ILC1s in mouse mammary pre-cancerous lesions exhibit potent cytotoxicity to limit tumor growth in response to IL-15 (116).

However, ILC1s also show a more complex behavior and display a detrimental role in cancer. Alteration of ILC subsets composition in patients with acute myeloid leukemia (AML), compared with the controls, could change the protective function of ILC1s by increasing their frequencies and reducing IFNγ and TNFα (118). Conversely, even though IFNγ is currently used as a clinical target for treatment of malignancies, enhanced IFNγ production by ILC1s is not associated with a better immune response but shows an immunosuppressive effect in the presence of IL-12-producing chronic lymphocytic leukemia (CLL) cells (117, 124). Moreover, ILC1s are highly abundant in colorectal cancer and produce IFNγ in response to IL-15 and IL-18, thus inducing an immunosuppressive environment (102, 133). These data suggest a dark side of IFNγ in tumor-promoting effects in cancer, and further investigation might help to understand the complex mechanisms of cancer progression in different contexts.

ILC2s

As an alarmin, IL-33 is released from nuclei to the extracellular milieu upon cell injury caused by cell stress or damage and activates ILC2s to initiate a type 2 immune response (120). IL-33-dependent tumor-infiltrating ILC2s mobilize from lung and recruit DCs to promote the adaptive T-cell response (119). ILC2 antitumor activity under IL-33 stimulation is also involved in recruiting eosinophils to limit tumor growth and in shaping chemokine profiles in the TME under IL-5 release (134). IL-33 promotes ILC2 secretion of CXCR2 ligands that bind to CXCR2-expressing tumor cells, reinforcing tumor cell-specific apoptosis independent of adaptive immunity (135). Secretion of IL-13 by ILC2s activates CD8$^+$ T cells through DC recruitment (120). In addition, ILC2s are major sources of IL-9, which can decrease tumor metastasis in a mouse model of melanoma (87).

Conversely, IL-13-producing ILC2s also show protumor immunity and are associated with a negative outcome in cancer. The ILC2/IL-13 axis modulates T cell-to-MDSC balance to drive an immunosuppressive microenvironment, whereas the inhibition of ILC2/IL-13 axis improves bladder cancer treatment (125). Similarly, the IL-33/ILC2 axis also accelerates tumor growth and promotes lung and liver metastasis by recruiting MDSCs to produce IL-13 in a mouse model of breast cancer (136), acute promyelocytic leukemia (APL), and prostate cancer (123). ILC2s impair IL-33-mediated tumor suppression by antagonizing the NK cell function during tumor growth via CD73 independently of adaptive immunity in a melanoma mouse model (119). Indeed, ILC2-derived IL-33 promotes tumor formation and metastasis in breast cancer by upregulating Treg cells through inducible co-stimulator (ICOS)/ICOS ligand (ICOSL) interaction (121), whereas IL-33 also activates ILC2s to recruit eosinophils through ICOS/ICOSL interaction during lung inflammation (137). Moreover, ILC2-derived AREG expression favors lung tumor growth and enhances resistance to apoptosis (126). Increased numbers of ILC2s were found in inflamed colonic tissue of patients with ulcerative colitis, a condition associated with a high risk for development of inflammation-driven colorectal cancer (122). Clinical studies have shown that ILC2s and their cytokines (IL-33 and IL-4) accumulate in peripheral blood and contribute to immune-suppressive environment formation in gastric cancer patients (138). The mediation of resistance to apoptosis indicates a poor prognosis in a number of cancers (139, 140).

All these data indicate that the function of ILC2s and their related cytokines are cell context dependent to facilitate either protumor or antitumor activities.

TABLE 1 | ILCs in different cancer types with anti-/pro-turmeric effects.

	Cancer types	Functions (anti-/pro-tumor)	References
ILC1s	Melanoma	Anti-tumor: ILC1s in response to IL-12, produce IFNγ and TFNα to limit tumor growth.	(115)
	Colorectal Cancer	Anti-tumor: intraepithelial CD127$^-$ILC1s (ielLC1) produce cytotoxic granules to repress tumor growth.	(102)
	Mammary pre-lesion	Anti-tumor: ILC1s number expansion exhibits potent cytotoxicity to limit tumor growth in response to IL-15.	(116)
	CLL	Pro-tumor: CLL cells induce ILC1s produce IFNγ and TNFα and form immunosuppressive environment.	(117)
	AML	Pro-tumor: reduce IFNγ and TNFα production.	(118)
ILC2s	Melanoma	Anti-tumor: IL-9-producing ILC2s inhibit tumor metastasis in mouse melanoma model.	(87)
	Lymphoma	Anti-tumor: IL-33 stimulates ILC2s to secrete CXCR2 ligands that bind to CXCR2-expressing tumor cells to induce tumor cell-specific apoptosis independently of adaptive immunity.	(119)
	Lung cancer	Anti-tumor: IL-33 dependent tumor-infiltrating ILC2s mobilize from lung and facilitate dendritic cells to promote adaptive T cell response. Pro-tumor: IL-33 actives ILC2s to recruit eosinophils through ICOS/ICOSL interaction.	(120) (121)
	Colorectal cancer	Pro-tumor: Increased ILC2 are associated with high risk for development of inflammation-related CRC.	(122)
	Breast cancer	Pro-tumor: IL-33/ILC2 axis facilitates tumor growth and metastasis by recruiting MDSCs to produce IL-13. Pro-tumor: ILC2s promote breast cancer growth by upregulating Tregs through ICOS/ICOSL interaction.	(123) (124)
	Bladder cancer	Pro-tumor: ILC2/IL-13 axis modulate T cell–to-MDSC balance driving an immunosuppressive TME.	(125)
	Gastric cancer	Pro-tumor: Accumulation of ILC2s and their cytokines, IL-33 and IL-4 contribute to immune-suppressive environment.	(126)
	Prostate cancer	Pro-tumor: ILC2 elevated tumor-derived PGD2 and B7H6 and activated MDSCs via IL-13 secretion to promote tumor growth.	(123)
	APL	Pro-tumor: ILC2 elevate tumor-derived PGD2 and B7H6 to active MDSCs via IL-13 thus inducing immunosuppressive effects.	(123)
ILC3s	Melanoma	Anti-tumor: IL-12 promotes NKp46$^+$CD49b$^-$RORγt$^+$ILC3 expansion and upregulation of VCAM1 to facilitate immune leukocyte infiltration resulting in tumor suppression.	(72)
	Lung (NSCLC)	Anti-tumor: NCR$^+$ILC3s produce IL-22, TNFα, IL-8 and IL-2 to activate endothelial cells forming protective tumor-associated tertiary lymphoid structures.	(127)
	Colorectal cancer	Pro-tumor: ILC3s sustain colon cancer via production of IL-22.	(128)
	Breast cancer	Pro-tumor: CCL21-mediated recruitment of ILC3 triggers CXCL13 secretion by TME stromal cells thereby enhancing tumor cell motility and promoting lymph node metastasis.	(74)
	Hepatocellular carcinoma	Pro-tumor: NCR$^-$ILC3s initiate IL-17 production upon IL-23 stimulation and promote hepatocellur carcinoma development.	(129)
	Cervical carcinoma	Pro-tumor: IL-17-producing ILC3s are also associated with poor survival in early stages.	(130)

ILC3s

Same as the other ILC subsets, ILC3s are also associated with both antitumor and protumor activities. NCR$^+$ILC3 concentrates in human NSCLC and associates with intratumoral lymphoid structures, which contribute to the formation of an antitumor environment and act as predictors of favorable clinical outcome (127). In a mouse model of melanoma, increased IL-12 promotes NKp46$^+$CD49b$^-$RORγt$^+$ILC3 expansion leading to upregulation of VCAM1 to facilitate leukocyte infiltration and the mediation of tumor suppression (72).

Conversely, ILC3s can be associated with tumor growth under different contexts. In a mouse model of colorectal cancer, NCR$^+$IL-22$^+$ILC3s, previously known as NK22 cells that are phenotypically distinct from LTis, sustain colon carcinogenesis in chronic inflammation (141). IL-22-producing ILC3s are crucial for the IL-22-mediated innate immune response. Notably, other subsets of ILC3s can also produce IL-22 such as CD4$^+$ILC3s, which lack NCR expression in the gut (142). NCR$^-$ILC3s promote hepatocellular carcinoma (HCC) development in response to IL-23 to produce IL-17, which directly limits CD8$^+$ T-cell immunity by enhancing lymphocyte apoptosis and inhibiting their proliferation (129). By using an immunodeficient Rag$^{-/-}$ mouse model, it has been shown that NKp46$^-$CD4$^-$ILC3 accumulation leads to IL-17 and IL-22 production, which promotes tumor development, whereas depletion of ILCs with anti-Thy1 mAbs limits tumor growth in colon cancer (141). Similarly, RORγt$^+$ILC3s are associated with an increased incidence of metastasis in human breast cancer, as it was shown that depletion of ILC3 using an anti-CD90.2 antibody reduced tumor cells metastasis to lymph nodes in a syngeneic 4T1.2 mouse model (74). IL-17-producing ILC3s are also associated with poor survival in early stages of squamous cervical carcinoma (130). Interestingly, one study showed that a previously uncharacterized ILC population, ILCregs, can produce IL-22 and exhibit low cytotoxicity with a gene expression profile that overlaps with NK cells and other ILCs showing the same immunosuppressive capacity as Treg cells in human ovarian cancer studies (39).

Nevertheless, emerging evidence indicates the balance of power of ILCs in the context of cancer, and many ILC functions appear to be regulated by mechanisms distinct from those of other innate and adaptive immune cells. Notably, many studies

of ILCs in cancer are based on the Rag-deficient mouse models lacking adaptive immunity. Although these experiments were crucial to analyze the fundamental features of ILC biology, these approaches have hampered the ability to study ILCs in the context of adaptive immunity and the potential interplay between ILCs and adaptive lymphocytes. The challenge now is to understand their roles and contribution within a complete immune system. The extent to which ILCs regulate the immune response (with antitumor or protumor activities) in a multitude of contexts need to be further analyzed. This is important for the understanding of their roles in cancer and for identifying targets for use in clinical treatments. In summary, although current studies reveal an important role of ILCs during tumor development, their indistinct functions in humans are incompletely understood.

INNATE LYMPHOID CELL HETEROGENEITY AND PLASTICITY

The pleiotropic roles of ILCs in cancer are dictated by their heterogeneity and plasticity, the noteworthy capacity of ILC subsets to convert into one another. Like helper T cells, the function and phenotype of ILCs can be modulated by extrinsic signals. In the context of cancer, mature ILCs exhibit substantial plasticity depending on the distinct cytokines present in the microenvironment milieus (143) (**Figure 5**).

NK Cells to ILC1s and ILC3s

In a mouse model of fibrosarcoma and melanoma, TGF-β in the TME induced mouse NK cells to convert into non-cytotoxic ILC1s, thus weakening the control over tumor growth and metastasis (63, 144, 145). Recent studies revealed that NK cells can permanently transform to Eomes$^-$T-bet-dependent ILC1-like cells independently of ongoing infection, which may explain the discrepancies of ILC1-like cells with either having antitumor function or as an inflammatory subpopulation. This exemplifies the current limitations of NK cells and ILC categorization on the basis of only their phenotypes as discrete ILC lineages (146). NK cells can also be converted into ILC3s. IL-1β stimulation prevents stage 3 NK cells from differentiating into stage 4 mature NK cells, thus keeping an ILC3-like phenotype with IL-22 production and IL-1R1 expression (147).

ILC1s to NK Cells, ILC2s, and ILC3s

In TGF-β knock-out mice, the ILC1 number decreased in salivary glands concurrent with increased expression of CD49a, CD103, and CD69, suggesting the potential for ILC1 to NK cell plasticity (148). IL-4 accumulation leads to a shift from ILC1s toward ILC2s, which can be reversed by IL-12, in patients with severe chronic obstructive pulmonary disease (COPD) or chronic rhinosinusitis with nasal polyps, suggesting the induction of IL-12 and IL-4 signatures in ILC1s and ILC2s, respectively (149). Differentiation of ILC1s to ILC3s is driven by IL-23 (103), and this can be enhanced by IL-1β and retinoic acid produced by CD14$^-$DC (103, 149, 150). The enhanced accumulation of ILC3s likely promotes tumorigenesis within different tissue locations (151).

ILC2s to ILC1s and ILC3s

Conversion of ILC2s into ILC1s has been shown in the intestines of patients with Crohn's disease (152) and in the lungs of patients with COPD (25). Inflammation may increase ILC plasticity, thereby increasing the risk of cancer development. IL-1β also converts ILC2s into ILC1s, resulting in the increase of T-bet and IL-12Rβ2 expression, which promotes ILC1s in response to IL-12 (153). Mouse lung inflammatory ILC2s, in response to IL-25, can differentiate into IL-17-producing ILC3s that play an important role in antihelminths and antifungal immunity (154).

ILC3s to NK Cells, ILC1s, and ILC2s

Human ILC3s treated with IL-15 and IL-12 initiate the expression of NK cell cytotoxicity markers (Eomes, CD94, CD56 NKG2A, and NKG2C), suggesting a beneficial role for ILC3-NK plasticity in cancer (155, 156). Plasticity between ILC3 and ILC1 mediated by IL-23 and also IL-12 produced by CD14$^+$DC promotes polarization from ILC3s toward ILC1s, which ultimately affects tumor immunosurveillance in response to various environmental changes (2, 103, 150). IL-23-induced activation of STAT4 in NCR$^+$ILC3s is a key determinant of plasticity from NCR$^+$ILC3s toward ILC1s, as demonstrated by a transcriptomic analysis (157). Moreover, ILC3-to-ILC1 transition was tissue dependent and relied on transcription factor Aiolos and T-bet cooperation to repress regulatory elements active in ILC3s. This highlights the relevance of the tissue niche in creating a microenvironment that promotes ILC diversity and functional adaptation to local stimuli (158). ILC3s convert into ILC2s after activation with IL-2/TLR2 signals. In humans, IL-23 stimulation induces IL-22 secretion by ILC3s, which display clonal heterogeneity for IL-13 and IL-5 production indicating polarization *in vivo* (159).

NCR$^-$ILC3s to NCR$^+$ILC3s

Mouse NCR$^-$ILC3s that differentiate into NCR$^+$ILC3s need Notch signals; conversely, TGF-β can convert NCR$^+$ILC3s into NCR$^-$ILC3s, indicating that bidirectional plasticity of ILC3s is regulated by the balance between the opposing effects of Notch and TGF-β signaling in the face of challenges (76).

Interestingly, genetic evidence shows that the differences in specific chromatin regions such as *cis*-acting enhancers and silencers that bind to transcription factors may determine the apparent plasticity of ILCs compared with the T-helper subsets. With the use of ATAC-sequencing to evaluate chromatin regions accessibility, the regulatory regions that control the distinct cytokine gene expression in ILCs exhibit poised or active status, whereas the identical regions in CD4 T helper cell counterparts become accessible only after stimulation (160, 161). These results indicate that ILC regulomes are prone to dynamic changes, and the resilient feature of ILCs directly affects their plasticity in response to the TME.

As discussed above, ILC plasticity, the ability to modify their functional phenotypes, is a fundamental phenomenon that can contribute to tumor escape mechanisms by altering the ILC-dependent tumor-surveillance system. Fully understanding the ILC plasticity control mechanisms is essential for manipulation of ILC function in the TME and designing novel therapeutic targets to prevent tumor metastasis.

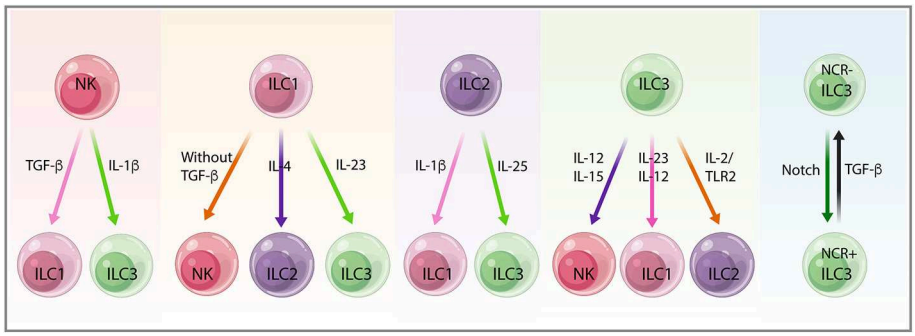

FIGURE 5 | The plasticity between innate lymphoid cell (ILC) subsets. Mature ILCs display substantial plasticity depending on the distinct cytokines, the microenvironment milieus, and the diverse context of cancer. Natural killer (NK) cells convert into non-cytotoxic ILC1s upon transforming growth factor-beta (TGF-β) stimulation in the tumor microenvironment (TME) in mouse fibrosarcoma and melanoma. NK cells also convert into ILC3s in response to IL-1β, preventing stage 3 NK cells from stage 4 mature NK cells and retaining ILC3-like phenotype. ILC1s show plasticity toward all other ILCs subtypes, including NK cells, ILC2s, and ILC3s with the absence of TGF-β, the accumulation of IL-4, and under the stimulation of IL-23, respectively. Conversion of ILC2s to ILC1s needs IL-1β, whereas ILC2s in response to IL-25 differentiate into IL-17-producing ILC3s. ILC3 treatment with IL-15 and IL-12 leads to NK cell conversion, whereas plasticity from ILC3s toward ILC1s and ILC2s depends on IL-23, IL-12, and IL-2/TLR2 signals. NCR^{-}ILC3 and NCR^{+}ILC3 conversion needs Notch and TGF-β signals, indicating the bidirectional plasticity of ILC3s in the face of different challenges.

TARGETING INNATE LYMPHOID CELLS FOR CANCER IMMUNOTHERAPY

Over the last two decades, cancer treatment has been revolutionized from targeting the tumor itself with traditional methods like surgery, chemotherapy, and radiotherapy to boosting the immune system coordinating with TIME to control cancers. Diverse approaches of immunotherapy including targeting cytokines, immune checkpoint blockade (ICB), and administration of depleting or agonistic antibodies have been used to enhance antitumor activities (27).

ILCs were first identified at the mucosal barriers, but more recent data confirm that ILCs exist in most, if not all, tissue types (75). ILCs are among the first immune cells to react to changes in environmental signaling and to shape TIME to coordinate the adaptive immune response, providing promising targets for cancer immunotherapy strategies.

Targeting Cytokines

As immunomodulators, ILCs produce their signature cytokines and respond to various cytokines produced from other cell types and the surrounding environments. Cytokine-based immunotherapies such as IL-2 family, IL-10, IL-12, TGF-β, and several other immunosuppressive cytokines are currently under ongoing clinical trials for cancer treatment (30, 162, 163). IL-2 is a key cytokine in promoting the expansion of cytotoxic lymphocytes. IL-2 receptors, CD25, and CD122 are expressed on mouse ILC2s, whereas human ILC2s and ILC3s show high expression of CD25 only. In response to IL-2 stimulation, ILCs can be expanded and activated to facilitate antitumor effects (128, 164). However, the systemic administration of this cytokine frequently causes grade 3 and 4 adverse effects owing to its toxic profile even at the recommended doses. Several second-generation IL-2-based compounds have been engineered to reduce the high-affinity binding to IL-2Rα/CD25 and increase the half-life in circulation (163). Directed conjugation of polyethylene glycol (PEGylation) generates an inactive cytokine with a long half-life in circulation. PEGylated IL-2 (NKTR-214) has been shown to preferentially activate CD122, and this modified cytokine is currently being evaluated in phase I/II clinical trials for the treatment of various tumors (27, 165). IL-15 and IL-15 super-agonist ALT803 exhibit capabilities to increase NK cell cytotoxicity in AML patients (166) and other tumor models such as advanced solid tumors (NCT01946789), multiple myeloma (NCT02099539), relapsed hematologic malignancy (NCT01885897) (27) and metastatic NSCLC (NCT02989844) (167). IL-10 is released by innate and adaptive immune cells to regulate pro-inflammatory cytokines activity. IL-10 exhibits a context-dependent outcome in cancer, and administration of PEGylated IL-10 increases its half-life to avoid grade 3–4 immune-related adverse effects. This feature has been evaluated in a phase I clinical trial in advanced, treatment-refractory tumors (NTC02009449) (163). The antitumor and protumor effects of TGF-β in cancer are based on the control of epithelial cell growth and promoting epithelial–mesenchymal transition (EMT), respectively. Although their efficacy as monotherapy agents is limited, combined blockade of TGF-β using small molecules or antagonistic monoclonal antibodies with immune checkpoint inhibitors such as PD-1/PD-L1 (NCT02423343 and NCT02734160) shows great potential for blocking the functions of this cytokine (163). TGF-β receptor type 1 (TGFBR1) inhibitors are currently used in phase I clinical trials to treat advanced solid tumors (NCT02160106) or in combination with pomalidomide for multiple myeloma (NCT03143985) (30). Because of the intimate links between cancer and inflammation, future cancer immunotherapies may benefit from repurposing of anti-inflammatory treatments that are already in human trials for chronic inflammatory diseases such as IBD. For instance, anti-IL-12 and IL-23 blocking antibody ustekinumab has been approved for clinical trials in Crohn's disease (168). Targeting

IL-17 or IFN-γ alone failed to show any effects in clinical trials, whereas targeting both provided favorable results in a preclinical model on IBD (77). Antibodies against IL-5 and anti-IL-4 receptor α subunit that block ILC2 function have provided encouraging results in patients with chronic rhinosinusitis (169). Similarly, inhibition of ILC2 function by chemoattractant receptor-homologous molecule expressed on Th2 cells (CTTH2) antagonists restores lung function in patients with asthma (170).

Cytokines are complex immune mediators with great potential in clinical cancer immunotherapy. The safety and effective use of cytokine-based drugs require a thorough knowledge of cytokine biology and advanced biotechnology in order to exploit their antitumor activity while minimizing their toxicity. In a combination with other immunomodulatory drugs such as immune checkpoint inhibitors and chimeric antigen receptor T cells, cytokine immunotherapy might become a more effective strategy in cancer treatment.

Immune Checkpoint Blockade

ILCs share some activating and inhibitory receptors with NK and T cells, suggesting potential targets for immunotherapeutic applications. ICB is an emerging antibody-based immunotherapy providing promising strategies for cancer treatment. Antibodies targeting immune checkpoints including CTLA-4, PD-1, and PD-L1 have now been approved and used in the clinics for diverse cancer treatments (171, 172). Activation of mouse ILCs shows upregulation of PD-1, whereas increased PD-1 expression on ILC2s and ILC3 has been found in human gastrointestinal tumors, and highly expressed PD-1 and CTLA-4 were shown in human breast cancer (133, 173). Strikingly, only a minority of treated patients have benefited from of PD-1 blockade, with some others displaying drug resistance. Therefore, understanding how to shape the TME in order to increase the sensitivity to PD-1 blockade becomes indispensable. The preclinical and clinical studies in TNBC have shown that low-dose chemotherapy with doxorubicin or cisplatin or irradiation induces a favorable TME that increases the sensitivity to nivolumab treatment for PD-1 blockade to stimulate an anticancer immune response (174).

Another immune checkpoint activation protein, inducible co-stimulator (ICOS) expressed on $CD4^+$T cells as well as on ILC2s and ILC3s, was identified as a crucial player in CTLA-4 blockade antitumor effects. Engagement of the ICOS pathway markedly enhances the efficacy of CTLA-4 blockade in cancer immunotherapy in a mouse model of melanoma and prostate cancer (175). Currently, an anti-ICOS agonist antibody (GSK3359609) is being used in a phase I clinical trial aimed at patients with solid tumors. In addition, therapeutic antibodies against other immune checkpoints have also been developed, such as TIM-3, LAG-3 (expressed on ILC1s only), and TIGIT (expressed on both ILC1 and ILC3s) (176). Moreover, monalizumab, an antibody against NK cell checkpoint protein NKG2A, showed the capability to enhance antitumor immunity by promoting both NK and $CD8^+$ T-cell function. Based on these findings, monalizumab in combination with cetuximab is being used in head and neck squamous cell carcinoma phase II clinical trial (177).

Depleting or Agonistic Antibody Application and Others

Both experimental mouse models and human samples showed that therapeutic targeting of ILC3 could be beneficial for autoimmune diseases. For example, depletion of ILCs by anti-Thy1 antibody improved the treatment for *Helicobacter hepaticus*-induced colitis (77). In the context of nonsolid tumors, human ILCs can be depleted before hematopoietic stem cell transplantation to treat acute leukemia (178).

Adenosine signaling belongs to a metabolic pathway that plays an important role in the immunosuppressive microenvironment favorable for cancer development. ILC1s express CD39 and CD73, activating the adenosine pathway in mouse salivary glands and increasing an immunosuppressive TIME for tumor growth, suggesting a potential target for therapeutic approaches (148).

So far, comprehensive information on the phenotypic and functional characterization of ILCs has been gathered. After the initial observation of the antitumor cytotoxicity of NK cells, other ILCs have become attractive targets for immunotherapy based on their abilities to rapidly sense and respond to signaling changes in the TME. The current challenge in cancer biology is to harness the cytokines, growth factors, and chemokines that regulate the specific function of ILCs within the TME, and further investigations will help to develop the exploitable targets for clinical applications.

CONCLUSIONS AND PERSPECTIVES

Despite advances in immunotherapies for cancer treatment, some patients still encounter few or no clinical benefits with the same treatments. Given the unique, non-redundant roles of ILCs within cancers, development of rational therapies that specifically target ILCs is likely to boost the immunotherapeutic effects in a larger group of cancer patients.

State-of-the-art technologies like single-cell RNA-sequencing (173) and high-dimensional mass spectrometry (CyTOF) analysis (179) have allowed us to investigate ILCPs, delineate distinct ILCs subsets and their developmental pathways, and identify novel mediators of antitumor immunity at single-cell resolution levels. These studies present new perspectives for exploring the complexity of ILCs in the diverse context of cancers and allow effective manipulation of ILC immunosurveillance to achieve optimal therapeutic opportunities.

AUTHOR CONTRIBUTIONS

ZA and TN conceived the manuscript. ZA reviewed the literature and wrote the manuscript. FF-B, SI, JD, and TN revised the manuscript.

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Eomes Expression Defines Group 1 Innate Lymphoid Cells During Metastasis in Human and Mouse

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Recent studies have attempted to uncover the role of Group 1 Innate lymphoid cells (ILCs) in multiple physiological contexts, including cancer. However, the definition and precise contribution of Group 1 ILCs (constituting ILC1 and NK subsets) to metastasis is unclear due to the lack of well-defined cell markers. Here, we first identified ILC1 and NK cells in NSCLC patient blood and differentiated them based on the expression of transcription factors, T-bet and Eomes. Interestingly, Eomes downregulation in the peripheral blood NK cells of NSCLC patients positively correlated with disease progression. Additionally, we noted higher Eomes expression in NK cells (T-bet^{+}Eomes^{hi}) compared to ILC1s (T-bet^{+}Eomes^{lo}). We asked whether the decrease in Eomes was associated with the conversion of NK cells into ILC1 using Eomes as a reliable marker to differentiate ILC1s from NK cells. Utilizing a murine model of experimental metastasis, we observed an association between increase in metastasis and Eomes downregulation in NKp46^{+}NK1.1^{+} Group 1 ILCs, which was consistent to that of human NSCLC samples. Further confirmation of this trend was achieved by flow cytometry, which identified tissue-specific Eomes^{lo} ILC1-like and Eomes^{hi} NK-like subsets in the murine metastatic lung based on cell surface markers and adoptive transfer experiments. Next, functional characterization of these cell subsets showed reduced cytotoxicity and IFNγ production in Eomes^{lo} ILC1s compared to Eomes^{hi} cells, suggesting that lower Eomes levels are associated with poor cancer immunosurveillance by Group 1 ILCs. These findings provide novel insights into the regulation of Group 1 ILC subsets during metastasis, through the use of Eomes as a reliable marker to differentiate between NK and ILC1s.

Keywords: Eomesodermin, Group 1 ILCs, Innate Lymphoid Cells, metastasis, non-small cell lung cancer

INTRODUCTION

Since the description of TRAIL^{+} NK cells in mouse liver in 2001 (1), extensive progress has been made in identifying the phenotype and function of recently discovered Innate Lymphoid Cells (ILCs) in health and disease (2–8). In both human and mouse, ILCs are the innate immune counterparts of T-cells, with ILC1, ILC2, and ILC3 sharing features with Th1, Th2, and Th17 subsets, respectively. Together with the previously identified and well-studied natural killer (NK) cells, ILC1s have been categorized as Group 1 ILCs. The NK cells represent the cytotoxic

counterparts of ILCs, bearing similarity to $CD8^+$ T cells. Based on parallels drawn from T cells, the Group 1 ILCs are known to depend on T-bet for their development, and produce IFNγ upon activation (9–11). While both murine and human ILC subsets are defined in a similar way, there is evidence suggesting differences in their precursor populations and pathways (12). The functions of Group 2 and Group 3 ILCs have been well-established through rigorous research efforts (13–20), however, that of Group 1 ILCs have remained somewhat unclear. This is, in part, due to lack of well-defined cell surface and intracellular markers to differentiate this heterogenous population into its subtypes—ILC1s and NK cells. As a result, most studies have referred to ILC1 subsets as tissue-resident NKs (tr-NK) or unconventional NKs, and this has led researchers to utilize markers common to both cell types, to define and study the roles of NK cells (21–23). Additionally, ILC1s and NK cells have been shown to exert different functions under different physiological conditions. Although, in the context of infectious diseases, the protective role of ILC1 has been uncovered (24), their contribution to tumor control and surveillance is controversial. While, it has been reported that loss of immune surveillance is associated with conversion of NK cells into ILC1s (25), another study has shown ILC1s to exhibit potent cytotoxicity against cancer cells (26). Furthermore, a high degree of plasticity amongst various ILC subsets (27, 28) makes it even more challenging to identify and examine the roles of different Group 1 ILC subsets in disease and pathology (25, 29, 30). Therefore, since it is now known that other subsets in addition to NK cells might exist, there is a need to revisit these studies and characterize the actual individual contribution of the Group 1 ILC subsets in order to reliably associate their specific functions to different diseases.

Early attempts to differentiate between NK cells and ILC1s utilized CD49a and CD49b (DX5) as two mutually exclusive markers (31). Classical NK cells in the bloodstream as well as in thymus, liver, skin, and uterus predominantly express $CD49a^-CD49b^+$ phenotype. On the other hand, ILC1s in the liver, skin and uterus are $CD49a^+CD49b^-$. However, a recent study has identified an intermediate ILC1 population which expresses $CD49a^+CD49b^+$ phenotype in the tumor microenvironment (25). Further, it is also noteworthy that the upregulation of CD49a and downregulation of CD49b occur under inflammation conditions in activated NK cells, thereby making these markers non-specific to the subsets (32). Therefore, alternative strategies have been tested to distinctly define the two subsets. Developmental dependence of NK cells on transcription factors such as NFIL3 (33) and that of liver ILC1s on Hobit (34) has been explored in attempts to study the individual function of these cells. However, the results from these trials have been rather unsatisfactory due to development of certain NK cells even in *Nfil3*$^{-/-}$ and ILC1s in *hobit*$^{-/-}$ mice in the presence of inflammatory stimuli. Likewise, a recent study identified CD200r1 as an ILC1 specific marker in the liver. However, its expression on ILC1s in other organs is unknown (35). For the purpose of this study, we defined Group 1 ILCs based on expression of T-bet and Eomesodermin (Eomes) in mouse and human. T-bet is a T-box transcription factor needed for the development of Group 1 ILC subsets while Eomes is needed for NK cell development, specifically (36). While T-bet is expressed on both ILC1s and NK cells (37), Eomes is seemingly expressed only on murine NK cells (9), thus making it a more reliable marker to differentiate ILC1s from NK cells (11, 38, 39).

Emerging studies have queried the involvement of novel Group 1 ILC subsets in disease and pathology (40–42), but little is known about their phenotype and function in cancer. Recently, Dadi et al. found an immuno-surveillance role for murine ILC1-like cells in genetic models of murine mammary carcinoma (26). On the other hand, an immune-suppressive role of human $CD56^+CD3^-$ Group 1 ILCs in Tumor Infiltrating Lymphocyte (TIL) culture has been reported (43). In the context of metastasis, while the role of NK cells is well-studied (44), that of recently identified ILC1 subsets is unknown (45–48). Here, we aimed to study Group 1 ILC subsets involved in metastasis by analyzing the profile of Group 1 ILCs in blood samples of NSCLC patients. We identified distinct ILC1 ($Eomes^{lo}$) and NK cells ($Eomes^{hi}$) in patient blood and observed Eomes downregulation in Group 1 ILCs (NK cells in particular), with the advancement of post-metastatic NSCLC. Similarly, using a mouse model of metastatic melanoma, we identified T-bet^+Eomes^{lo} and T-bet^+Eomes^{hi} subsets within $NKp46^+NK1.1^+$Group 1 ILCs. Subsequent *ex vivo* analysis of the Group 1 ILC subsets showed increased cytotoxicity with increased Eomes expression. Based on our findings, we propose that the Eomes levels regulate the response of Group 1 ILCs to metastasis. Furthermore, the weakening of Group 1 ILC anti-tumor response was associated with Eomes downregulation, which could contribute to worse clinical outcomes in cancer metastasis.

MATERIALS AND METHODS

Patient Samples

All patient samples used in this study were collected from the National University Hospital (NUH), Singapore, approved under DSRB number 2016/00698 and were taken after patient written informed consent at least 24 h before the surgery or on the day of the consultation. Five milliliter of peripheral blood was collected from NSCLC patients before the treatment was started. Stages I and II samples were collected from patients undergoing surgical resection of lung mass while Stages III and IV were collected from patients consulting with National University Cancer Institute (NCIS) at NUH. De-identified patient information is provided in **Table S1**. Blood specimens were diluted 1X with HBSS and layered onto ficoll-paque media (GE Healthcare) and centrifuged at 400 g for 40 min at 20°C without brake and acceleration, after which the PBMC ring was collected into a fresh tube. The cells were then washed twice, counted and shifted to ice for immunostaining and flow cytometry.

Flow Cytometry of Human PBMCs

Cells were resuspended in 1 ml PBS and spun down at 500 g for 5 min at 4°C. The cells were then stained for 30 min with a live-dead stain, Fixable Viability Dye (FVD)-506 at 1:1000 dilution in 100 µl PBS. Then, the cells were washed and stained for cell-surface markers. In order to improve the antibody binding, a blocking antibody (Biolegend) was used

at 1:200 dilution. A lineage panel consisting of the following antibodies was included to allow for clear identification of ILCs—FITC-conjugated anti-CD3 (OKT3), anti-CD19 (H1B19), anti-CD11b (M170), anti- CD11c (3.9). To this mix, the following antibodies from Biolegend were added at 1:50 dilution: APC-Cy7-conjugated anti-CD45(2D1), PerCP-conjugated anti-CD56 (CMSSB), PE-Cy7-conjugated anti-CRTH2 (BM16), PacBlue-conjugated anti-CD117 (104D2) and Qdot-605-conjugated anti-CD127 (A019D5). Cells were incubated with the antibodies for 30 min on ice. This was followed by fixation permeabilization for detection of intranuclear T-bet and Eomes markers. For this, eBioscience Foxp3 transcription factor staining kit was used (#005523), following which the cells were stained with PE-conjugated anti-T-bet (4B10) and APC-conjugated anti-Eomes antibody (WD1928) at room temperature. Intranuclear staining with anti T-bet and Eomes antibodies was carried out 1 h before running the samples on flow cytometer. The cells were resuspended in 500 µl 2% FBS in PBS and centrifuged at 8,000 g to remove the supernatant. To the pellet, 400 µl of PBS was added before the suspension was filtered through 70 µm filter and run on flow cytometer. Fixed samples, prior to intracellular staining were stored overnight at 4°C. Samples were run on BD LSR Fortessa flow cytometer and analyzed using Flowjo V10. Fluorescence compensation data were acquired using single stained compensation beads (Thermofisher Scientific) and applied to the samples. For gating of positive and negative populations, Fluorescence Minus One (FMO) controls were used. For additional clarity, internal staining controls were used, wherever mentioned. For data presentation and statistical analysis, graphs were plotted using GraphPad Prism 5.01.

Mice Models and Cell Lines

The experiments and breeding of mice were performed under Institutional Animal Care and Use Committee (IACUC approved protocols: R17-0209 and BR-1142, respectively). All the mice used in this study were housed at Comparative Medicine at MD1, National University of Singapore. Throughout this study, C57BL/6J female wild type (WT) mice between 6 and 8 weeks of age were used. T-bet KO and CD45.1 congenic mice were purchased from Jacksons lab while Eomes-GFP reporter mice were a kind gift from Dr. Thierry Walzer, Centre International de Recherche en Infectiologie, Inserm, Lyon, France. B16F10 melanoma cells were purchased from ATCC and were maintained in DMEM containing 10% FBS. Mouse melanoma B16F10 cells were tested to be mycoplasma-free. To set up a model of pulmonary metastasis in B6 mice, 0.2 million B16F10 cells in PBS were administered intravenously through the tail vein. Subsequently, the mice were euthanized, and lung and spleen tissues were harvested at different time points. The cells were then isolated and characterized as discussed below.

Cell Isolation

White blood cells were isolated from mouse lungs, spleen, and liver using enzymatic and mechanical dissociation. After slicing the spleen into small fragments of ∼2 mm, in PBS, a 10 ml syringe and plunger was used to release the cells further. For lung, tissue chunks were first incubated in 0.5 mg/ml Collagenase D and 20 U/ml DNase (Merck) for 20 min, followed by mechanical dissociation using Miltenyi tissue dissociator. Liver was perfused with 1 mM EDTA in PBS, isolated, and mechanically homogenized by using Miltenyi tissue dissociator. Dissociated tissue samples were then filtered through a 70 µm nylon filter (Miltenyi Biotec). For lungs and liver, the cells were resuspended in 40% Percoll PLUS density gradient medium (GE Healthcare) and overlaid on 70% Percoll Plus medium and centrifuged at 500 g for 30 min at 20°C The interphase containing lymphocytes was collected, washed and subjected to lysis of red blood cells using ACK lysis buffer, together with splenic cells. The isolated cells were then stained as described below.

Flow Cytometry and Sorting of Group 1 ILCs

For staining of mouse samples, the following antibodies were used: From eBioscience: PacBlue-conjugated anti-CD45.1 (104), PE-Cy7-conjugated anti-NKp46 (29A1.4), APC/APC-Cy7-conjugated anti-NK1.1 (PK136), APC-conjugated anti-CD49b (DX5), PerCP-cy5.5-conjugated anti-CD11b (M1/70), AF488-conjugated anti-CD27 (LG.7F9), APC-conjugated anti-CD62L (MEL-14), FITC-conjugated anti-CD44 (IM7), PerCP-cy5.5-conjugated anti-Ki67 (So1A15), AF488-conjugated anti-IFNγ (XMG1.2), PerCP-cy5.5-conjugated anti-TNFα (MP6-XT22), PE conjugated anti-T-bet (4B10), PE-TexasRed-conjugated anti-Eomes (Dan11mag). PerCP-cy5.5-conjugated anti-CD49a (Ha31.8) was from BD Biosciences. A cocktail of biotin-tagged antibodies (eBioscience) containing anti-mouse CD3, CD19, CD5, γδTCR, TER119, Gr-1, F4/80 was used to separate NK cells from other immune cells. Fixable Viability Dye (FVD)-506 (65-0866, eBioscience) was used to separate live from dead cells, and cells were fixed using Foxp3 Fix/perm kit (88-8824-00, eBioscience). Flow cytometry of the cells was performed with BD LSR Fortessa and data were analyzed using FlowJo V10. In order to sort ILC1s/NKs, spleen from multiple Eomes-GFP reporter mice were pooled and processed to isolate single cells. For depletion of lineage cells, streptavidin beads were used to remove lineage-positive cells stained with biotin antibodies (StemCell™ Technologies, Catalog #19860). The cells were stained with PacBlue-conjugated anti-CD45.1 (104), PE-Cy7-conjugated anti-NKp46 (29A1.4), APC-conjugated anti-NK1.1 (PK136) and Eomes was detected using GFP expression. After staining, cells were sorted on BD FACS Aria and $Eomes^{lo}$ and $Eomes^{hi}$ Group 1 ILC fractions were collected in complete RPMI. MFIs (mean fluorescence intensity) of pre- and post-sorted cells were compared and a 99% pure population of $Eomes^{hi}$ cells was isolated.

Adoptive Transfer

2×10^5 $Eomes^{hi}$ cells were adoptively transferred into CD45.1 mice at day 4 post-B16F10 cancer cell injection. The lungs were then harvested and analyzed at day 10 using flow cytometry,

and donor cells in the recipient CD45.1 mouse were detected as $CD45.2^+CD45.1^-$.

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

Flash frozen lung lobes from mice were thawed on ice and 500 µl Trizol was added to carry out RNA extraction. The lung lobe was finely excised and homogenized using a tissue dissociator (GentleMACS c tube, Miltenyi). Chloroform-isopropanol extraction was used to precipitate the RNA followed by another round of extraction with Trizol to achieve higher purity. The RNA pellet obtained thereafter was re-suspended in water and reverse-transcribed using superscript cDNA kit (ThermoFisher). The cDNA obtained was diluted 5 times and qRT-PCR using Promega master-mix was performed. All measurements were relative to reference gene, *Rpl27*, which was used as an internal loading control. The PCR primers for *Melan-A* were: F- 5′ GAGAAATCCCATCAGCCCGT 3′ and R- 5′ AGCGTTCTCAGGAGTTTCCC 3′, and for *Pmel* were: F- 5′ GCCACATGGTAGCACTCACT 3′ and R- 5′ AACAAAAGCC CTCCCGCAAG 3′.

Ex vivo Stimulation and Intracellular Staining

IFNγ and TNFα production by murine Group 1 ILCs was measured through intracellular cytokine staining after *ex vivo* stimulation with 25 ng/ml Phorbol Myristate Acetate (PMA, Sigma) and 500 ng/ml ionomycin (Thermofisher Scientific) in complete RMPI at 37°C for 5 h. Secretion of the cytokine was blocked by the addition of GolgiPlug™ (Beckton-Dickinson) to the media. Cells were then fixed using Foxp3 Fix/perm kit (88-8824-00, eBioscience) for 30 min on ice, and stained with anti-IFNγ AF488 and anti-TNFα antibody.

Co-culture of Group 1 ILCs With Cancer Cells

For co-culture of mouse Group 1 ILC fractions with B16F10 cells, flow sorted $Eomes^{hi}$ and $Eomes^{lo}$ Group 1 ILC subsets were resuspended in RPMI and co-cultured with B16F10 cells in a 4:1 effector to target ratio. For measurement of Eomes MFI, cells cultured without B16F10 cells were used as controls and cytotoxicity was normalized against spontaneous cell death in "B16F10 only" wells. Cells were harvested onto ice at different time points and cytotoxicity was detected through 7AAD staining. The Eomes levels were measured through GFP expression. The cytotoxicity was determined by calculating [% dead cells / (% dead cells + % live cells)].

Cytokine Administration and *in vivo* NK Cell Expansion

Intranasal administration of cytokines was carried out to evaluate the response of Group 1 ILC subsets to *in situ* stimulation. For this, the experimental mice were given 50 µl of 0.5 µg IL-12 and 1.0 µg IL-18 in 1x PBS while the control group received 50 µl of 1x PBS only. The mice were anesthetized in the gas chamber using isofluorane. This was done on days 1, 3, and 5, followed by harvesting and isolation of cells on day 7.

Statistical Tests

Prism (GraphPad) was used for statistical analysis. Individual statistical tests used are described in the corresponding figure legend. *p*-values are shown in figures or included in the figure legend. For all animal studies, points represent biological replicates, for co-culture analysis technical replicates were used and representative experiments are shown. For NSCLC patient sample analysis, each point represents unique patient data. Bar position represents the mean, and error bars represent ± s.e.m.

RESULTS

Eomes Downregulation in Circulating NK Cells Accompanied NSCLC Progression

To investigate the role of Group 1 ILCs in cancer, we analyzed peripheral blood samples of NSCLC patients across various stages of cancer prior to treatment initiation. Group 1 ILCs in the blood were broadly classified as $CD45^+Lin^-c\text{-}kit^-CRTH2^-$ live cells and were subdivided into ILC1s and NK cells. ILC1s were defined as $CD127^+CD56^-$ and NK cells as $CD127^-CD56^+$, as described previously (49) (**Figure 1A**). Since the expression of T-bet and Eomesodermin (Eomes) on human Group 1 ILCs is not well-defined (39, 50, 51), we analyzed the profile of these transcription factors in circulating ILC1s and NKs in NSCLC. Interestingly, we noticed a decrease in the expression levels of Eomes post-metastasis (Stages III and IV) compared to early stage (Stages I and II), while T-bet levels did not change significantly (**Figures S1A,B**). We also noted a concomitant increase in the frequency of $Eomes^{lo}$ cells (among the $T\text{-}bet^+$ Group 1 ILCs) with NSCLC advancement (**Figure 1B**). This suggested downregulation of Eomes in Group 1 ILCs, particularly NK cells, during metastasis (**Figures S1C,D**). We also investigated Eomes levels in individual ILC1 and NK cell subsets and observed significantly elevated Eomes expression in NK cells, compared to ILC1s (**Figure 1C**). While T-bet was highly expressed in both NK cells and ILC1s, there was a notably higher expression in NK cells compared to ILC1s (**Figure 1D**). Based on this, we propose that circulating ILC1s exhibit $T\text{-}bet^+Eomes^{lo}$ profile while NK cells express $T\text{-}bet^+Eomes^{hi}$ phenotype during NSCLC progression (**Figure S1E**). Interestingly, we did not observe any significant difference in the ratio of NK cells to ILC1s with cancer advancement (**Figure S1F**).

Murine $Eomes^{lo}$ Group 1 ILCs Accumulated in the Lung and Spleen During Metastatic Progression

In light of these findings, we questioned whether Eomes downregulation in Group 1 ILCs during tumor progression was associated with acquisition of ILC1-like phenotype in NK cells. Furthermore, using a model of murine metastasis, we asked whether Eomes could also be used as a reliable marker to differentiate ILC1s from NK cells in the tumor

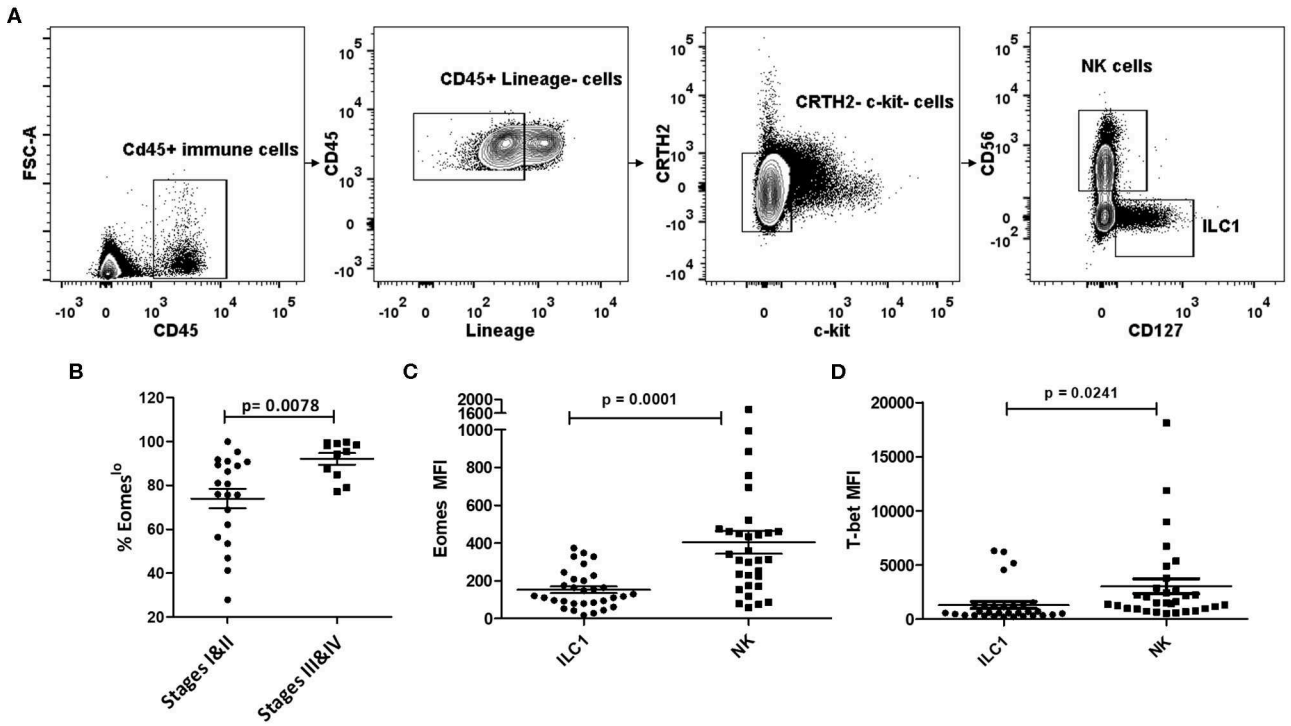

FIGURE 1 | Human Group 1 ILCs expressed Eomes differentially during NSCLC progression. **(A)** Gating for identification of Group 1 ILC subsets in peripheral blood of NSCLC patients. Group 1 ILCs were gated as CD45$^+$ Lineage (CD3, CD19, CD11b, CD11c)$^-$c-Kit$^-$CRTH2$^-$; ILC1s were further gated as CD127$^+$CD56$^-$ and NK cells as CD127$^-$CD56$^+$ **(B)** Percentage of Eomes^{lo} subset gated over CD45$^+$ Lineage (CD3, CD19, CD11b, CD11c)$^-$c-Kit$^-$ CRTH2$^-$T-bet$^+$ cells at early stage (Stage I and II) vs. late stage (III and IV). Cancer progression is accompanied with increase in frequency of cells expressing lower Eomes levels **(C)** Quantification of Eomes expression in Group 1 ILC subsets, viz, ILC1, and NK cells. Peripheral blood NK cells showed higher Eomes levels compared to circulating ILC1s **(D)** Quantification of T-bet expression in Group 1 ILC subsets, viz, ILC1, and NK cells. Peripheral blood NK cells showed higher T-bet levels compared to circulating ILC1s. MFI is Mean Fluorescence Intensity, $n = 16$ for stage I, $n = 4$ for stage II, $n = 4$ for stage III, $n = 7$ for stage IV. Data are presented as mean ± s.e.m.; significance was tested using unpaired two tailed students' *t*-test.

microenvironment. After observing the loss of expression of Eomes in NK cells and increase in Eomes^{lo} ILC1s in human peripheral blood with cancer progression, we queried the profile of murine Group 1 ILCs during metastasis using a mouse model of B16F10 metastatic melanoma. To this end, we injected B16F10 cancer cells into the tail vein and monitored tumor development over 21 days (**Figure 2A**). The tumor burden in the lungs was quantified using qRT-PCR, based on *Melan-A* and *Pmel*, which are two melanocyte-specific markers with zero basal expression in mock-treated lungs (52). These genes were expressed at higher levels over time, indicating increased tumor burden in the lungs (**Figure 2B**). Next, we investigated the profile of pulmonary Group 1 ILCs during metastatic establishment and colonization. For this, we used the NK cell surface markers, NKp46 and NK1.1, to define Group 1 ILCs (9). Since Group 1 ILCs consist of ILC1s and NK cells which share similar phenotypic profile, we used Eomes as a marker to differentiate ILC1s and NK cells. Like with human samples, we probed for Group 1 ILC subsets in mice tissues based on Eomes and T-bet levels, revealing two different Group 1 ILC subsets: T-bet$^+$Eomes^{lo} and T-bet$^+$Eomes^{hi} cells in the lung and spleen (**Figures 2C,E**). Since ILC1s are generally Eomes$^-$ but NK cells in most organs express Eomes (38), and our findings in human patient samples show that ILC1 expressed lower levels of Eomes compared to NK cells, we hypothesized that the pulmonary and splenic Eomes^{lo} and Eomes^{hi} subsets represent ILC1 and NK cells, respectively. Quantification of pulmonary and splenic Eomes^{lo} and Eomes^{hi} NK cells showed accumulation of these subsets with increase in tumor progression (**Figures 2D,F**). Furthermore, analysis of the ratio of Eomes^{hi} to Eomes^{lo} cells at different time points showed decrease in Eomes^{hi} cells with increase in metastatic burden, suggesting increase of the number of Eomes^{lo} cells in the lungs (**Figure S2A**). However, due to reduced infiltration of CD45.2$^+$ immune cells into the lungs, we did not detect any Group 1 ILCs at day 21, coinciding with massive tumor burden at that time point (**Figure S2B**).

Eomes^{lo} Group 1 ILC Subset Is Not Derived From Eomes^{hi} Cells in the Tumor Microenvironment

Cancer cells are known to polarize the tumor milieu in order to dampen the effector function of various immune cells (53). Since we identified increase in the frequency of T-bet$^+$Eomes^{lo} population in the lung and spleen, we questioned whether the

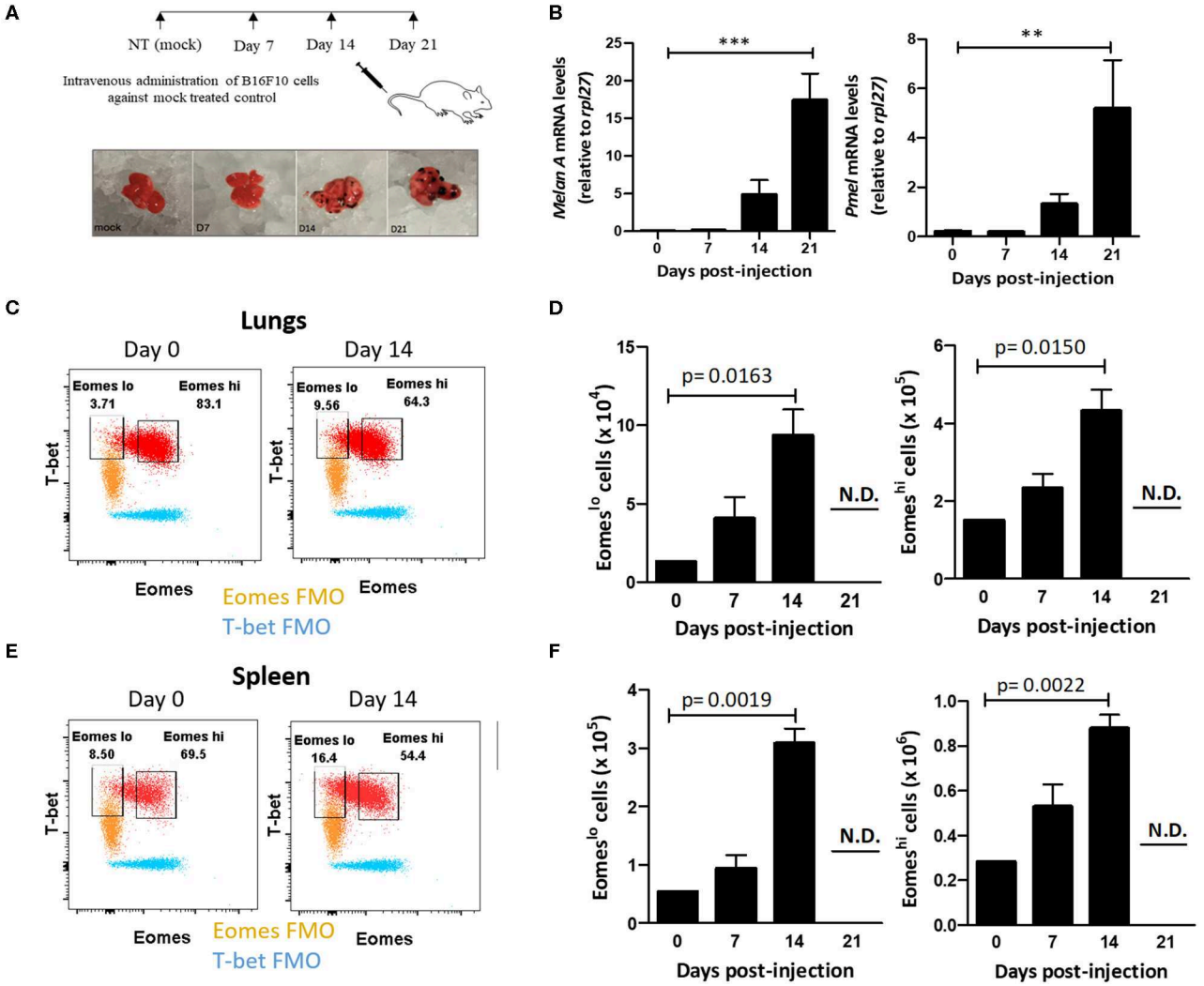

FIGURE 2 | Eomes^{lo} Group 1 ILCs accumulate in the lung during B16F10 metastatic progression. **(A)** Schematic for setting up B16F10 murine metastatic melanoma study model in B6 mice. Mice were sacrificed and lungs were harvested at various time points. **(B)** Quantification of tumour burden in the lungs using melanocyte-specific genes *Melan-A* and *Pmel* relative to *Rpl27* housekeeping gene. **(C)** Identification and representative flow plot of pulmonary Eomes^{lo} and Eomes^{hi} cells in the lung at day 0 and day 14. **(D)** Quantification of Eomes^{lo} and Eomes^{hi} cell numbers with increase in metastatic burden in the lungs. Both these cell subsets were not detectable (N.D.) at day 21. **(E)** Identification and representative flow plot of splenic Eomes^{lo} and Eomes^{hi} cells at day 0 and day 14. **(F)** Increase in splenic Eomes^{lo} and Eomes^{hi} numbers with increase in metastasis. Both these cell subsets were not detectable (N.D.) at day 21. Cell populations were gated over NKp46^{+}NK1.1^{+}CD45.2^{+} live Group 1 ILCs for **(C–F)**. For **(C,E)**, FMO controls are overlaid. MFI is Mean Fluorescence Intensity, $n = 4$ for each group. Data are representative of three independent repeats; Data are presented as mean ± s.e.m.; statistical significance was tested using one-way ANOVA **(B)** two-tailed students' *t*-test **(D,F)**. $^{**}p < 0.005$ and $^{**}p < 0.0005$.

increase in Eomes^{lo} subset is due to Eomes downregulation in the tumor microenvironment, viz, could they have arisen from Eomes^{hi} cells? To this end, we first adoptively transferred FACS-sorted Eomes^{hi} cells isolated from the spleen of Eomes GFP reporter mice (CD45.2) into congenic CD45.1 mice at day 4 post-injection of B16F10 cells or PBS (mock). Since we observed an increase in Eomes^{lo} cells as early as day 7 after injection of B16F10 cells, we followed a similar timeline for adoptive transfer experiment. At day 10 after B16F10 injection, we analyzed the cell frequency and population (**Figures 3A,B**). However, we did not observe any decrease in Eomes expression (based on GFP MFI) in the adoptively transferred cells isolated from tumor-bearing recipient mice at day 10 compared to mock naïve recipient mice (**Figure 3C**). Likewise, we did not observe any increase in the frequency of Eomes^{lo} cell population upon the transfer of Eomes^{hi} cells into tumor-bearing mice, suggesting that Eomes^{lo} cells did not arise from Eomes^{hi} cells at day 10 (B16F10 vs. mock) (**Figure 3D**). This suggests that Eomes downregulation did not occur as a result of the transformation of Eomes^{hi} into Eomes^{lo} cells in the tumor microenvironment, which could indicate that Eomes^{lo} and Eomes^{hi} cells perhaps belonged to different lineages. Since ILC1s need T-bet for development and NK cells rely on

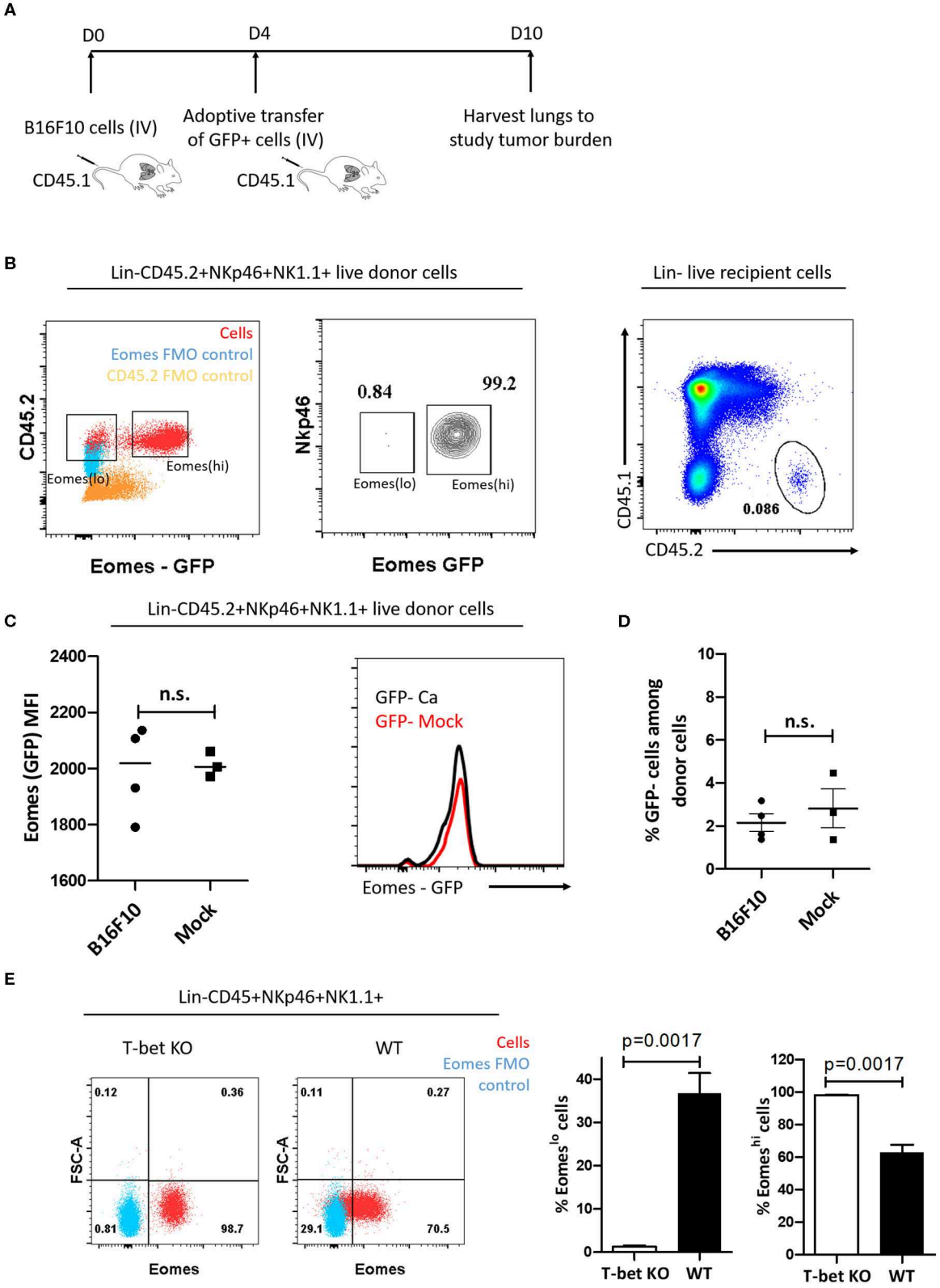

FIGURE 3 | Eomes^{lo} and Eomes^{hi} subsets correspond to different cell lineages. **(A)** Scheme of adoptive transfer of Eomes^{hi} cells into CD45.1 mice bearing cancer (relative to mock). After injection of B16F10 cells (day 0), Eomes^{hi} cells were harvested from donor Eomes-GFP mice and adoptively transferred into recipient mice at

(Continued)

FIGURE 3 | day 4. The mice were then sacrificed at day 10 and lungs were harvested and analyzed. **(B)** Eomes^{lo} and Eomes^{hi} cells in Eomes-GFP mice (Red) overlaid with Eomes FMO (blue) (left panel). Two distinct populations can be seen upon running live cells under flow cytometer; Sorting efficiency ∼99% middle panel); Donor cells after transfer of CD45.2^{+} Eomes^{hi}-GFP^{+} cells into CD45.1 mice (right panel). **(C)** Eomes (MFI) after transfer of donor derived Eomes^{hi} GFP^{+} cells in cancer-bearing (B16F10) and cancer-lacking (mock) hosts at day 10. No significant difference in Eomes MFI was observed between cancer and mock mice. **(D)** Frequency of Eomes^{lo} GFP^{-} cells amongst donor NK cells at day 10 post-injection of B16F10 cells **(E)** Flow plots and graphs show near absence of Eomes^{lo} cells in T-bet KO mice (∼0.8%) compared to WT mice (∼29.1%), indicating that T-bet is needed for development of Eomes^{lo} cell development (Red—NKp46^{+}NK1.1^{+} cells; Blue—Eomes FMO). MFI is Mean Fluorescence Intensity, $n = 3$–4 for each group. Results are representative of three independent repeats; data are presented as mean ± s.e.m.; Significance was tested using two-tailed students' *t*-test.

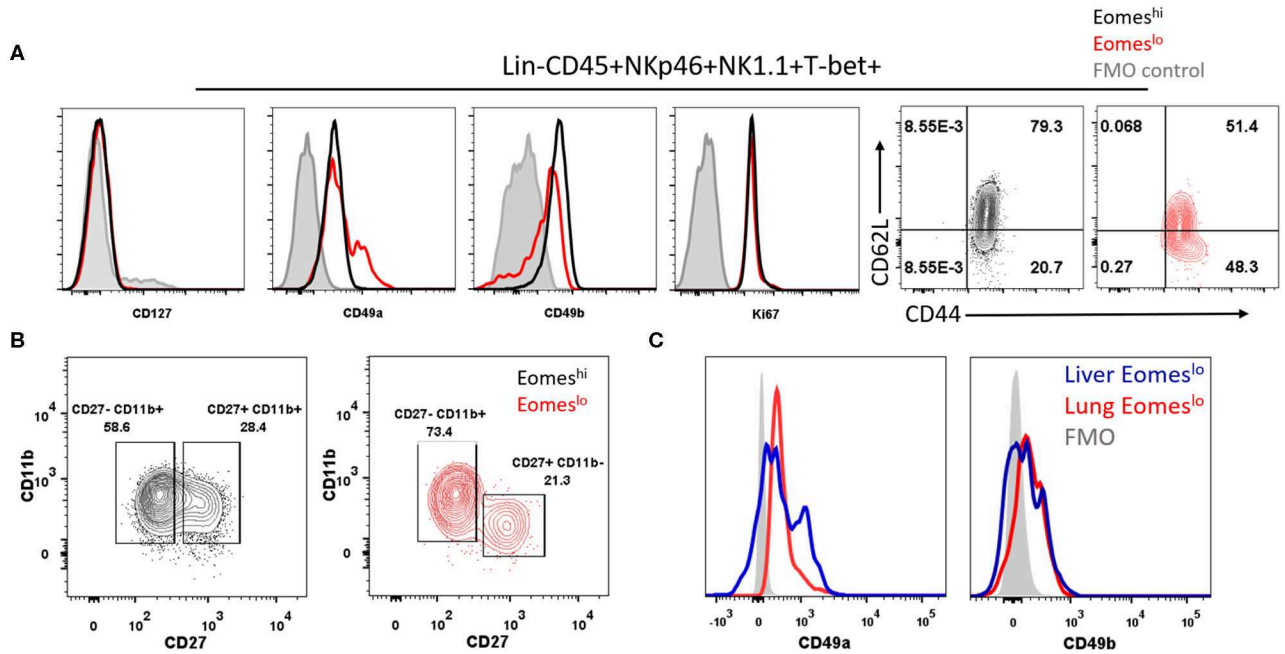

FIGURE 4 | Eomes^{lo} Group 1 ILCs represent both cNK and tr-NK (ILC1) phenotype and function. **(A)** Representation of CD127, CD49a, CD49b expression in Eomes^{lo} and Eomes^{hi} subsets. Ki67 (proliferation marker) and CD44, CD62L (endothelial cell interaction/recruitment marker) levels on Eomes^{lo} and Eomes^{hi} cells were measured relative to FMO controls; red—Eomes^{lo}, black—Eomes^{hi}, gray—control. **(B)** Maturation status of NK cells using Cd11b and CD27 markers, with gating based on FMO controls; red—Eomes^{lo}, black—Eomes^{hi} **(C)** CD49a and CD49b expression levels in lung Eomes^{lo} compared to liver Eomes^{lo} cells relative to FMO (Fluorescence Minus One) controls. MFI is Mean Fluorescence Intensity, $n = 4$ for each group. Data are representative of three independent repeats.

it for maturation, we would anticipate the absence of ILC1s in T-bet knockout mice whereas immature NKs would be present. To test this hypothesis and to assess the source of Group 1 ILC subsets, we checked the profiles of Eomes^{lo} and Eomes^{hi} subsets in T-bet knockout mice. Since accumulation of these two subsets peaked at day 14 (**Figure 2D**), we compared cell numbers in T-bet knockout and wild type mice at this time point. Interestingly, we observed a significant reduction of Eomes^{lo} cell population in the lungs while Eomes^{hi} cells were the majority, supporting the hypothesis that Eomes^{lo} cells represent ILC1s while Eomes^{hi} cells are NK cells (**Figure 3E**).

Murine Eomes^{lo} and Eomes^{hi} Group 1 ILC Subsets Share Phenotypic Similarities With ILC1s and cNKs, Respectively

After establishing that Eomes^{lo} subset did not arise from Eomes^{hi} Group 1 ILCs, we sought to determine whether the T-bet^{+}Eomes^{hi} and T-bet^{+}Eomes^{lo} subsets represent conventional NK cells (cNK) and unconventional tissue resident NK cells (tr-NK)/ILC1, respectively, as in the liver and uterus (36, 54). To this end, we screened these subsets for various cell surface markers. Both of these subsets lacked the expression of CD127 (**Figure 4A**), a subunit of IL-7 receptor, suggesting that IL-7 was not needed for their maintenance. Next, we noted that Eomes^{lo} cells expressed higher levels of CD49a than Eomes^{hi} subset, pointing at an ILC1-like phenotype of Eomes^{lo} subset. Conversely, Eomes^{hi} cells expressed higher levels of CD49b than Eomes^{lo} subset suggesting an NK-like phenotype of Eomes^{hi} cells. Quantification of the percentage of CD49a^{+} and CD49b^{+} cells showed similar results (**Figures S3A,B**). We also measured cell proliferation and recruitment markers, Ki67 and CD44, CD62L, respectively (**Figure 4A**). We did not find any major difference in Eomes^{lo} and Eomes^{hi} subsets except that Eomes^{hi} cells comprised of 79.3% of double positive CD62L^{+}CD44^{+} cells, suggesting their potential recruitment from other organs or the bloodstream while Eomes^{lo} cells consisted of 51.4% double positive population. Therefore, compared to Eomes^{lo}

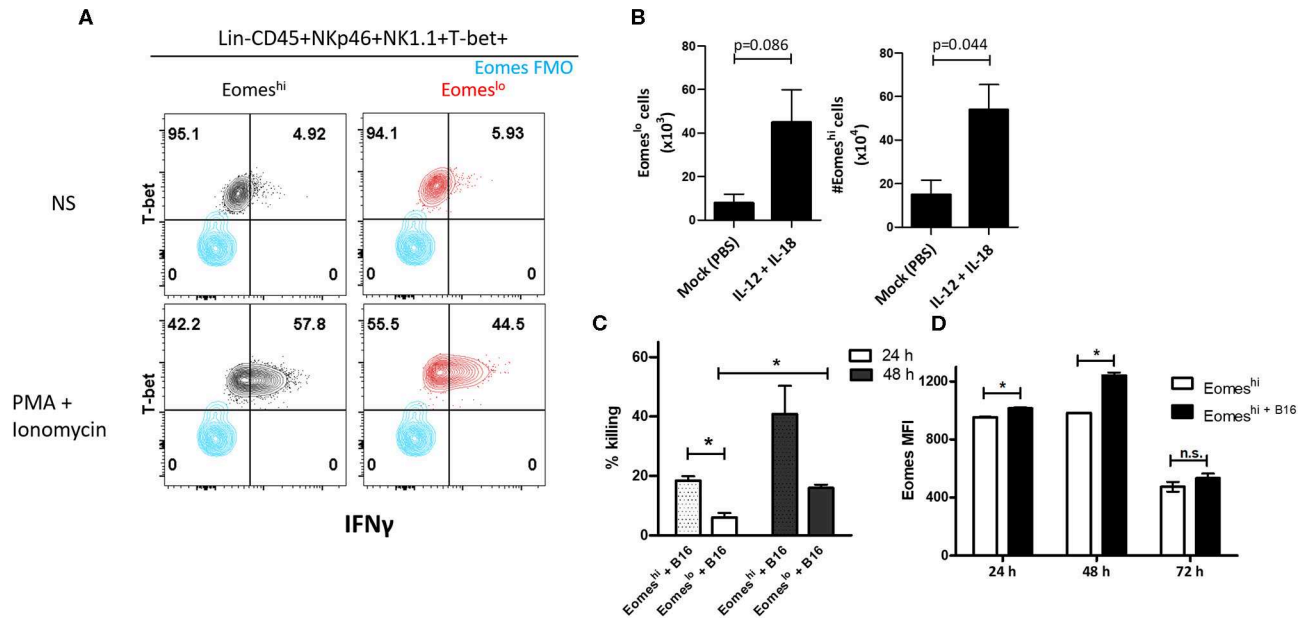

FIGURE 5 | Eomes upregulation is correlated with Group 1 ILC-mediated cytotoxicity. **(A)** Representation of IFNγ expression in Eomes^{lo} and Eomes^{hi} subsets relative to FMO controls; red—Eomes^{lo}, black—Eomes^{hi}; Cells were isolated and stimulated *ex vivo* with PMA and Ionomycin (NS = non-stimulated) **(B)** Increase in Eomes^{lo} and Eomes^{hi} cell numbers in response to IL-12 and IL-18 stimulation *in vivo*. The lungs were harvested, and cells were counted at day 7. Here, $n = 4$ biological replicates. **(C)** Percentage killing of B16F10 melanoma cells by Eomes^{hi} subset relative to Eomes^{lo} cells at 24 and 48 h, normalized to spontaneous death (B16F10 only). **(D)** Measurement of Eomes MFI across 24, 48, and 72 h in flow-sorted murine Eomes^{hi} Group 1 ILC in co-culture with B16F10 cells compared to Eomes^{hi} subset alone. Cells were isolated from Eomes-GFP reporter mice; Eomes MFI is indicative of GFP MFI in the cells. Target to Effector ratio (T:E) was maintained at 4:1. MFI, Mean Fluorescence Intensity, $n = 3$ technical replicates, Data are representative of three independent repeats; data are presented as mean ± s.e.m.; Significance was tested using two-tailed students' *t*-test; n.s., not significant $^*p < 0.05$.

cells, the Eomes^{hi} subset showed larger proportion of cells with circulating cell phenotype. These observations are in alignment with an ILC1-like phenotype the Eomes^{lo} subset and NK-like phenotype for Eomes^{hi} cells as suggested by other studies (31). Next, in order to rule out the possibility that these Eomes^{lo} cells were immature NK cells, we examined the expression of NK maturation markers, CD11b and CD27, on these cells. While both Eomes^{lo} and Eomes^{hi} subsets consist of various fractions of immature NK cells (indicated by CD11b$^-$CD27$^+$ and CD11b$^+$CD27$^+$), the majority of Eomes^{hi} and Eomes^{lo} cells represented terminally mature cells (CD11b$^+$CD27$^-$) (**Figure 4B**), precluding the possibility that Eomes^{lo} subset cells were immature NK cells. Similar analysis of splenic Eomes^{lo} and Eomes^{hi} subsets showed a comparable profile for CD49b and CD49a (**Figure S3C**), whereas liver cell subsets showed higher expression of CD49a in liver Eomes^{lo} cells (**Figure 4C**).

Eomes^{lo} Subset Has Reduced Effector Function Compared to Eomes^{hi} Group 1 ILCs

In order to characterize the function of the two subsets, we measured IFNγ and TNFα produced by these cells. We did not observe measurable levels without cell stimulation, however, upon stimulation with PMA and Ionomycin, Eomes^{lo} cells produced significantly lower IFNγ compared to Eomes^{hi} cells, at day 14 (**Figure 5A**). Similarly, a larger fraction of Eomes^{hi} cells produced IFNγ compared to that of Eomes^{lo} cells (**Figure S3D**). On the other hand, TNFα did not show any difference in MFI (**Figure S3E**). This could be due to inherent differences in the nature of these cells or change in the activation status as a result of the polarization of the tumor microenvironment toward a pro-tumor milieu, albeit transient. Next, since Group 1 ILCs are activated by cytokines IL-12, IL-15, and IL-18 (8), we checked the response of NKp46$^+$NK1.1$^+$ subsets to these cytokines as per the treatment scheme (**Figure S4A**). Eomes^{hi} cells were more sensitive to the stimulation, and the cell numbers increased significantly compared to Eomes^{lo} cells upon stimulation with IL-12 + IL-18 (**Figure 5B**, **Figure S4B**). Since murine Eomes^{lo} ILC1-like subset produced lower IFNγ production compared to Eomes^{hi} subset, we next queried the role of Eomes in cytotoxicity of Group 1 ILCs. For this, we performed FACS sorting of Eomes^{hi} and Eomes^{lo} Group 1 ILCs and independently co-cultured them with B16F10 cells *ex vivo*. Interestingly, Eomes^{hi} cells were more cytotoxic compared to Eomes^{lo} at 24 and 48 h time points (**Figure 5C**). Furthermore, we observed an increase in the killing ability of Eomes^{hi} from 24 to 48 h with increase in intracellular Eomes expression (**Figures 5C,D**). This indicates that Eomes^{lo} ILC1s are less cytotoxic than Eomes^{hi} NK cells and cell cytotoxicity is positively associated with Eomes expression. Overall, we conclude that Eomes^{lo} and Eomes^{hi} Group 1 ILCs

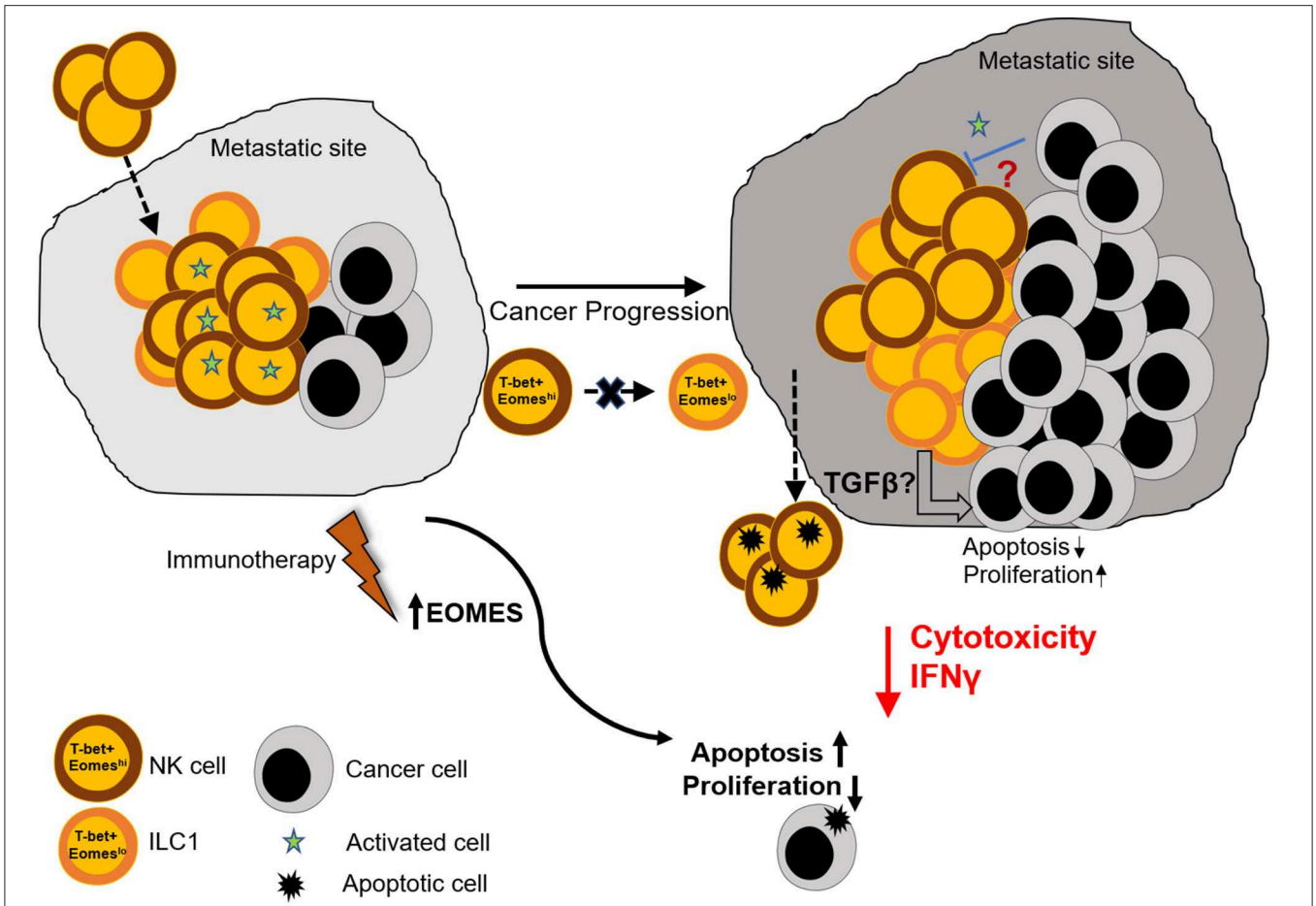

FIGURE 6 | Graphical summary. Naïve lungs consist mainly of Eomes^{hi} NK cells. However, metastatic colonization leads to increase in the number of Eomes^{lo} ILC1. This increase in Eomes^{lo} ILC1s is associated with increased metastatic burden due to lower cytotoxic potential and reduced IFNγ production by this subset of ILC1s, compared to NK cells. This in turn supports proliferation of cancer cells and prevents apoptosis and cancer cell death. However, despite lowering of Eomes levels in Group 1 ILCs, Eomes^{lo} ILC1s did not seem to be derived from Eomes^{hi} NK cells, thus eliminating the possibility of plasticity under the conditions tested. Therefore, we propose that (i) the reduction in Eomes levels is associated with a worse metastatic burden and (ii) this could be due to production of immune-suppressive factors by Eomes^{lo} cells (iii) immunotherapeutic targets designed to augment Eomes levels could prove useful in the treatment of metastasized cancers.

are fundamentally different and represent ILC1s and NK cells. **Figure 6** illustrates our findings in a hypothetical model.

DISCUSSION

While Group 1 ILC subsets are considered to play an important role in cancer regulation due to their similarity to CD4 Th1 cells and production of IFNγ, their involvement and function in metastasis is rather unclear. This is due to phenotypic similarity to the well-known NK cells as well as evidence of plasticity among ILC subsets. In this study, we first identified ILC1 and NK cells in NSCLC patient blood using CD127 and CD56 markers. We further noted lower levels of Eomes in ILC1s compared to NK cells. Additionally, Eomes levels in NK cells were reduced with increase in disease severity. This prompted us to fully characterize Group 1 ILCs in murine models based on the expression of Eomes. Since Eomes and T-bet have been shown to be reliable in differentiating various subsets of Group 1 ILCs, we used these markers to study pulmonary Group 1 ILCs using a mouse model of B16F10 experimental metastasis. Since we observed a drop in Eomes levels in NK cells, post-metastasis (Stages III and IV), this murine model of metastasis was adopted to mirror similar conditions to confirm the role of Group 1 ILCs in cancer metastasis. Like in human cells, we noticed a decrease in the frequency of murine Eomes^{hi} NKp46^{+}NK1.1^{+} cells with increase in tumor burden, giving rise to T-bet^{+}Eomes^{lo} and T-bet^{+}Eomes^{hi} ILC1 subsets in the lungs. Further phenotypic and functional characterization of these subsets revealed an ILC1-like signature for Eomes^{lo} subset and NK-like properties for Eomes^{hi} cells. Interestingly, none of the cellular subsets showed specificity for NK or ILC1 markers, thus resulting in an "intermediate" ILC1 population as has been reported recently (29). In alignment with this, adoptive transfer of Eomes^{hi} subset did not give rise to Eomes^{lo} subset, suggesting different lineage of the two cell types. Our findings are also in line with a study showing immune-evasion by cancer cells through conversion of NK cells

into ILC1-like cells where Eomes^{lo} ILC1s produced significantly lower IFNγ and had reduced cytotoxicity compared to Eomes^{hi} NK cells (25). While we did not observe conversion of Eomes^{hi} cells into Eomes^{lo} cells under the conditions analyzed, the increase in the number of murine Eomes^{lo} ILC1s positively correlated with metastatic advancement. This observation, coupled with Eomes downregulation in human Group 1 ILCs with NSCLC progression, suggests that loss of Eomes is associated with a reduction in the anti-cancer effector function of Group 1 ILCs. Our findings provide an avenue for future elucidation of the molecular mechanism through which Eomes modulates cancer cell death. Additionally, it is interesting to note that the presence of B16F10 cells on the *in vivo* assays did not increase the levels of Eomes on ILC1 and NK cells (**Figure 3C**) but led to increased Eomes expression *ex vivo* in the killing assay (**Figure 5D**). We speculate that this perceived differences in the expression level could be due to different time points at which the cells were being analyzed. Eomes levels in **Figure 3C** were measured 6 days after adoptive transfer (at which point the Eomes levels could have stabilized) while in **Figure 5D**, the increase in eomes expression is detected at 24 and 48 h. It is also important to note that at 72 h time point in the killing assay, there was no observable change in Eomes levels, which further corroborates the *in vivo* data. Furthermore, while we observed near absence of Eomes^{lo} group 1 ILCs in T-bet KO mice, suggesting ILC1-like behavior, it is important to note that because of the compensatory nature of T-bet and Eomes, mice deficient in T-bet may have upregulated Eomes, which could in turn lead to their mis-identification as Eomes^{hi}. Therefore, to ultimately define the developmental profile and origin of these cells, lineage tracing with knockout mice deficient in transcription factors crucial for development of ILC1 but not for NK cells must be carried out in future.

In the context of cancer, while it is conceivable that various group 1 ILC subsets play an anti-cancer effector function due to their IFNγ production, caution is needed since the modulation of the immune cell response might occur in the tumor microenvironment, thus changing the role of these cells from anti- to pro-tumor phenotype as reported for other immune cell types (55, 56). Furthermore, whether these murine Eomes^{lo} and Eomes^{hi} subsets are similar to human Eomes^{lo} ILC1s and Eomes^{hi} NK cells remains to be confirmed. While the phenotype and profile of various Group 1 ILC subsets (e.g., cNKs and ILC1), have been identified in the past decade in liver, thymus, kidney, uterus and skin, such information on lung Group 1 ILCs in metastasis is hitherto unavailable. Therefore, to our knowledge, this is the first study on the role of ILC1s in metastasis in human and mouse. Finally, although our studies do suggest that the phenotype of Eomes^{lo} and Eomes^{hi} cells resembles that of ILC1 and NK cells, respectively, we cannot preclude the possibility that these cells could be under different activation states of the same cell types which developed out of distinct lineages. Future studies involving determination of the lineage would help to further elucidate the development and function of these cells.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Domain Specific Review Board (DSRB Reference number 2016/00698) affiliated to the National Healthcare Group (NHG), Singapore. The patients/participants provided their written informed consent to participate in this study. Animal Studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore, under Research Protocol R17-0209 and Breeding Protocol BR15-1142.

AUTHOR CONTRIBUTIONS

RV planned and performed the experiments. RV, JE, and RP analyzed the data. JS obtained patient consent and coordinated sample transfer between hospital and laboratory. RS, HM, and JT provided NSCLC samples and clinical information. RV, JE, and JD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01190/full#supplementary-material

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The Role of NK Cells and Innate Lymphoid Cells in Brain Cancer

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The brain is considered an immune privileged site due to the high selectivity of the blood-brain barrier which restricts the passage of molecules and cells into the brain parenchyma. Recent studies have highlighted active immunosurveillance mechanisms in the brain. Here we review emerging evidence for the contribution of innate lymphoid cells (ILCs) including natural killer (NK) cells to the immunosurveillance of brain cancers focusing on glioblastoma, one of the most aggressive and most common malignant primary brain tumors diagnosed in adults. Moreover, we discuss how the local tissue microenvironment and unique cellular interactions influence ILC functions in the brain and how these interactions might be successfully harnessed for cancer immunotherapy using insights gained from the studies of autoimmunity, aging, and CNS injury.

Keywords: clinical trial, immunotherapy, brain cancer, innate lymphoid cell, NK cell

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INTRODUCTION

The global age-adjusted incidence of brain tumors is 5.57 per 100,000 people with over half being astrocytic tumors (1). Of astrocytomas, the most malignant form, glioblastoma (GBM), is diagnosed at a much higher frequency than lower grade astrocytomas. Even with an aggressive treatment regime, comprised of maximal safe resection, radiotherapy, and administration of the DNA alkylating agent, temozolomide (TMZ), the mean GBM survival time ranges between 12 and 15 months. Whilst recent advances in cancer immunotherapy have enhanced expectations for improved patient outcomes, current GBM treatment options remain limited and the mean overall survival of GBM patients has failed to improve over the last decade. The unique anatomy of the brain, the exclusive nature of the blood-brain barrier (BBB), and a poorly immunogenic, complex, and immunosuppressive tumor microenvironment (TME) represent major challenges in treating malignant brain cancers. Here, we review emerging evidence for brain tumor immunosurveillance by NK cells and ILC subsets.

Natural Killer Cells

NK cells are large granular lymphocytes considered the innate counterparts of cytotoxic T lymphocytes (CTL) due to their spontaneous ability to lyse malignant and virus-infected cells, whereas ILC1, ILC2, and ILC3 mirror adaptive T helper subsets (**Figure 1A**). NK cells respond to "stressed" cells that downregulate MHC class I (MHCI) to evade CTL recognition and are therefore critical for anti-tumor immunity whenever CTL are compromised (3). NK cells are present at lower frequencies in the brains of naïve mice (4) (**Figure 1B**), but during neuropathological conditions, such as virus infection or autoimmunity, the BBB can become permeable, allowing NK cell migration into the CNS (5–9).

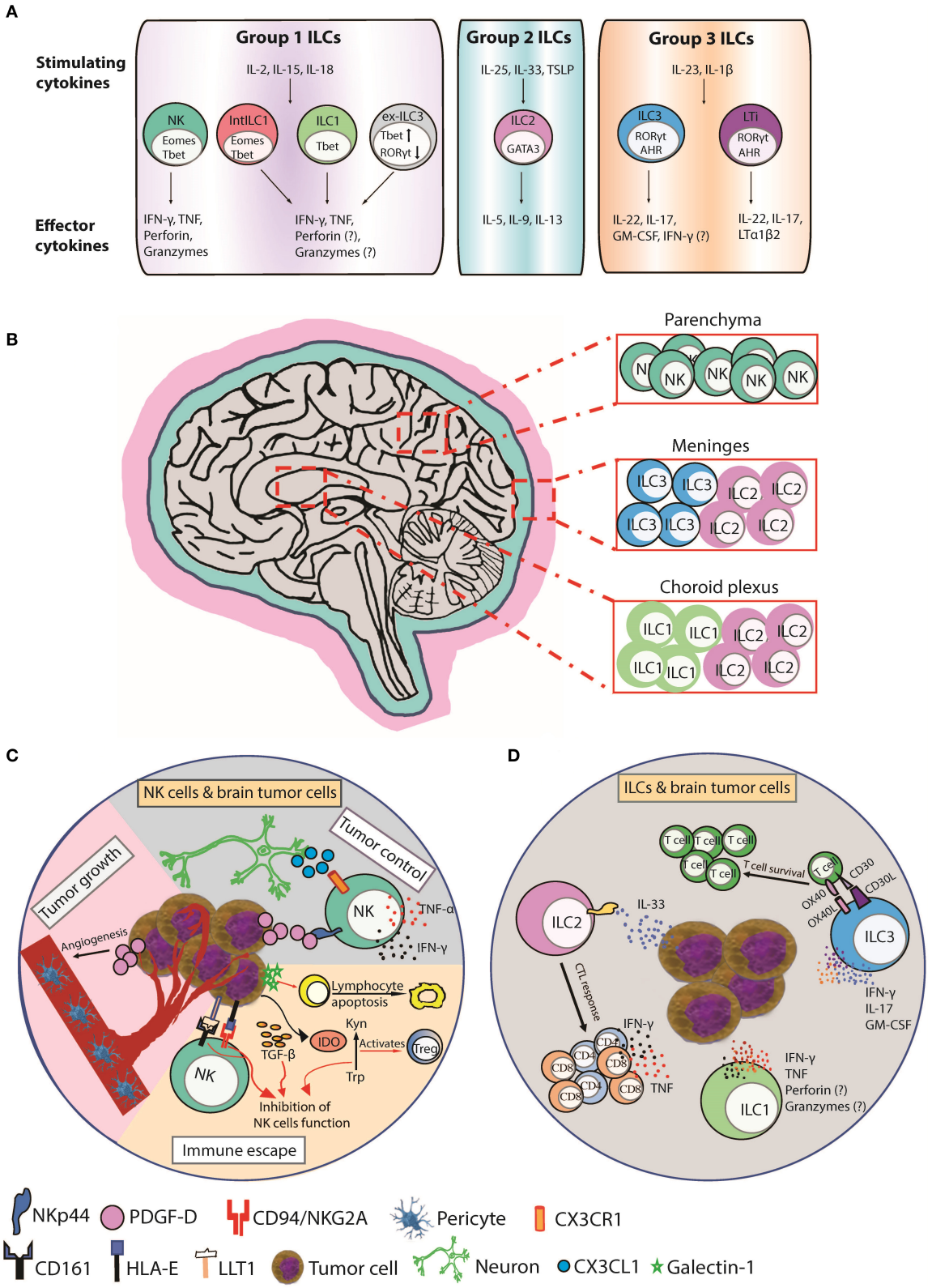

FIGURE 1 | Function, distribution, and anti-tumor responses of CNS ILC subsets. **(A)** Group 1 CNS ILCs have thus far been shown to include NK cells, intermediate ILC1s (intILC1s), ILC1s, and "ex-ILC3s." NK cells express T-bet and Eomes and secrete IFN-γ and TNF in response to IL-2, IL-15, and IL-18, and lyse malignant cells *(Continued)*

FIGURE 1 | via perforin, and granzymes. ILC1s express T-bet and produce IFN-γ and TNF in response to IL-2, IL-15, and IL-18 to promote type I immunity. Intermediate ILC1 (IntILC1) represent an intermediate phenotype between NK and ILC1 and express T-bet and Eomes. Ex-ILC3s are former ILC3 that have upregulated T-bet and downregulated RORγt to differentiate into ILC1-like cells. ILC2s express GATA3 and secrete IL-5, IL-9, and IL-13 in response to IL-25, IL-33, and TSLP to promote type II immunity. Group 3 ILCs include ILC3s and LTi cells. ILC3s express RORγt and AHR and produce IL-17, IL-22, and GM-CSF in respond to IL-23 and IL-1β stimulation to counteract extracellular bacterial and fungal infections. LTi cells also express RORγt and AHR and produce IL-17, IL-22, and lymphotoxin ($LT\alpha1\beta2$). LTi cells trigger lymphoid tissue organogenesis during development. **(B)** NK cells are mainly present in the brain parenchyma. ILC1s are enriched in choroid plexus, whereas ILC2s accumulate in the choroid plexus and meninges and ILC3s accumulate in the meninges. **(C)** $CX3CR1^+$ NK cells can infiltrate the brain in response to CX3CL1 chemokine produced by neurons. (Tumor control) PDGF-D expressed by tumor cells binds to the activating NKp44 receptor expressed on activated NK and induces the secretion of IFN-γ and TNF that inhibits tumor cell proliferation. (Tumor growth) PDGF-D enhances tumor growth by promoting pericyte recruitment and tumor angiogenesis. (Immune escape) Tumor cells upregulate IDO, which inactivates NK cells and activates immunosuppressive regulatory T cells (Tregs) by depletion of Trp and accumulation of Kyn. Tumor cells also secrete galectin-1 that induces lymphocyte apoptosis. Tumor cells supress NK cells function by inducing HLA-E and LLT1 ligands, which are ligands for NK cell inhibitory receptors CD94/NKG2A and CD161, respectively. TGF-β also inhibits NK cells function. **(D)** A hypothetical scheme showing the possible role of ILCs in brain cancer. Human ILC1s and ILC3s can express NKp44 and secrete IFN-γ and/or TNF in response to PDGF-DD to promote anti-tumor immunity (2). ILC2s enhance CTL responses to control the spread of tumors in response to IL-33 produced by tumor cells. ILC3s can also produce proinflammatory cytokines IFN-γ, IL-17, and GM-CSF and express the costimulatory molecules CD30L and OX40L that can promote T cell survival and function. AHR, aryl hydrocarbon receptor; CTL, cytotoxic T lymphocyte; EOMES, eomesodermin; GATA3, GATA-binding protein 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; IDO, indoleamine 2,3-dioxygenase; Kyn, kynurenine; LLT1, Lectin-like transcript-1; LTi, Lymphoid tissue-inducer; PDGF-D, Platelet Derived Growth Factor D; RORγt, retinoic acid-related orphan receptor gamma t; T-bet, T-box expressed in T cells; Trp, tryptophan; TSLP, thymic stromal lymphopoietin.

NK Cells in Brain Cancer

The CX3CL1 chemokine (also known as fractalkine) produced by neurons mediates $CX3CR1^+$ NK cell recruitment to the brain and is associated with a favorable glioma prognosis (10) (**Figure 1C**). In one study, NK cells constituted the highest percentage of lymphocytes infiltrating GBM compared to breast cancers or melanomas suggesting a prominent role for NK cells in brain cancer surveillance (11). NK cells infiltrate meningiomas and metastatic brain neoplasms (12–14) and lyse GBM and medulloblastoma tumor cell-lines *in vitro* (15–22). NK cell receptors are linked with brain tumor surveillance and an allele of the activating KIR2DS4 receptor is associated with control of cytomegalovirus (CMV)-positive GBMs (23). CMV-induced expression of platelet-derived growth factor D (PDGF-D) enhanced GBM growth by promoting pericyte recruitment and tumor angiogenesis (24) (**Figure 1C**). PDGF-D is expressed by most GBMs and binds to the activating NKp44 receptor to stimulate cytokine secretion from NK cells and ILCs to control tumor growth, which was associated with improved survival of GBM patients (2) (**Figure 1C**). These studies implicate NK cells engage in brain tumor surveillance that impacts prognosis (14).

Several computational-based studies show that glioma patients expressing activated NK cell transcriptional signatures (TS) have improved prognosis (2, 25–29). Studies in patients and mouse models support these findings (10, 30, 31), with one human study showing a remarkable relationship between the presence of activated NK cells and improved survival in GBM (32). Another study showed that activated NK cells were higher in low grade compared to high grade gliomas suggesting reduction in activated NK cells is associated with transition from low to high grade brain cancers (27). NK cells may therefore play a detrimental role in brain tumor progression and heterogeneity. Expression of B7-H6, a ligand for the activating NKp30 receptor, is elevated in human glioma and associated with tumor progression (33). Whilst NK cells efficiently lyse undifferentiated GBM cancer stem cells (CSC), NK cell-derived IFN-γ promotes GBM CSC differentiation and decreased susceptibility to NK cell cytotoxicity (34, 35). In GBM, CSCs that survive therapy are a source of tumor recurrence/relapse. Influencing the balance of NK cell-mediated lysis of CSCs or opposing the pro-tumorigenic effect of NK cell-IFN-γ-induced CSC differentiation will be an important mechanism to decipher and target. Interestingly, IFN-γ-induced CSC differentiation concomitantly enhances tumor susceptibility to chemotherapy, suggesting NK cell-based therapies can be combined with other therapeutic strategies for more effective clinical outcomes (36, 37).

NK Cell-Based Immunotherapies for Brain Cancer

CNS tumors are often poorly immunogenic and highly immunosuppressive which imposes barriers to successful immunotherapy (38). A summary of current research and clinical trials into NK cell immunotherapies for malignant CNS tumors is provided (**Table 1**). Whilst NK cell cytotoxicity is facilitated by an array of activating receptors (62, 63) the chief inhibitory signal for NK cells, MHC class I (MHC-I), can be overexpressed in CNS malignancies and suppresses NK cell activity (64, 65). Strategies to enhance NK cell anti-tumor function include activating the DNA damage response (DDR) to induce ligands for activating NK cell receptors (66–68). The proteosomal inhibitor bortezomib (BTZ) activates the DDR and sensitizes GBM cells to NK cell killing by inducing ligands for the activating receptors, NKG2D (39, 59, 69, 70) and DNAM-1 (40). BTZ treatment with autologous NK cells suppressed tumor growth and prolonged survival in 25% of test animals (70). However, appropriate BTZ scheduling with NK cell transfer remains to be optimized to prevent sensitization of NK cells themselves (70). GBM patients have increased expression of NKG2D ligands (NKG2DL) following TMZ therapy and TMZ-induced activation of the DDR improved survival in a mouse model of GBM that was NKG2D-dependent (41).

Anti-cancer treatments, such as chemotherapy, selective inhibitors of oncogenic signaling pathways, or oncolytic virotherapy can promote tumor cell death and enhance immunogenicity, which holds great potential when combined

TABLE 1 | Summary of current academic investigations and clinical trials into NK cell immunotherapy directed against malignant tumors of the CNS.

Therapeutic Strategy	Treatment	Results summary	Model (tumor type)	Reference/Trial ID
Combinational therapy NK cells and chemotherapy	NK cells infusion with: Sulindac; MAPK and cyclin-dependent kinase 4/6 inhibitors (38)	Reduces VEGF secretion and increases NK cell cytotoxicity; Suppresses tumor proliferation and increases NK cell cytotoxicity	Human (GBM, Lung cancer)	(37, 38)
Sensitization of tumors to NK cell cytotoxicity	NK cell infusion with BTZ	Predisposes tumor to NK natural cytotoxicity and TRAIL/DR5; BTZ and NK cell infusion increased tumor elimination	Mouse (BG7); mouse (U87)	(39, 40)
Virotherapy	Triple therapy (NK cell infusion, BTZ, oHSV)	Tumor clearance tumor bearing mice; combinational therapy with BTZ and oHSV enhances tumor death and NK cell activation	Mouse (GBM30)	(41, 42)
	TGF-β and oHSV infection	Modulated NK cell immune response to oHSV infected cells and improved anti-GBM effect of the oHSV treatment	Mouse (GB30)	(43)
Overcoming immunosuppressive TME	siRNA interference of TGF-β signaling; infusion of TGF-β receptor negative NK cells	Reduction of tumorigenic profile of glioma; NK cells were resistant to TGF-β inhibition	Mouse (LNT229); *in vitro*; *in vitro*	(44–46)
Toll-like receptor agonism	CpG-ODN DNA TLR-9 agonism	Clinical trials failed to recapitulate results of encouraging *in vitro* TLR-9 agonism	Clinical trial (GBM); mouse (GL621)	(47–50)
	Poly-ICLC TLR-3 agonism with bevacizumab (a-VEGF)	Poly-ICLC adjuvant to tumor associated antigens mixed with Bevacizumab—study unfinished, promising *in vitro* results	Human trial (GBM)	NCT02754362 (withdrawn—personnel changes)
Directing NK cell immunity toward brain tumor antigens	Infusion of monalizumab (a-NKG2A)/cetuximab (a-EGFR)	NKG2A blockage potential to boost ADCC against GBM. Cetuximab treatment increased ADCC mediated by CD16+ NK cells (IL-2 or lectin-activated)	Human GBM stem cells *in vitro*	(51)
	Infusion of CAR NKs engineered to be specific for EGFR, EGFRVIII, Erbb2	EGFR, EGFRvIII CAR NK cells suppressed tumor growth and significantly prolonged survival; CXCR4 transduction enhanced antitumor responses of EGFR CAR NK cells; Erbb CAR NK cells	Mouse (GB19 xg); mouse (U87 xg); mouse (GL621 xg)	(52–54)
	Infusion of a-NG2/CSPG4 Ab	NK cell directed ADCC and proinflammatory tumor environment enhancing survival	Rat (U87); mouse (GBM)	(55, 56)
Immune checkpoint blockade	Infusion of a-PD-1 and a-CTLA-4 Ab	Interference with peripheral immune cell inhibition potentiates intracranial immune response; immune checkpoint blockade antibodies improved survival in models	Mouse(GL621); mouse (B16)	(57, 58)
	Blockade of interactions of HLA-E:CD94/NKG2A or LLT1/CD161 with siRNA or blocking Ab	Blockade promoted NK cell lysis	Human (GBM) *in vitro*	(59, 60)
Circumventing the BBB	Infusion of a-CTLA-4/a-PD-1 Ab conjugated to biopolymer scaffold	Treatment able to cross BBB. Prolonged survival of mice compared to free a-CTLA-4 and a-PD-1 Ab	Mouse (GL261)	(61)
Autologous NK cell infusion expanded *ex vivo*	Artificial antigen-presenting cells	Promising *in vitro*, results forthcoming	Human trial (Recurrent MBM)	NCT02271711 (active)
	Cytokines/Feeder cells + infusion recombinant human interleukin-15	Promising *in vitro*, results forthcoming	Human (Solid brain tumors, SCM, NBM)	NCT01875601 (complete)
	Cancer/testis antigens presented by DNA-demethylated T_H cells	Labeled cells infiltrated tumor site (SPECT imaging). Reduced disease burden 5 out of 10 patients. Long term survival in 3 of 10. However, slow kinetics of induced antitumor response	Human trial (GBM)	NCT01588769 (complete)
	Genetically modified feeder-cells	Promising *in vitro*, results forthcoming	Human trial (GBM)	NCT04254419 (not yet recruiting)
Allogeneic (HLA)-haploidentical hematopoietic cell transplantation	Allo HTC and infusion with donor NK cells	Promising *in vitro*, results forthcoming	Human trial (eSCM, rSCM, oSCM, NBM)	NCT02100891 (recruiting)

Abbreviated cancers: GBM, glioblastoma; MBM, medulloblastoma; NBM, neuroblastoma; e, Ewing; SCM, sarcoma; r, Rhabdomyosarcoma; o, Osteosarcoma. Other abbreviations: xg, Xenograft; VEGF, vascular endothelial growth factor; BTZ, bortezomib; oHSV, oncolytic herpes simplex virus; TGF-β, transforming growth factor-beta; EGFR, epidermal growth factor receptor; Ab, antibody.

with immunotherapy (42, 71). Using a triple therapy approach, BTZ treatment combined with an oncolytic Herpes Simplex Virus (oHSV) strain sensitized GBM to adoptive NK cell therapy (40). BTZ enhanced expression of ligands for activating NK cell receptors, such as DNAM-1, whilst oHSV infection induced NK cell secretion of IFN-γ and TNF, which enhanced tumor cell death and improved survival of athymic nude mice transplanted with GBM tumors (40). Conversely, other studies claim NK cells limit oncolytic virotherapy by curbing virus infection of tumor cells. Transient TGF-β delivery or NK cell depletion increased oHSV titers, suppressed tumor growth, and prolonged survival in mouse GBM models (43, 72). TGF-β is considered a pro-tumor cytokine (73, 74) that suppresses NK cell function by downregulating activating NK cell receptors (75–77) or their ligands on brain tumor cells (78) (**Figure 1C**), reducing NK cell proliferation and converting NK cells into pro-tumor ILC1-like cells (79), or upregulating immunosuppressive extracellular matrix TME components, such as the galectins (44). Neutralizing TGF-β in the brain TME rescued NK cell anti-tumor function in glioma or medulloblastoma patients (45, 46) and expression of a dominant negative TGF-β receptor lacking the kinase domain (80) restored NK cell cytotoxicity against GBM and medulloblastoma cells in the presence of TGF-β *in vitro* (46, 81).

Brain tumors also secrete other soluble immunosuppressive factors, such as the carbohydrate-binding protein, galectin-1, that reduced lymphocyte viability (82) (**Figure 1C**). Galectin-1-deficient gliomas are more susceptible to NK cell lysis and were eradicated by NK cells before adaptive anti-tumor immune responses (17). Alternatively, glioma cells upregulate indoleamine 2,3-dioxygenase (IDO), a key rate-limiting enzyme of tryptophan (Trp) metabolism. IDO is involved in tumor-derived immunosuppression through Trp depletion and accumulation of the metabolite kynurenine that inactivated NK cells and promoted immunosuppressive regulatory T cells (Tregs) (83) (**Figure 1C**).

Brain tumor cells also modulate their cell-surface to suppress NK cell function. Gain-of-function mutations in isocitrate dehydrogenases (IDH1 and IDH2) in diffuse gliomas promotes epigenetic reprogramming of a number of immune genes including NKG2DL downregulation and resistance to NK cell-mediated lysis (60). Decitabine (a hypomethylating compound) increased NKG2DL expression and restored NK cell-mediated lysis of IDH mutant cells in an NKG2D-dependent manner. In addition to downregulating activating NK cell surface interactions, brain tumors also promote inhibitory NK cell surface interactions. Malignant gliomas induce HLA-E or Lectin-like transcript-1 (LLT1), which can induce NK cell inhibition by binding CD94/NKG2A and CD161, respectively (51, 84–86) (**Figure 1C**). Blockade of HLA-E:CD94/NKG2A or LLT1/CD161 inhibitory interactions using small interfering RNA or blocking antibodies promoted NK cell lysis of glioma cells (65, 87). Interestingly, a humanized anti-NKG2A antibody, "monalizumab," in combination with the anti-epidermal growth factor receptor (EGFR) "cetuximab," is effective in promoting antibody-dependent cellular cytotoxicity (ADCC) against cetuximab-coated head and neck squamous carcinoma (88). EGFR is a prime target for therapy across a broad variety of tumor types including gliomas, suggesting NKG2A blockade with monalizumab has potential to boost NK cell-mediated ADCC against gliomas, particularly those resistant to TMZ (89).

Cancer adjuvants provide other means of reinvigorating anti-tumor immune responses. Oligodeoxynucleotides containing unmethylated cytosine-guanosine motifs (CpG-ODNs) mimic pathogen-associated molecular patterns (PAMPs) that bind to Toll-like Receptor 9 to induce type-I interferon (IFN-I) production from plasmacytoid dendritic cells (pDCs) (90). IFN-I enhances NK cell anti-tumor functions (47, 48) and CpG-ODN stimulation and Treg ablation unleashed NK cell cytotoxicity toward intracranial tumors (49). Despite encouraging results *in vitro*, human trials of CpG-ODN treatment in patients with primary (50) and recurrent GBM (52, 91) reported no benefit in CpG-ODN therapy, suggesting combination treatments are necessary, e.g., Treg depletion that may improve clinical responses. Intriguingly, depletion studies have implicated NK cells as the predominant anti-tumor effector cell in murine models of glioma following repeated low dose administration of CpG-ODN (53). However, tumor-infiltrating NK cells remained susceptible to suppression both locally and systemically, reinforcing the need for more effective methods of augmenting NK cell function in the brain TME.

Chimeric Antigen Receptors (CARs) are tumor-specific antibody single-chain variable fragments (scFvs) fused by a transmembrane linker domain to the CD3ζ signaling chain of the T cell receptor that can be transduced into autologous cytotoxic T lymphocytes (CAR T) or NK cells (CAR NK), respectively (54). CAR NK cells are attractive because they can be engineered to respond to a tumor antigen whilst retaining capacity for natural cytotoxicity. CAR NK cells directed toward EGFR or the constitutively activated mutant EGRFvIII GBM tumor antigen (55, 56) and ErbB2 (92) have shown potent cytotoxicity toward primary GBM tumor cells and cell lines *in vitro*. Some studies targeting GBM antigens show NK cells are important regulators of proinflammatory environments through IFN-γ secretion rather than cytotoxicity (57, 58). Transfer of NKG2D-CAR T cells combined with radiotherapy exhibited therapeutic synergy in mice bearing orthotopic tumors of the murine glioma cell line, GL261, although mice receiving intratumoral vs. intravenous CAR T cells were more likely to survive, reiterating the poor infiltration of intravenously administered CAR T cells into the brain parenchyma (61). Such studies highlight the need to develop immunological and mechanical adjuvants in concert with NK cell therapy to improve delivery to the brain parenchyma.

The BBB is a selectively permeable membrane that excludes harmful material from the parenchyma but impedes delivery of immunotherapeutic agents and cells to brain tumors. Anti-PD-1 and anti-CTLA-4 checkpoint blockade therapy increased NK cell and $CD8^+$ T cell infiltration to the CNS and improved survival in models of GBM and melanoma brain metastases (93, 94). The conjugation of poly(β-L-malic) acid to anti-PD-1 and anti-CTLA-4 facilitated NK cell infiltration and survival in mice bearing intracranial GL261 tumors (95, 96). Combining these approaches with techniques to prolong persistence and

enhance cytolytic potential of adoptively transferred NK cells may assist in overcoming the BBB and the immunosuppressive brain TME (97).

Group 1 Innate Lymphoid Cells (ILC1)

ILC1 express the transcription factor T-bet and secrete IFN-γ in response to IL-12, IL-15, and IL-18, facilitating control of intracellular pathogens by classical macrophage activation (98) or limiting local viral replication (99) (**Figure 1A**). It has been challenging to distinguish tissue ILC1s from NK cells because they share common functions and markers. The CNS contains NK cells, ILC1s, intermediate ILC1s, and "ex-ILC3" in the brain parenchyma, meninges, and choroid plexus (CP) (100). In contrast to CNS-NK cells, CNS-ILC1s are enriched in the CP (**Figure 1B**) (100). ILC1 functions in brain tumors awaits in depth evaluation but were investigated in the context of autoimmune encephalomyelitis (EAE) (100). Steady-state CNS-NK cells and CNS-ILC1s expressed similar amounts of IFN-γ, whilst CNS-ILC1s produced more TNF. In EAE, NK cells had increased IFN-γ and TNF expression whilst ILC1s maintained stable levels. Interestingly, CNS-ILC1s from naïve and EAE mice express granzyme B and perforin and degranulated suggesting anti-tumor cytotoxic potential.

NK cells, ILC1s, and intermediate ILC1s accumulated in the brain parenchyma as EAE progressed. Unlike NK cells, ILC1s and intermediate ILC1s did not proliferate *in situ*, suggesting entry into the brain parenchyma via meninges or CP. ILC1 distribution and response during EAE strongly suggests they can regulate neuroinflammation. CNS-ILC1 were $CD49a^{+}CD49b^{-}$ $T\text{-}bet^{+}Eomes^{-}$ whilst CNS-NK cells were $CD49a^{-}CD49b^{+}$ $T\text{-}bet^{+}Eomes^{+}$. These markers provide a useful foundation to study ILC1 and NK cell contributions to anti-tumor immunity in mouse models of brain cancer. The dynamics and cytotoxic potential of ILC1s observed in the context of EAE suggests a potential role for CNS-ILC1s in restricting the growth of a brain tumors by lysing tumor cells or secreting immunoregulatory cytokines (100). NKp44 is also expressed by human ILC1 and ILC3 and activated by PDGF-DD, a pathway implicated in greater GBM survival (**Figure 1D**). It will be interesting to delineate the relative contributions of human ILC1, ILC3, or NK cells to anti-tumor immunity in brain cancers, such as GBM (2).

Group 2 Innate Lymphoid Cells (ILC2)

ILC2 express GATA3 and respond to IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) by secreting IL-5, IL-9, and IL-13 to promote type II immunity (**Figure 1A**) (101). ILC2s are found in large numbers in the meninges of naïve mice enriched around the dural sinuses (**Figure 1B**). After spinal injury, meningeal ILC2s are activated by IL-33 to produce IL-5 and IL-13 and partially improve recovery following spinal cord injury (102). ILC2s accumulate in the CP of aged mice and represent the major lymphocyte subset in aged mice and humans (103). ILC2s in the aged brain produced large amounts of IL-5 and IL-13 in response to IL-33. Treatment with IL-5 or IL-33 or adoptive transfer of activated ILC2s into the brain improved neurogenesis and cognitive function by reducing TNF secretion from brain-resident $CD8^{+}$ T cells. ILC2s may therefore play a neuroprotective role by orchestrating appropriate CNS immune responses (**Figure 1D**).

Many reports suggest type-II immunity downregulates anti-tumor immunity by hindering CTL. Mice genetically lacking ILC2s had markedly increased tumor growth rates and higher frequencies of circulating tumor cells and brain metastases (1,000-fold) (104). Tumor cell-derived IL-33 stimulated IL-13 secretion by ILC2s that enhanced DC antigen presentation and generation of anti-tumor CTL. The authors proposed a model where ILC2s mobilized from the lungs and other tissues enter tumors at distal sites to engage in immunosurveillance (104). It will be interesting to determine the relative contribution of meningeal ILC2s or ILC2s mobilized from tissues, such as the lungs, in restricting brain metastases and tumor growth (102). Interestingly, the primary male sex hormone testosterone, influenced ILC2 numbers and function and promoted and sustained a non-pathogenic TH2 myelin-specific response in EAE. These results suggest sexual dimorphism in ILC2 numbers or function could influence ILC2 brain tumor surveillance in addition to GBM invasiveness (105, 106). The protective role of activated ILC2s that observed during CNS injury, EAE, and restriction of brain metastases, suggests a potential role for activated meningeal ILC2s in suppressing brain tumor progression by enhancing CTL activity in response to elevated levels of IL-33 that are observed in brain cancers, such as glioma (107).

Group 3 Innate Lymphoid Cells (ILC3)

ILC3 express RORγt and respond to IL-23 and IL-1β stimulation by secreting IL-17 and IL-22 that induce epithelial defense mechanisms and granulocytic responses to counteract extracellular bacterial and fungal infections (98) (**Figure 1A**). Lymphoid tissue-inducer (LTi) cells that trigger lymphoid tissue organogenesis during development are $ROR\gamma t^{+}$ and produce IL-17 and IL-22 are categorized as ILC3 (**Figure 1A**) but emerge from the common lymphoid progenitor prior to ILCs (108). The role of ILC3 in brain cancer has not been extensively investigated. $ROR\gamma t^{+}$ ILC3s are residents of the meninges in naïve B6 mice (109). LTi characteristically express c-Kit whereas expression of NKp46 differentiates ILC3 subtypes. A population of $NKp46^{+}CD4^{+}$ ILC3s was detected in the meninges but not in the CNS, whereas more $c\text{-}Kit^{+}$ ILC3s were observed in the meninges than the CNS (**Figure 1B**).

In EAE, ILC3 numbers in the meninges and CNS increased. Meningeal ILC3s produced proinflammatory cytokines (IFN-γ, IL-17, and GM-CSF), and the costimulatory molecules (CD30L and OX40L) that promoted $CD4^{+}$ memory T cell survival and function, which may impact the recognition of MHC class II-restricted neoantigens and response to immunotherapy TME (110). Moreover, $c\text{-}Kit^{+}$ LTi cells, which can promote ectopic lymphoid follicle development, a hallmark of autoimmune diseases, were reduced in the meninges of EAE-resistant c-Kit mutant mice. Importantly, disease-induced trafficking of adoptively transferred wild type T lymphocytes to the meninges was impaired in ILC3-deficient $Rorc^{-/-}$ mice showing ILC3s sustain neuroinflammation by supporting T cell survival and reactivation in the meninges (**Figure 1D**).

Another study found T-bet-dependent NKp46$^+$ ILCs, which encompass NK cells, ILC1, and the NKp46 subset of ILC3s, were localized to the meninges and produced various inflammatory mediators that disrupted the BBB and facilitated infiltration of myelin-reactive TH17 cells into the brain parenchyma (111). Selective loss of T-bet in NKp46$^+$ ILCs resulted in the reduction of NK cells and ILC1s in the meninges and production of IFN-γ by NKp46$^+$ ILC3s, which impaired TH17 invasion of the CNS and protected from EAE disease. Importantly, NK cell-sufficient and NK cell-deficient mice showed similar levels of paralysis, suggesting NK cells do not play a major role in EAE immunopathogenesis and the pathogenic role of T-bet maps to the NKp46$^+$ ILC3s and/or ILC1. Collectively, these findings suggest meningeal ILC3s could promote anti-tumor immunity in brain cancer by facilitating the infiltration of T lymphocytes into the brain and supporting their survival. It will be interesting to determine if the NKp44$^+$ cells located in the brain parenchyma and associated with improved survival in GBM are derived from meningeal ILC3 populations or from other regions of the brain (2) (**Figure 1D**).

CONCLUDING REMARKS

Transition of low-grade glioma to high-grade glioma, including transition to GBM, as well as post-treatment relapse remain major causes of treatment failure, and improved strategies to eradicate brain tumor cells are required. Understanding the functions of NK cells and ILCs in both healthy and tumorigenic brain is necessary for developing strategies for effective immunotherapy, including enhanced activation of brain resident NK cells and ILCs and transfusion and mobilization of engineered NK cells, e.g., CAR NK (112). Existing evidence, presented in this review, demonstrates a rapidly evolving NK cell and ILC research field and proposes that continued research efforts will lead to the development and refinement of NK- and ILC-based therapies which can be used in combination with existing standard and novel oncolytic virus and drug-based therapies to meaningfully enhance brain cancer patient outcomes.

AUTHOR CONTRIBUTIONS

AS, NG, and PC drafted the manuscript. TM and AB critically reviewed the manuscript for important intellectual content and approved it for publication. All authors contributed to the article and approved the submitted version.

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A Transcriptional Signature of PDGF-DD Activated Natural Killer Cells Predicts More Favorable Prognosis in Low-Grade Glioma

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The binding of platelet-derived growth factor D (PDGF-DD) to the NKp44 receptor activates a distinct transcriptional program in primary IL-2 expanded human natural killer (NK) cells. We were interested in knowing if the PDGF-DD-NKp44 pathway of NK cell activation might play a clinically relevant role in anti-tumor immunity. In order to address this question, we determined transcriptional signatures unique to resting, IL-2 expanded, and PDGF-DD activated, NK cells, in addition to different T cell subsets, and established the abundance of these immune cell phenotypes in The Cancer Genome Atlas (TCGA) low-grade glioma (LGG) dataset using CIBERSORT. Our results show that LGG patient tumors enriched for either the PDGF-DD activated NK cell or memory $CD8^+$ T cell phenotypes are associated with a more favorable prognosis. Combined cell phenotype analyses revealed that patients with LGG tumors enriched for the PDGF-DD activated NK cell phenotype and the $CD4^+$ T helper cell phenotype had a more favorable prognosis. High expression of transcripts encoding members of the killer cell lectin-like receptor (KLR) family, such as KLRK1 and KLRC2, KLRC3 and KLRC4 in LGG tumors were associated with more favorable prognosis, suggesting that these NK cell family receptors may play a prominent role in LGG anti-tumor immunity. Finally, many of the TCGA findings were reciprocated in LGG patients from the Chinese Glioma Genome Atlas (CGGA) dataset. Our results provide transcriptomic evidence that PDGF-DD activated NK cells and KLR family receptors may play an important clinical role in immune surveillance of LGG.

Keywords: NK cell, low grade glioma, NK cell receptor, anti-tumor immunity, The Cancer Genome Atlas

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INTRODUCTION

Diffuse and infiltrative low-grade gliomas (LGGs) are derived from the malignant transformation of astrocytes or oligodendrocytes (1). Whilst grade I LGGs are readily resectable benign tumors, grade II LGG display pathologic traits and inexorably progress to high grade gliomas, such as glioblastoma (GBM), with terminal neurological decline (1, 2). Early surgical excision and temozolomide treatment followed by radiotherapy underpin current standards of care but are not curative (3–5). Despite growing understanding of LGG pathogenesis, clinical outcomes have failed to improve particularly for young adults (6). Furthermore, variable rates of progression to lethal disease impede timely clinical intervention and make accurate prognoses difficult (7). Thus, there is an urgent need to understand effective anti-tumor immunity in LGG. Whilst the phenotype and function of tumor-infiltrating lymphocytes (TILs) have been explored for high grade gliomas (8), the prognostic value of TIL subsets and the molecular pathways of tumor recognition for LGG remain unclear.

NK cells preferentially eliminate nascent tumors that have downregulated MHC class-I (MHC-I) (9–11). In homeostasis, NK cells are retained in the tissues surrounding the brain parenchyma by the blood-brain-barrier (BBB) (12, 13). Whilst NK cells have been identified in brain tumors and the surrounding tissue microenvironment (14–16), the mechanisms facilitating NK cell transmigration across the BBB and activation within the brain are poorly defined, although the BBB is more permeable under inflammatory conditions (17–20). Glioma cell lines are readily susceptible to NK cell lysis *in vitro* (21, 22). However, *in vivo* studies reveal a highly vascularized tumor microenvironment that actively subverts immune control (23, 24) and so the significance of NK cell surveillance for gliomas remains to be fully understood (25). Defining immune surveillance mechanisms in those LGG patients with enhanced survival will therefore be critical for the development of novel cancer immunotherapies.

Among others, germline-encoded activating receptors, such as KLRK1 (also known as NKG2D) and the Natural cytotoxicity receptors (NCRs), such as NKp46 (NCR1), NKp44 (NCR2), and NKp30 (NCR3), can synergize to overcome inhibitory thresholds and evoke NK cell anti-tumor functions (26–28). As such, NK cell anti-tumor activity is sensitive to activating receptor surface phenotype and the expression of tumor ligands (29). KLRK1 recognizes a range of ligands upregulated by transformed cells, such as MHC class I chain-related sequence (MIC) A and MICB, which are major determinants of NK cell tumor cytolysis in humans (30–33). Of the NCRs, NKp44 also recognizes a range of cellular and tumor-associated surface ligands, such as Nidogen-1 (34), the heparan sulfate proteoglycan, Syndecan-4 (35), a subset of HLA-DP molecules (36), a splice variant of the mixed lineage leukemia 5 (MLL5) gene (37), and proliferating cell nuclear antigen (PCNA) (38), that have all been reported to positively or negatively regulate NK cell function (39, 40). Recently, PDGF-DD was shown to induce signaling from the activating NKp44 immunoreceptor (41).

The platelet-derived growth factor (PDGF) family are comprised of four polypeptides that assemble into five dimeric isoforms, PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD. PDGFs play essential roles in embryonic development, cell proliferation, cell migration, survival and chemotaxis by engaging PDGF receptors (PDGFRs) that are mostly expressed by mesenchymal cells (42). PDGF-DD is a potent mitogen that plays an important role in wound healing and blood vessel maturation during angiogenesis by inducing PDGFR-β signaling on mesenchymal cells (43, 44). In brain cancer, PDGF-DD binding to PDGFR-β can induce pro-tumorigenic signaling that drives glioma progression (45–49).

PDGF-DD stimulation of NKp44 induced NK cell secretion of IFN-γ and TNF that arrest tumor cell proliferation *in vitro* and may confer a survival benefit in GBM (41). In support of this, PDGF-DD is abundantly expressed in GBM, suggesting a novel mode of NK cell tumor surveillance (50), but the clinical significance of the NKp44-PDGF-DD pathway for anti-tumor immunity in many other human cancers including LGG remains unclear.

Here, we employed a computational approach to investigate the clinical impact of the relative enrichment of resting, IL-2 expanded, and PDGF-DD activated NK cell phenotypes in the LGG tumor microenvironment. To achieve this, we used transcriptional signatures from the three NK cell activation states and estimated their relative abundance in LGG tumor specimens from TCGA database and tested the association with curated progression-free survival (51).

METHODS

Material Availability

The R codes for the analyses presented in this study are available at RAGG3D/LGG_SPANK (github.com). An overview of the methods used in this study are shown in **Supplementary Figure 1**.

Data Collection

Gene transcript-abundance and patient clinical information were collected from TCGA through the GDC Data Portal (52) and the CGGA (53–56). Progression-free survival information was used as a measure of clinical outcome (51). The cell-type specific transcriptional signatures were derived from a large collection of RNA sequencing samples spanning a wide range of cell types. For NK cells, experimentally derived dataset for IL-2 expanded (27 biological replicates), PDGF-DD activated *via* NKp44 signaling (4 biological replicates), and resting (25 biological replicates from 6 datasets) (41) were included. For other cell types, the data collected was from the following datasets: BLUEPRINT (57), Monaco et al. (58), ENCODE (59), Squires et al. (60), GSE77808 (61), Tong et al. (62), PRJNA339309 (63), GSE122325 (64), FANTOM5 (65), GSE125887 (66), GSE130379 (67), GSE130286 (68).

Transcriptional Signatures

In order to derive transcriptional signatures of 21 cell types (memory B cell, naive B cell, immature dendritic myeloid cell, immature dendritic myeloid cell, endothelial, eosinophil, epithelial, fibroblast, macrophage M1 and M2, mast cell,

monocyte, neutrophil, ReNK, IL2NK, SPANK, memory CD4 T cell, memory CD8 T cell, naive CD8 T cell, gamma-delta T cell and helper T cell), a total of 592 highly curated (i.e. for which identity was confirmed in the literature), non-redundant biological replicates (including 25 ReNK samples, 27 IL2NK samples and 4 SPANK samples), have been used. Due to the sparse nature of heterogeneous data sets, the expected value and variability of gene transcription abundance was inferred for each cell type using a publicly available Bayesian statistical model (github: stemangiola/cellsig), based on a negative binomial data distribution (69). This model allows to fit incomplete data (e.g. transcript abundance of one gene for which data is available in a subset of reference biological replicates) and calculate theoretical data distributions of cell-type/gene pairs. The cell-type transcriptional marker selection was based on the pairwise comparison of each cell type within cell-type categories along a cell differentiation hierarchy (**Supplementary Figure 2**) (70). For example, all cell-type permutations from the root node of level one (including epithelial, endothelial, fibroblasts and immune cells) were interrogated in order to select the genes for which the theoretical transcript abundance distribution (data generated from the posterior distribution) was higher for one cell type compared to another. This was executed calculating the distance of the upper and lower 95% credible intervals, respectively (obtained from cellsig). From each comparison, the top 5, 10 and 20 genes per cell-type pair were selected from levels 1, 2, and 3 (**Supplementary Figure 2**), and the union of all genes was taken as overall marker gene list. This hierarchical approach favors the identification of marker genes that distinguish broad cell-type categories as well as specific activation phenotypes.

Estimation of the Association of Cell-Type Abundance With Relapse-Free Patient Survival

In order to estimate the cell type relative abundance for each biological replicate, we used the algorithm CIBERSORT (71) with our RNA sequencing-derived gene marker signature. In order to estimate the clinical relevance of NK activation phenotypes (72) (73),for each cancer-type/cell-type pair, Kaplan-Meier (KM) survival curves (74) were calculated from the median split CIBERSORT-inferred proportions through the R framework tidybulk. Percent survival vs time-to-event statistics were calculated by the Log-rank (Mantel-Cox) Test (75). Statistics of KM curves were performed by log-rank test then adjusted by the Benjamini-Hochberg (BH) procedure. A table of all p-values prior to adjustment is provided in **Supplementary Table 1**.

Data analysis and visualization were performed using the R environment in RStudio (76). Packages include tidyverse (77), tidybulk (73), tidyHeatmap (78), survminer (79), survival (80, 81), foreach (82), org.Hs.eg.db (83), cowplot (77), ggsci (84), GGally (85), gridExtra (86), grid (76), reshape (87), Hmisc (88), and viridis (89).

Benchmark of the Transcriptional Signatures

In order to visually evaluate the ability of the marker gene selection in segregating cell types, we first performed principal component analyses (PCA) (90) for three levels of cell differentiation: (i) the NK activated states, (ii) all NK cells, and (iii) all cell types. Briefly, the raw read counts were normalized by trimmed mean of M values (TMM) using tidybulk function *scale_abundance*, whilst CGGA transcripts were already normalized by transcripts per kilobase of exon model per million mapped reads (TPM) *via* RNA-seq by expectation Maximization (RSEM). PCA analyses were performed by *reduce_dimensions(method="PCA")* (73). To directly test whether the selected signature for PDGF-DD activated NK cells was suitable to accurately detect changes in cell abundance across samples with a censored time-to-event, we implemented a test on simulated data. First, for a selected number of patients N, we sampled the progression-free survival time from the clinical annotation of the LGG patient cohort. Cell type proportions were simulated using a Dirichlet distribution, according to a linear model with a slope value S and a progression-free survival as factor of interest. For each simulated dataset, the slope S was assigned to only one cell type, and the slope of 0 to all the others (i.e. only one cell type changing for each simulated tissue mixture). The intercept (baseline proportion) was defined to be the same for all cell types. The simulated proportions were used to compose the in-silico mixtures. For each simulated dataset, the transcriptional profile of each cell type was sampled at random from the reference dataset. In order to test the accuracy of our method against the presence of foreign cell types (of which transcriptomic signature was not included in our reference set), a proportion P of neural cells was added to the mixture. The framework tidybulk was used to infer the cell type proportions through CIBERSORT and perform a multiple cox regression on the predicted proportions (logit-transformed) (81), with progression-free survival censored time as a covariate. That is, for half of the N samples, the survival time was censored to half of its value. The significance calls were compared with the ground truth to generate a receiver operating characteristic (ROC) curve. For each simulation condition (values of N, S, and P), 63 test runs were performed with one variable cell type each. A range of simulation conditions were tested, ranging N from 250 to 1000, S from 0.2 to 1, and P from 0 to 0.8.

RESULTS

NK Cell Phenotypes Have Unique Transcriptional Profiles

Given the innate ability of NK cells to lyse tumor cells and secrete potent anti-tumor cytokines, such as IFN-γ and TNF, prior to immunization, we hypothesized that NK cells of unique phenotype may infiltrate different cancers and confer anti-tumor immunity. Specifically, we were motivated by the recent discovery of PDGF-DD as a ligand for the activating NK cell receptor NKp44 (41) and whether this mechanism of NK cell stimulation might constitute a clinically relevant pathway of anti-tumor immunity. In order to answer this question, we gathered publicly available RNA sequencing data from 21 different

immune and stromal cell types (see Methods) in order to define marker genes that may distinguish resting (91), IL-2 expanded, and PDGF-DD activated, NK cell phenotypes (41).

We next performed a principal component analysis (PCA) to determine the ability of these marker genes to segregate all three NK cell phenotypes from each other and from other cell types, such as T cells (**Figure 1A**). NK cell activation states are associated with the first principal components (**Figure 1A**, left and middle panels), and NK cells overall are defined by a unique cluster when compared with other major cell types (**Figure 1A**, right panel). NK cell phenotypes segregated from other cell types and from each other by PCA.

We next performed a benchmark for the inference of changes in the relative abundance of PDGF-DD activated NK cells in association with survival information for artificial tissue mixtures built from our reference data set (see Methods). This benchmark measured the ability of the PDGF-DD activated NK cell signature to extract clinically-relevant information from TCGA whole tissue RNA sequencing data (**Figure 1B**). The benchmark showed a high accuracy (area under curve) across simulation settings including magnitude of variability, sample-size, and proportion of unknown cells (please see Methods). An accuracy of 0.75 (representing the area under the ROC curve) was reached for simulation settings that match our findings on TCGA data (slope and sample size; **Figures 1C, D**). We refer to the different NK cell phenotypes as: resting NK cells (ReNK), IL-2 expanded NK cells (IL2NK), and the signature of PDGF-DD activated NK cells (SPANK), respectively, and the transcript

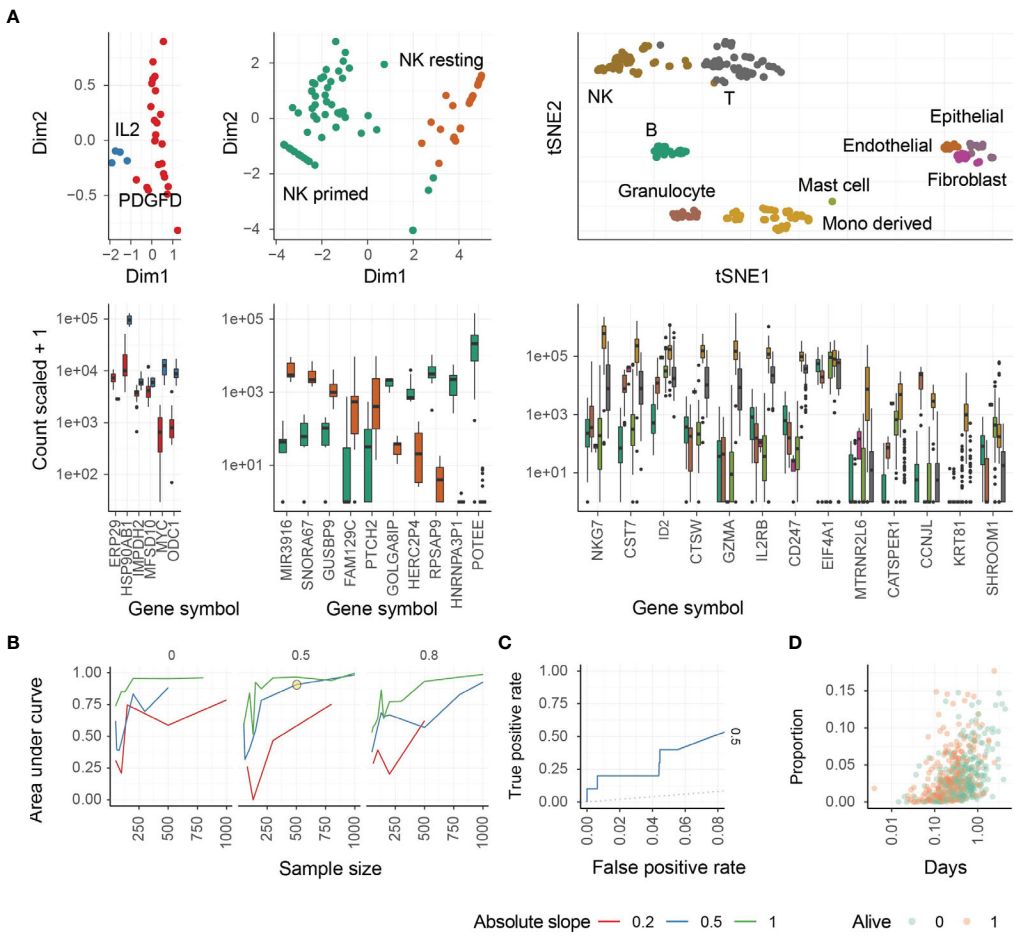

FIGURE 1 | Identification of different NK cell phenotypes using transcriptional signatures. **(A)** Study of transcriptomic signatures for activated NK cells (left), all NK cell phenotypes (middle), and all cell types (right). On the top the principal components or the t-distributed stochastic neighbor embedding dimensions for the biological replicate within the reference dataset are shown. On the bottom, the relative transcriptional abundance is shown for marker genes. **(B)** Test for accuracy of the inference of PDGF-DD activated NK cell proportion from simulated mixtures. This plot represents the accuracy of the combination of CIBERSORT with Cox-regression by inferring associations between convoluted tissue composition and survival time. The three facets represent low-to-high proportion of missing information (proportion of total cells being of neural origin in the simulated mixtures, for which signature was not present in the reference). Data was simulated across a range of sample-size and slopes. A simulation condition that represents the associations we detected in the TCGA database is circled. **(C)** Receiver Operating Characteristic (ROC) curve, measuring the accuracy (true-positive and false-positive) for the simulated mixture circled in panel **(C, D)** The underlying association between the positively associated cell types with survival days, of the simulated dataset circled in panel **(C)**.

abundance of each marker gene in these NK cell phenotypes are shown in **Supplementary Table 2**.

The SPANK Is Associated With Improved Prognosis in TCGA LGG Dataset

Previous studies have implicated NK cells in immune responses to glioma (15, 92–95). We next sought the association of the ReNK, IL2NK, and SPANK phenotypes with LGG. The SPANK was more abundant than either the ReNK or IL2NK phenotypes in LGG tumors from TCGA (**Figure 2A**). LGG tumors enriched for the SPANK were associated with greater overall patient survival compared to the ReNK or IL2NK phenotypes (**Figure 2B**). These results show that tumor abundance of a distinct NK cell phenotype is associated with cancer patient survival, such as LGG. Moreover, these data also suggest that LGG tumors express PDGF-DD which may activate pro-tumorigenic pathways.

PDGFD Expression Is Associated With LGG Invasion and Poor Prognosis

In contrast to evoking NK cell anti-tumor functions through PDGF-DD binding to NKp44 (41), PDGF-DD (encoded by *PDGFD*) binding to PDGFR-β (encoded by *PDGFRB*) induces pro-tumorigenic signaling pathways that are detrimental for cancer patient survival (42, 47, 96, 97). Expression of a three-gene signature, comprised of *TGFBI*, *IGFBP3*, and *CHI3L1*, has previously been associated with glioma tumor cell invasion and migration and poor patient survival (98). Tumor expression of *TGFBI*, *IGFBP3*, and *CHI3L1* were positively correlated with *PDGFD* and *PDGFRB* expression in TCGA LGG dataset, respectively (**Figure 3A**) (98).

Since *PDGFD* and *PDGFRB* were associated with genes involved in glioma tumor cell invasion and migration, we next examined the relationship between tumor expression of *PDGFD* or *PDGFRB* and LGG patient survival. LGG patients with low tumor expression of *PDGFD* had more favorable prognosis compared to LGG patients with high tumor expression of *PDGFD* (**Figure 3B**). Higher LGG tumor expression levels of *PDGFRB* alone displayed a trend towards poor survival (**Figure 3B**) when *PDGFD* expression was low, but this was not statistically significant (**Figure 3C**). These data show that high tumor expression of *PDGFD* is primarily associated with poor prognosis compared to *PDGFRB* expression in TCGA LGG dataset.

SPANK Abundance Mitigates the Pro-Tumorigenic Effects of PDGFD in TCGA LGG

Anti-tumor immunity would be expected to curtail pro-tumorigenic factors and benefit patient survival. In addition to pro-tumor functions, we hypothesized that tumors enriched for the SPANK would contribute to anti-tumor immunity resulting in a more favorable TCGA LGG prognosis (41). In order to assess whether the abundance of these NK cell phenotypes

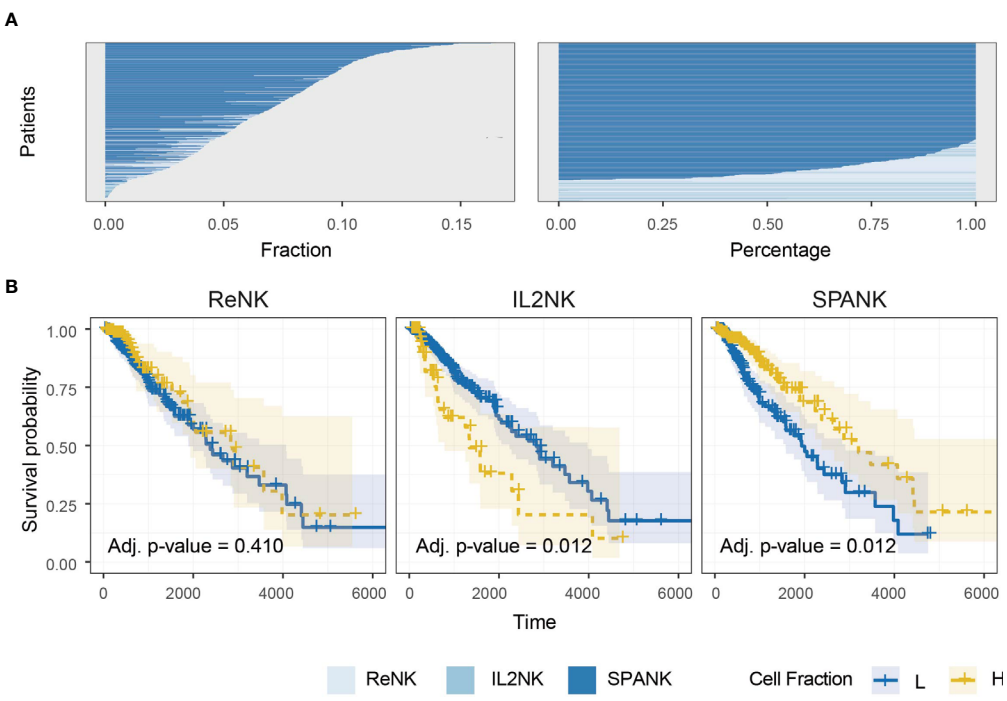

FIGURE 2 | Abundance of NK cell phenotypes and association with survival in the TCGA LGG dataset. **(A)** Abundance of NK cell phenotypes (fraction and percentage) for TCGA LGG cohort, the SPANK is the most abundant NK cell phenotype in LGG. **(B)** KM curves for all three NK cell phenotypes for TCGA-LGG; high tumor abundance of SPANK is associated with a beneficial LGG patient outcome compared to ReNK and IL2NK.

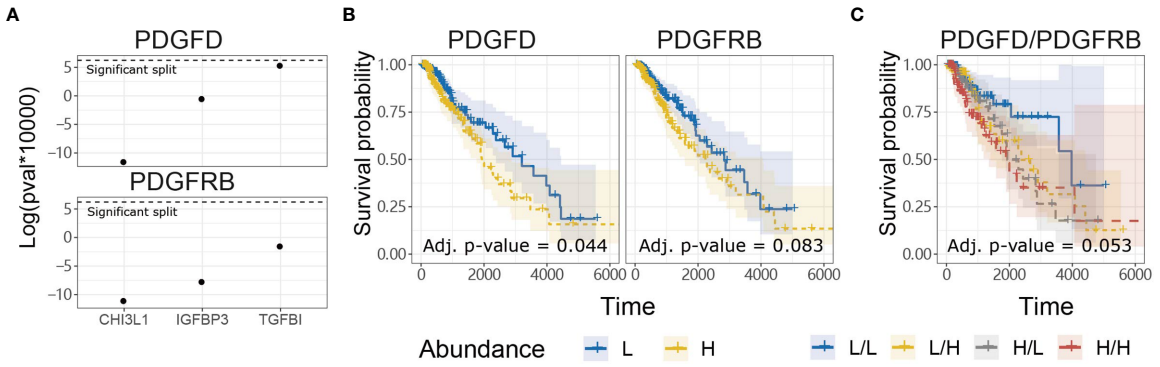

FIGURE 3 | *PDGFD* expression is associated with tumor invasion and poor prognosis compared to *PDGFRB* in TCGA LGG dataset. **(A)** Tumor expression of *PDGFD* and *PDGFRB* were positively correlated with the expression of *TGFBI, IGFBP3, and CHI3L1* which play important roles in glioma invasion and migration. **(B)** KM survival curves constructed for *PDGFD* or *PDGFRB* expression in LGG tumors. **(C)** KM curves constructed for combinations of *PDGFD* and *PDGFRB* abundance in LGG tumors.

counteracted the pro-tumorigenic expression of *PDGFD* and thus improve TCGA LGG prognosis, we next determined the progression-free survival of LGG patients with tumors stratified for *PDGFD* expression and abundance of either the ReNK, IL2NK, or SPANK phenotypes. When LGG tumors were stratified for PDGFD expression, patients with tumors enriched for the SPANK had a more favorable prognosis compared to LGG patients with a lower tumor abundance of SPANK (e.g. compare H^{PDGFD}/H^{SPANK}, red KM curve, to H^{PDGFD}/L^{SPANK}, grey KM curve and compare L^{PDGFD}/H^{SPANK}, yellow curve, to L^{PDGFD}/L^{SPANK}, blue curve) (**Figure 4**). In contrast, this was not observed for either ReNK or IL2NK (**Figure 4**). These results show that LGG tumors enriched for the SPANK may mitigate the detrimental effect of *PDGFD* expression on the prognosis of TCGA LGG patient cohort compared to the ReNK or IL2NK phenotypes.

Memory CD8$^+$ T Cell Abundance Mitigates the Pro-Tumorigenic Effects of PDGFD in TCGA LGG

For a given cancer, it is likely that immune subsets other than NK cells infiltrate the tumor microenvironment to elicit anti-tumor immunity, particularly T cells. We were interested in knowing whether the abundance of a given T cell subset in the LGG tumor microenvironment is associated with anti-tumor immunity. TCGA LGG tumors enriched for the memory CD8$^+$ T cell phenotype were associated with improved prognosis, but not the naïve, γδ, CD4$^+$ memory, or Helper, T cell phenotypes (**Figure 5A**). Our analyses

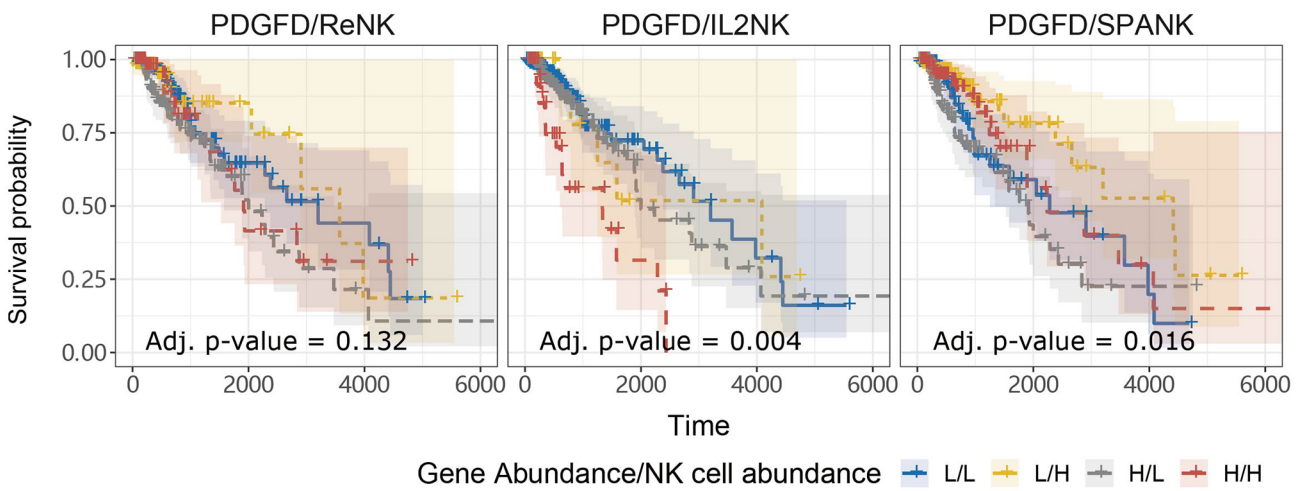

FIGURE 4 | SPANK abundance alleviates the pro-tumorigenic effects of *PDGFD* on TCGA LGG prognosis. Combined LGG patient survival analysis stratified for tumor expression of *PDGFD* and each NK cell phenotype, ReNK, IL2NK, and SPANK, respectively. KM curves display LGG patient survival plotted in all four combinations for each stratum, respectively (L/L, L/H, H/L, and H/H). For LGG tumors with either high or low *PDGFD* expression, a high tumor abundance of SPANK is associated with improved LGG prognosis.

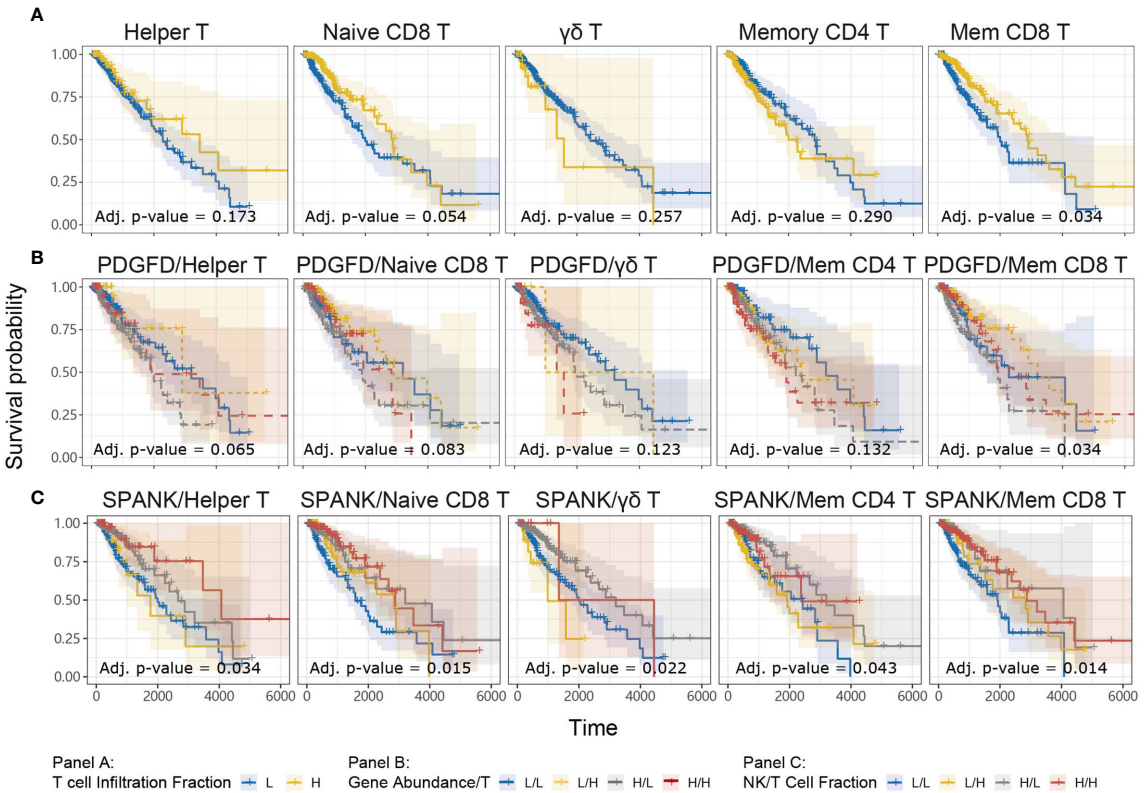

FIGURE 5 | Tumor abundance of memory CD8$^+$ and Helper T cells influences TCGA LGG prognosis KM curves displaying the survival of LGG patients split by median fraction into low (L) and high (H) tumor expression of **(A)** each T cell phenotype alone, **(B)** *PDGFD* and each T cell phenotype, respectively, or **(C)** each NK cell and each T cell phenotype, respectively. KM curves display the survival of LGG patients plotted in all combinations for each stratum.

show that TCGA LGG patients enriched for the memory CD8$^+$ T cell phenotype have improved prognosis.

In order to determine whether each T cell phenotype might counteract the detrimental expression of *PDGFD* on LGG prognosis, similarly to the SPANK, we next determined progression-free survival for LGG patients with tumors stratified for expression of *PDGFD* and the abundance of each T cell phenotype, respectively (**Figure 5B**). Using this approach, LGG tumors enriched for the memory CD8$^+$ T cell phenotype were associated with improved prognosis (**Figure 5B**). These results show that LGG tumors enriched for the memory CD8$^+$ T cell phenotype mitigate the pro-tumorigenic effects of *PDGFD* because they are associated with improved prognosis.

The SPANK and Helper T cell Phenotype Are Associated With Improved LGG Prognosis

Given that the abundance of NK and T cell phenotypes differ markedly in LGG tumors (**Supplementary Figure 3**), we were interested in understanding the relative contribution of SPANK and T cell phenotypes for LGG prognosis, respectively. We therefore determined patient survival for TCGA LGG tumors stratified for the abundance of SPANK and each respective T cell subset (**Figure 5C**). Interestingly, LGG tumors enriched for the SPANK and CD4$^+$ T helper phenotypes (TH) had improved prognosis compared to other strata e.g. compare H^{SPANK}/H^{TH} to either H^{SPANK}/L^{TH} or L^{SPANK}/H^{TH} or L^{SPANK}/L^{TH} (**Figure 5C**, column 1). In contrast, LGG tumors enriched for the SPANK and memory CD8$^+$ T cell phenotypes (CD8mem) did not further improve LGG patient survival compared to other strata e.g. compare H^{SPANK}/H^{CD8mem} to either L^{SPANK}/H^{CD8mem} or H^{SPANK}/L^{CD8mem} (**Figure 5C**, column 5). We conclude that LGG tumors enriched for the SPANK and CD4$^+$ T helper cell phenotypes are associated with improved LGG prognosis in TCGA. These results provide new insights into the possible cooperation between different NK and T cell subsets for LGG anti-tumor immunity which may inform adoptive cell therapies.

Critical Role for Killer Cell Lectin-Like Receptor Family Members in LGG Anti-Tumor Immunity

NK cells express a family of germline-encoded activating and inhibitory surface receptors that engage in cancer immune surveillance, which can also be expressed by memory CD8$^+$ T cells. However, the NK cell family receptors most critical for anti-tumor immunity in LGG remain unclear. Given that LGG patients in TCGA with tumors enriched for the SPANK or memory CD8$^+$ T cells were associated with improved prognosis,

we were interested in analyzing whether tumor expression of transcripts encoding NK cell family receptors was also associated with improved prognosis for TCGA LGG patients. LGG tumors with high expression of the *KLRK1*, *KLRC1*, *KLRC2*, *KLRC3*, or *KLRC4* transcripts encoding the NKG2D, NKG2A, NKG2C, NKG2E, and NKG2F NK cell receptors, respectively, were associated with improved prognosis (**Figure 6A**). In contrast, high LGG tumor expression of *CD226*, *CD244*, *CRTAM*, *KIR2DL4*, *NCR1*, or *NCR3* encoding the DNAM-1, 2B4, CRTAM, NKp46 and NKp30 NK cell receptors, respectively, were not associated with prognosis (**Supplementary Figure 4**). Moreover, expression of the *KLRK1*, *KLRC1*, *KLRC2*, *KLRC3*, and *KLRC4* receptor genes were overwhelmingly positively correlated with the SPANK and memory CD8$^+$ T cell phenotypes in TCGA LGG tumors (**Figure 6B**). These results show that high expression of the Killer cell lectin-like receptor (KLR) family in LGG tumors is associated with improved prognosis, suggesting that expression of KLR receptors may be critical for regulating anti-tumor immunity in LGG.

The SPANK and Memory CD8$^+$ T Cell Phenotypes Are Associated With KLRC2 Expression and More Favorable Prognosis in CGGA LGG Patients

Since LGG tumors enriched for expression of the SPANK and KLR family receptors were associated with improved prognosis in the TCGA LGG patient cohort, we next sought to validate these findings using another glioma patient dataset, such as the CGGA (**Figure 7**). Similar to TCGA LGG patient cohort, high tumor expression of *PDGFD* in CGGA LGG patients was associated with a poor prognosis (**Figure 7A**). Moreover, LGG tumors enriched for the SPANK and memory CD8$^+$ T cell phenotypes were associated with improved prognosis when CGGA tumors were also stratified for *PDGFD* expression compared to the IL2NK phenotype (**Figure 7B**). In contrast to TCGA LGG dataset, high tumor expression of *KLRC1* and *KLRC2* in CGGA LGG patients were associated with improved prognosis, but not *KLRC3*, *KLRC4* or *KLRK1* (**Figure 7C**). Like TCGA, high expression of *KLRC2*, which encodes the activating NKG2C receptor, was also associated with the SPANK and memory CD8$^+$ T cell phenotypes in LGG tumors (**Figure 7D**). However, in contrast to TCGA, expression of *KLRC1*, which encodes the inhibitory NKG2A receptor, was associated with the ReNK phenotype and not the SPANK or memory CD8$^+$ T cell phenotypes in CGGA LGG tumors (**Figure 7D**). Similar to TCGA, these results show that high tumor expression of *PDGFD* is associated with poor CGGA LGG prognosis, and tumors enriched for the SPANK and memory CD8$^+$ T cell phenotypes have improved prognosis. Moreover, like TCGA, high LGG tumor expression of *KLRC2* is also associated with the SPANK and memory CD8$^+$ T cell phenotypes and improved prognosis of CGGA LGG patients.

DISCUSSION

The clinical relevance of NK cells in cancer immune surveillance, particularly for solid tumors, remains unclear. We hypothesized that differential tumor enrichment of NK cells in different

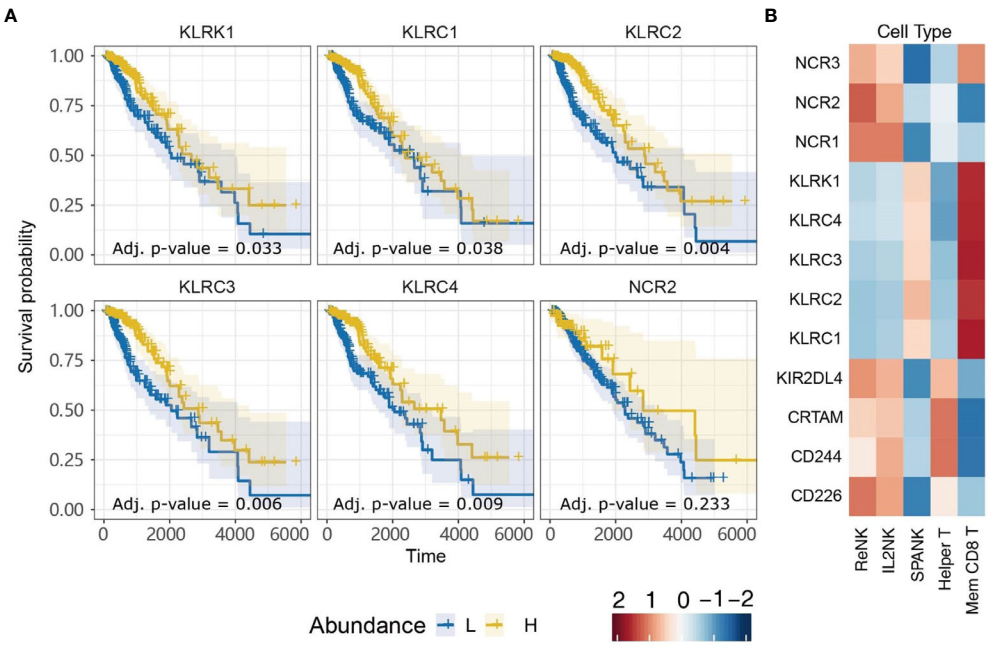

FIGURE 6 | Killer cell Lectin-like Receptor family expression is associated with more favorable TCGA LGG prognosis. **(A)** KM plots displaying progression-free survival of LGG patients split by median fraction into low (L) and high (H) tumor expression for the NK cell receptor genes: *KLRK1, KLRC1, KLRC2, KLRC3, KLRC4*, or *NCR2*. **(B)** Heatmap displaying correlations between expression of each NK cell receptor transcript (y-axis) and each NK cell and T cell phenotype (x-axis), respectively.

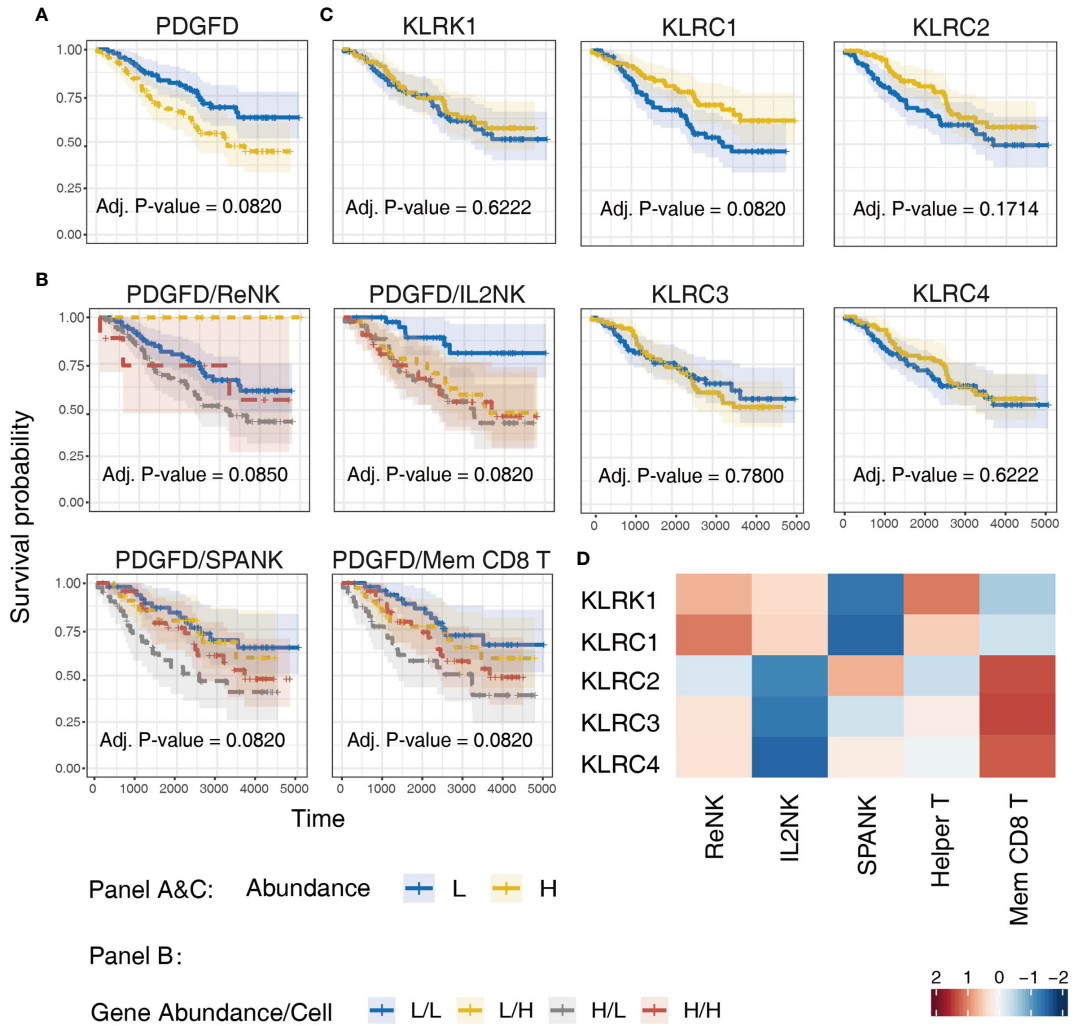

FIGURE 7 | Validation of TCGA findings using the CGGA LGG dataset. KM curves displaying the survival of CGGA LGG patients split by median fraction into low (L) and high (H) tumor expression of: **(A)** *PDGFD*, **(B)** *PDGFD* and either NK cell or memory CD8$^+$ T cell phenotypes, respectively, and **(C)** NK cell receptor genes, *KLRK1, KLRC1, KLRC2, KLRC3*, or *KLRC4*, respectively. KM curves display the survival of LGG patients plotted in all combinations for each stratum. **(D)** Heatmap displaying correlations between the expression of each NK cell receptor transcript (y-axis) and NK cell and T cell phenotypes (x-axis) in CGGA LGG tumors, respectively.

activation states may contribute to anti-tumor immunity. To investigate this important question, we determined TS from experimental RNA-seq datasets derived from NK cells in three different functional states; ReNK, IL2NK, and SPANK. Using this unbiased approach, we found that enrichment of the SPANK phenotype in LGG tumors was associated with improved prognosis in TCGA and CGGA datasets. The SPANK was derived from RNA-seq data from NK cells that had been stimulated with PDGF-DD, suggesting that PDGF-DD is expressed in the LGG tumor microenvironment.

In addition to activating NK cells, PDGF-DD binding to PDGFR-β can induce pro-tumorigenic signaling pathways. We reasoned that high expression of the genes for PDGF-D and PDGFR-β in LGG might predict poor LGG cancer prognosis (42, 47, 96, 97). In support of this, *PDGFD* and *PDGFRB* were positively correlated with a three-gene signature (*TGFBI*, *IGFBP3*, and *CHI3L1*) associated with glioma tumor cell invasion and migration and poor patient survival (98). Indeed, our analysis shows that LGG patients with high tumor expression of *PDGFD* had a poor prognosis in both TCGA and CGGA cohorts (42, 47, 96, 97). This model awaits confirmation in mouse models of glioma to determine whether PDGF-DD/PDGFR-β signaling can be targeted to restrict glioma tumor cell migration and invasion or even progression to higher glioma grades.

Given that the expression of *PDGFD* was primarily associated with pro-tumor pathways and poor prognosis, we further hypothesized that enrichment of the SPANK phenotype in LGG tumors may be associated with effective anti-tumor immunity and improved prognosis. High tumor abundance of the SPANK was associated with improved prognosis when LGG

tumors were stratified for the expression of *PDGFD* in both TCGA and CGGA patient cohorts. Our data show that the relative abundance of the SPANK may counteract the pro-tumorigenic properties of *PDGFD* expression and improve LGG prognosis. Interestingly, Nidogen-1 (34), the heparan sulfate proteoglycan, Syndecan-4 (35), a subset of HLA-DP molecules (36), a splice variant of the MLL5 gene (37), and PCNA (38), have all been reported to bind and regulate NKp44 signaling, and it will be interesting to determine whether expression of these latter genes in the LGG tumor microenvironment can influence the association between the NK cell phenotypes that we describe and LGG prognosis.

It is very likely that NK cells are not the sole mediators of anti-tumor immunity *in vivo* and our analyses revealed that enrichment of the $CD8^+$ memory T cell phenotype in LGG tumors was also associated with improved prognosis when LGG tumors were stratified for high or low tumor expression of *PDGFD* in both TCGA and CGGA patient cohorts. Interestingly, stratifying LGG tumors for T cell subsets and NK cells phenotypes revealed new insights into potential cooperation between these innate and adaptive immune cell subsets that was not revealed from the analysis of these immune cell phenotypes alone. For example, LGG patients with tumors enriched for the SPANK and $CD4^+$ T helper phenotypes had improved survival suggesting that adoptive transfer of NK cells with $CD4^+$ T helper cells may represent a novel therapeutic approach for LGG. Again, these computational results await confirmation in pre-clinical mouse models of glioma to determine whether the adoptive transfer of NK cells with $CD4^+$ T helper cells can restrict glioma tumor cell migration and invasion or possibly even progression to higher glioma grades.

Finally, high tumor expression of the NK cell receptor genes; *KLRK1*, *KLRC1*, *KLRC2*, *KLRC3*, and *KLRC4* that encode the NKG2D, NKG2A, NKG2C, NKG2E and NKG2F, respectively, were all associated with improved prognosis and positively correlated with the SPANK and memory $CD8^+$ T cell phenotypes, suggesting expression of these KLR NK cell receptor family gene products are important for LGG anti-tumor immunity in TCGA patient cohort.

Interestingly, the KLRC1 receptor, also known as NKG2A, is expressed as a heterodimer with CD94 on the surface of NK cells and T cells (99). CD94/NKG2A can bind to HLA-E as ligand to negatively regulate signaling from other activating KLR family members including KLRC2, known as NKG2C, which also heterodimerizes with CD94 to bind HLA-E (100, 101). Interestingly, KLRC1 has recently been shown to function as a checkpoint inhibitor that when blocked can promote NK cell and $CD8^+$ T cell-mediated anti-tumor immunity (102, 103). Since NK cell effector function is regulated by the balance of signaling from an array of germline-encoded activating and inhibitory receptors (29, 104), it is possible that signaling from the activating KLRK1 (NKG2D), NCR2 (NKp44) and KLRC2 (NKG2C) receptors, which are all associated with improved survival (**Figure 6A**), may cooperate to overcome any inhibitory threshold set by KLRC1 (NKG2A) in TCGA LGG patient cohort (103).

High tumor expression of *KLRC1* and *KLRC2* were also associated with improved survival in the CGGA LGG patient cohort, but not KLRK1, KLRC2 or KLRC4. However, the SPANK and $CD8^+$ T cell phenotypes were associated with expression of *KLRC2* and not *KLRC1* in the CGGA LGG patients, suggesting the balance of signaling may favor KLRC2 activation in the CGGA LGG patient cohort compared to TCGA LGG cohort. Given our results and those from other laboratories, it will be interesting to determine the expression of HLA-E in LGG and KLR family receptors on glioma-infiltrating NK cells and $CD8^+$ T cells in different ethnic groups and to test whether blocking the inhibitory function of KLRC1 can enhance the anti-tumor activity of NK cells and $CD8^+$ T cells in LGG and other brain cancers (102, 103). Finally, using CIBERSORT, we have uncovered an intriguing association between tumor expression of *PDGFD* and tumor enrichment of the SPANK and T helper and memory $CD8^+$ T cell signatures, that may be important for LGG patient survival. However, it is premature to conclude that the SPANK or the T cell signatures play a definitive role in LGG survival and future studies will aim to determine the biological significance of the SPANK and different T cell phenotypes and NK cell receptors, such as the KLR family, in LGG patient survival.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

AB, SM, YS, and YP contributed to the conception and design of the study. YS and SM performed computational and statistical analysis. AB and YS wrote the first draft of the manuscript. SM and AS wrote sections of the manuscript. AB and SM contributed equally and are joint senior authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.668391/full#supplementary-material

Supplementary Figure 1 | Computational pipeline. In addition to the three NK cell functional phenotypes, bulk RNA-seq data was collected from open online resources for 21 immune and stromal cell types including five T cell subsets, two B cell subsets, two dendritic cells subsets, two macrophage subsets, eosinophils,

mast cells and neutrophils, fibroblasts, endothelial and epithelial cells. Secondly, we selected marker genes by pairwise comparison and CIBERSORT to create our transcriptional signature (TS). Thirdly, we input our TS and RNA-seq matrix of TCGA cancer patients into CIBERSORT to estimate the proportion of each cell type in each patient. Finally, we performed a series of statistical analysis using the immune cell type profiles and clinical results of all TCGA cancer patients.

Supplementary Figure 2 | The Cell differentiation hierarchy used in marker gene selection. The higher levels are differentiated from cells in the lower levels (L1 differentiates into L2, L2 differentiates into L3, and L3 differentiates into L4).

Supplementary Figure 3 | Profiles of NK and T cell subset phenotypes in LGG patients. **(A)** Profiles showing original fractions of NK and T cell phenotypes in LGG tumors **(B)** Proportions of total NK and T cell abundance in LGG tumors.

Supplementary Figure 4 | Kaplan-Meier curves of NK cell receptor family genes in LGG patients. Survival analysis of receptor genes *CD226* (encoding DNAM-1), *CD244* (encoding 2B4/SLAMF4), *CRTAM, KIR2DL4, NCR1* (encoding NKp46) and *NCR3* (encoding NKp30) in TCGA-LGG. High expression of CD226 gene is significantly related to poor survival. Low (L) and high (H) expression groups were split by median TMM normalized counts. Statistics was performed by log-rank test.

Supplementary Table 1 | Log rank p values and Benjamini-Hochberg corrected p values. Log rank p-values of Kaplan-Meier curves were all corrected by BH procedure (/ = unchanged).

Supplementary Table 2 | NK phenotype TS transcripts. The abundance of each marker gene in the TS of NK cell phenotypes.

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A Transcriptional Signature of IL-2 Expanded Natural Killer Cells Predicts More Favorable Prognosis in Bladder Cancer

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Activation of natural killer (NK) cell function is regulated by cytokines, such as IL-2, and secreted factors upregulated in the tumor microenvironment, such as platelet-derived growth factor D (PDGF-DD). In order to elucidate a clinical role for these important regulators of NK cell function in antitumor immunity, we generated transcriptional signatures representing resting, IL-2-expanded, and PDGF-DD-activated, NK cell phenotypes and established their abundance in The Cancer Genome Atlas bladder cancer (BLCA) dataset using CIBERSORT. The IL-2-expanded NK cell phenotype was the most abundant in low and high grades of BLCA tumors and was associated with improved prognosis. In contrast, *PDGFD* expression was associated with numerous cancer hallmark pathways in BLCA tumors compared with normal bladder tissue, and a high tumor abundance of *PDGFD* transcripts and the PDGF-DD-activated NK cell phenotype were associated with a poor BLCA prognosis. Finally, high tumor expression of transcripts encoding the activating NK cell receptors, KLRK1 and the CD160–TNFRSF14 receptor–ligand pair, was strongly correlated with the IL-2-expanded NK cell phenotype and improved BLCA prognosis. The transcriptional parameters we describe may be optimized to improve BLCA patient prognosis and risk stratification in the clinic and potentially provide gene targets of therapeutic significance for enhancing NK cell antitumor immunity in BLCA.

Keywords: NK cell, bladder cancer, prognosis, NK receptors, TCGA, anti-tumor immunity

INTRODUCTION

Bladder cancer (BLCA) is a disease of the elderly in the developed world (1, 2). An aging population, industrialization, and endemic tobacco smoking in developing nations mean that global BLCA diagnoses are estimated to double (2). The first-line management of high-grade non-muscle-invasive BLCA involves transurethral resection of the bladder tumor (3) and administration of an induction course of intravesical bacille Calmette–Guerin (BCG) vaccine. Longer-term BCG

maintenance therapy retards the progression and recurrence of BLCA disease (4, 5), but toxicity and intolerance, albeit rare, confer considerable risks to BLCA patients (6, 7).

Transition to a BCG-refractory high-grade BLCA is associated with poor survival outcomes (8), and radical cystectomy (RC) for patients that fail intravesical immunotherapy is the current gold standard treatment (9). Nonetheless, RC and chemotherapy are costly (10), highly invasive, and are associated with significant side effects (11) and impaired quality of life (12). Limited therapeutic options beyond systemic chemotherapy have resulted in dire outcomes for patients with metastatic BLCA disease (11, 13). Thus, there is an urgent need for less invasive, tolerable, and durable alternatives for intractable BLCA.

Early incursions into immune checkpoint blockade (ICB) in BLCA have yielded promising results (14–19). However, only 30% of bladder cancer patients with metastatic disease respond to ICB therapy. To circumvent tumor incompatibility (20) and acquired resistance to ICB (21), a more detailed characterization of tumor immune surveillance pathways will undoubtedly inform more effective immunotherapies for BLCA patients (22–25). Natural killer (NK) cells are innate lymphocytes that produce IFN-γ but are distinct from other innate lymphoid cells because they specialize in the cytolysis of malignant and infected cells and are thus considered the innate counterparts of cytotoxic T lymphocytes (26). While NK cell cytotoxicity is known to contribute to BCG therapeutic benefit (27, 28), immune surveillance of malignant uroepithelial tissue by NK cells is understudied (29).

NK cell cytotoxicity is regulated by antagonistic signaling networks moderated by an array of activating and inhibitory cell surface receptors (30, 31). The downregulation of ligands, such as MHC-I, for inhibitory receptors in conjunction with the abundant expression of ligands recognized by activating receptors, such as killer cell lectin-like receptor K1 (KLRK1) (32) and the natural cytotoxicity receptors (NCRs) (33), predisposes tumor cells to NK cell elimination (34). Human KLRK1, also known as NKG2D, is expressed by NK cells and $CD8^+$ T lymphocytes and recognizes a range of stress-inducible ligands expressed on malignant cells, such as MICA, MICB, and the ULBP-binding proteins 1–6 that are collectively known as "NKG2D ligands" (NKG2D-L) (35, 36). Indeed, *in vitro* assays suggest that NKG2D recognition of stress-inducible ligands is a prominent mode of BLCA tumor cell recognition (37). In contrast, the NCR NKp44 can bind to platelet-derived growth factor D (PDGF-DD), which is overexpressed by many solid tumors including BLCA, and may activate NK cell antitumor functions to control tumor growth (38). NK cells express many other activating and inhibitory receptors that are likely to cooperate to elicit maximal NK cell activity in the tumor microenvironment (39). In-depth analysis of the immune cell phenotypes and receptors associated with improved BLCA patient prognosis will shed light on the tumor surveillance pathways that may be enhanced for improved BLCA immunotherapy (40).

Like NKG2D, TNFRSF14 is expressed by NK cells and $CD8^+$ T lymphocytes and has multiple ligands, such as TNFSF14 (also known as LIGHT), lymphotoxin-α (LTA), CD160 (also known as natural killer cell receptor BY55), and B- and T-lymphocyte-associated protein (BTLA). TNFRSF14, also known as herpes virus mediator of entry (HVEM), can convey either lymphocyte activation or inhibition depending on *cis* and *trans* interactions with the ligand (41). For example, acting as a receptor for TNFSF14 or LTA, TNFRSF14 can stimulate downstream NF-κB signaling to promote NK cell and T-lymphocyte proliferation, IFN-γ production, and tumor cell clearance (42–45). CD160 is also expressed by NK cells and $CD8^+$ T cells as GPI-anchored and transmembrane forms. Binding of TNFRSF14 to the transmembrane form of CD160 delivers an activating signal that can promote NK cell cytotoxicity and IFN-γ production (46). In contrast, TNFRSF14 binding *in cis* to BTLA inhibits *trans* interactions with LIGHT, LTA, or CD160, to maintain NK cells and T lymphocytes in a resting state, thus tuning lymphocyte activation to the surrounding tumor microenvironment (47). Interestingly, TNFRSF14 is also expressed by tumor cells and TNFRSF14 ligation can inhibit bladder cancer cell proliferation by inducing apoptosis (48).

Here, we investigated the clinical impact of the abundance of resting, IL-2-expanded, and PDGF-DD-activated NK cell phenotypes and the receptors they express in the BLCA tumor microenvironment. To achieve this, we generated transcriptional signatures representing the latter NK cell activation states to estimate their relative abundance in The Cancer Genome Atlas (TCGA) BLCA dataset and tested the association with curated progression-free survival (49).

METHODS

Material Availability

The R codes for the analyses presented in this study are available at RAGG3D/BLCA_IL2NK (github.com).

Data Collection and Validation of Functional NK Cell Datasets

Gene transcript-abundance and patient clinical information were collected from TCGA through the GDC Data Portal (50). Progression-free survival information was used as a measure of clinical outcome (49). The cell-type-specific transcriptional signatures were derived from a large collection of RNA sequencing samples spanning a wide range of cell types. For NK cells, an experimentally derived dataset for IL-2-expanded [27 biological replicates (38)], PDGF-DD activated *via* NKp44 signaling [4 biological replicates (38)] and resting (25 biological replicates from six datasets) were included. For resting NK cells and other cell types, the data collected were from the following datasets: BLUEPRINT (51), Monaco et al. (52), ENCODE (53), Squires et al. (54), GSE77808 (55), Tong et al. (56), PRJNA339309 (57), GSE122325 (58), FANTOM5 (59), GSE125887 (60), GSE130379 (61), and GSE130286 (62).

In order to validate the functional status of the RNA-seq datasets curated in this study, we determined the expression of transcripts for surface proteins commonly upregulated during

IL-2 expansion of NK cells, such as CD69, CD25, CD70, and NKp44 (63–67). With the exception of *CD69*, transcripts for *IL2RA*, *CD70*, and *NCR2* were significantly upregulated in IL-2-expanded and PDGF-DD-activated NK cells compared with resting NK cells (**Supplementary Figure 1**), which we conclude sufficiently validates the use of these curated datasets to generate transcriptional signatures representative of "resting" and "IL-2-expanded" NK cell phenotypes.

Generation of Transcriptional Signatures

In order to derive transcriptional signatures of 24 cell types (memory B cell, naive B cell, immature dendritic myeloid cell, mature dendritic myeloid cell, endothelial, eosinophil, epithelial, fibroblast, macrophage M1 and M2, mast cell, monocyte, neutrophil, resting NK cells, IL-2-expanded NK cells, PDGF-DD-activated NK cells, central memory CD4 T cell, effector memory CD4 T cell, central memory CD8 T cell, effector memory CD8 T cell, naive CD8 T cell, gamma-delta T cell, helper T cell, and regulatory T cell), a total of 592 highly curated (i.e., for which identity was confirmed in the literature), non-redundant biological replicates (including 25 resting NK cell samples, 27 IL-2-expanded NK cell samples, and 4 PDGF-DD-activated NK cell samples) have been used. Due to the sparse nature of a heterogeneous set of datasets, the expected value and variability of gene transcription abundance was inferred for each cell type using a publicly available Bayesian statistical model (github: stemangiola/cellsig), based on a negative binomial data distribution (68). This model allows to fit sparse data (e.g., transcript abundance of one gene for which data are available in a subset of reference biological replicates) and calculate theoretical data distributions of cell-type/gene pairs. The cell-type transcriptional marker selection was based on the pairwise comparison of each cell type within cell-type categories along a cell differentiation hierarchy (**Supplementary Figure 2**) (69). For example, all cell-type permutations from the root node of level 1 (including epithelial, endothelial, fibroblasts, and immune cells) were interrogated in order to select the genes for which the transcript abundance distribution (data generated from the posterior distribution) was higher for one cell type compared with another. This was executed calculating the distance of the upper and lower 95% credible intervals, respectively (obtained from cellsig). From each comparison, the top 5, 10, and 20 genes per cell-type pair were selected from levels 1, 2, and 3, respectively (**Supplementary Figure 2**). The marker gene list is composed by the union of genes for all levels. This hierarchical approach favors the identification of marker genes that distinguish broad cell-type categories as well as specific activation phenotypes.

The top marker genes (upregulated) that segregate IL-2-expanded NK cells from PDGF-DD-activated NK cells are ERP29, IMPDH2, and MFSD10. The top markers (upregulated) that segregate activated from resting NK cells are RPSAP9, POTEF, POTEE, GOLGA8IP, HERC2P4, and HNRNPA3P1. The top markers (upregulated) that segregate NK cells from other major immune cells are CD247, CTSW, HOPX, GZMA, ID2, IL2RB, SHROOM1, CD74, NKG7, CATSPER1, CCNJL, MTRNR2L6, CST7, EIF4A1, KRT81, PPP1R9A, and SH2D2A. The full signature matrix is provided as supplementary material (**Supplementary File 1**).

Benchmark of the Transcriptional Signatures

To test whether the selected signature for IL-2-expanded NK cells was suitable to accurately detect changes in cell abundance in association with progression-free survival, we implemented a benchmark on simulated tissue mixtures. This benchmark was organized in 81 simulation conditions, each of which included 63 test runs. The simulation conditions were i) the amount of tissue mixture (replicates) from 250 to 1,000, ii) the degree of change (slope) from −1 to 1, and iii) the proportion of a foreign cell type, whose signature was not included in our reference (i.e., neural cells), from 0 to 0.8 (80%). For each test run, a number of tissue mixtures (replicates) were simulated. One mixture is created as the sum of the transcriptional profiles for each cell type, weighted by a proportion array (summing to one) that represents the relative amount of cell types within the tissue. The transcriptional profiles were samples at random from our reference database. The proportion arrays (for each run) were built according to a linear model, correlating the cell-type proportion with progression-free survival. For example, in case T cells were to be positively associated with progression-free survival, the tissue mixtures (i.e., patients) with bigger progression-free survival would be characterized by a larger proportion of T cells. The values of progression-free survival were simulated according to real-world data. We sampled the progression-free survival time from the TCGA BLCA patient cohort. For each test run (including several simulated mixtures), only one cell type was set up as being associated with progression-free survival.

The cell-type proportion associations were estimated for each test run. The estimation included two steps: deconvolution and Cox regression of the estimated cell-type proportions. To simulate censored data (partial follow-up for progression-free survival time), the Cox regression was provided with halved the time-to-event for half of the tissue mixtures. We classified IL-2-expanded NK cells as changing or not-changing based on a *p*-value threshold of 0.05. The framework tidybulk was used to infer the cell-type proportions through CIBERSORT and perform a multiple Cox regression on the predicted proportions (logit-transformed) (70), with progression-free survival censored time as a covariate. The significance calls were compared with the ground truth to generate a receiver operating characteristic (ROC) curve.

Estimation of the Association of Cell-Type Abundance With Relapse-Free Patient Survival

To estimate the cell-type abundance for each biological replicate, we used CIBERSORT with our RNA sequencing-derived gene marker signature. In order to estimate the clinical relevance of NK activation phenotypes, we produced a Kaplan–Meier estimator (71) based on the median proportion split of each cell type. Percent survival *vs.* time-to-event statistics were

calculated by the log-rank (Mantel–Cox) test (72). Statistics of Kaplan–Meier curves were performed by the log-rank test then adjusted by the Benjamini–Hochberg (BH) procedure. A table of all *p*-values prior to adjustment is provided in **Supplementary Table 1**. Data analysis was performed using the R environment in R Studio (73). Packages include tidyverse (74), tidybulk (75), survminer (76), survival (70, 77), foreach (78), org.Hs.eg.db (79), cowplot (80), ggsci (81), GGally (82), gridExtra (83), grid (73), reshape (84), Hmisc (85), tidyHeatmap (86), and viridis (87).

Functional Enrichment Analysis

To identify the unique protumorigenic pathways associated with *PDGFD* expression in BLCA tumors compared with normal bladder tissue, we utilized the functional enrichment analysis for the top 1,000 co-expressed genes of *PDGFD* in both TCGA BLCA tumors and TCGA BLCA normal tissues datasets, which we obtained from the GEPIA2 web server (88). The comparative functional enrichment analysis was performed in Gitools v1.8.4 (89) utilizing the modules constructed from the Gene Ontology (90) Biological Process (GOBP), Bioplanet pathways (91), KEGG pathways (92), Reactome pathways (93), and Wikipathways (94) databases. During the analysis, the resultant *p*-values of the enriched terms were adjusted using the multiple test correction approach outlined in the Benjamini–Hochberg's false discovery rate (FDR) method, and we only considered those enriched pathways/terms significant which have an FDR *q*-value <0.05. From the enrichment results, we sorted and grouped the significant pathways/terms manually based on the associated protumorigenic hallmarks and immune responses.

RESULTS

IL-2-Expanded NK cells Are Associated With a More Favorable BLCA Prognosis

We hypothesized that NK cells of unique phenotype can infiltrate different cancer types to confer antitumor immunity. To answer this question, we performed a benchmark for the inference of changes in the abundance of IL-2-expanded NK cells in artificial tissue mixtures built from our reference dataset (see *Methods*) to determine the ability of the IL-2-expanded NK cell signature to provide an identifiable biologically relevant signal from whole tissue RNA sequencing data (**Figure 1A**). This benchmark showed a high accuracy (area under the curve) across simulation settings including magnitude of variability, sample size, and proportion of unknown cells (please see *Methods*). An accuracy of 0.75 was reached for simulation settings that match our findings on TCGA data (slope and sample size; **Figures 1B, C**). These data show that our NK cell signatures have the potential to uncover clinically relevant associations from TCGA-derived whole tissue RNA sequencing data. We then defined marker genes for transcriptional signatures representing resting NK cells (ReNK) (95), IL-2-expanded NK cells (IL2NK), and a signature of PDGF-DD-activated NK cells (SPANK) (96), respectively, and established the transcript abundance of these NK cell phenotypes in TCGA BLCA cohort using CIBERSORT (please see *Methods*). Using this approach, we found that the IL2NK phenotype was more abundantly expressed in BLCA tumors compared with the ReNK or SPANK phenotypes (**Figure 1D**). Interestingly, the IL2NK phenotype, but not the ReNK or SPANK, was associated with improved BLCA patient prognosis (**Figure 1E**). In contrast to IL2NK, high tumor abundance of the ReNK phenotype was associated with poor prognosis, while abundance of the SPANK was not associated with prognosis (**Figure 1E**). The tumor abundance of signature T-cell phenotypes was also not associated with prognosis (**Supplementary Figure 3**). These results show that a high infiltration of the IL2NK phenotype in BLCA tumors is associated with improved BLCA prognosis.

Abundance of NK Cell Phenotypes in Different Clinical Grades of BLCA Tumors

Since a high tumor abundance of the IL2NK phenotype was associated with a more favorable BLCA prognosis, we next asked whether a particular NK cell phenotype was preferentially associated with a different clinical grade of the BLCA tumor. BLCA tumors were partitioned into either low or high grade and the abundance of the ReNK, IL2NK, and SPANK NK cell phenotypes was determined, respectively (**Figure 2**). In both low- and high-grade BLCA tumors, the IL2NK phenotype was the most abundant followed by the SPANK and then ReNK phenotypes (**Figure 2**). Remarkably, abundance of either the ReNK, IL2NK, or SPANK NK cell phenotypes did not differ between low- and high-grade BLCA tumors (**Supplementary Figure 4**). These results show that the IL2NK phenotype is more abundant in low and high BLCA tumor grades, followed by the SPANK and then ReNK phenotypes.

PDGFD Expression Is Associated With Cancer Hallmarks and Poor BLCA Prognosis

PDGF-DD is produced by many aggressive cancers and binds to PDGFR-β expressed on tumor cells to induce protumorigenic signaling pathways that are thought to be associated with poor patient outcome (38–42, 97). Even though the SPANK and IL2NK represent activated NK cell phenotypes, our initial analysis showed that tumor abundance of the SPANK was not associated with BLCA patient prognosis, unlike IL2NK (**Figure 1B**). We hypothesized that protumorigenic pathways associated with *PDGFD* expression might mask any antitumor functions of PDGF-DD-activated NK cells on BLCA patient prognosis. We downloaded the top 1,000 transcripts from the GEPIA2 website associated with *PDGFD* expression in BLCA and in normal bladder tissue (please see *Methods*) and performed gene enrichment analysis to identify enriched protumorigenic pathways (**Figure 3**). Many protumorigenic pathways associated with *PDGFD* expression that represent core cancer hallmarks (98, 99) were enriched in BLCA but not in normal bladder tissue (**Figure 3A**). These pathways include PDGF signaling and response to growth factor stimulus (**Supplementary Figures 5A, B**). These data show that protumorigenic pathways representing key cancer hallmarks are associated with *PDGFD* expression in BLCA tumors.

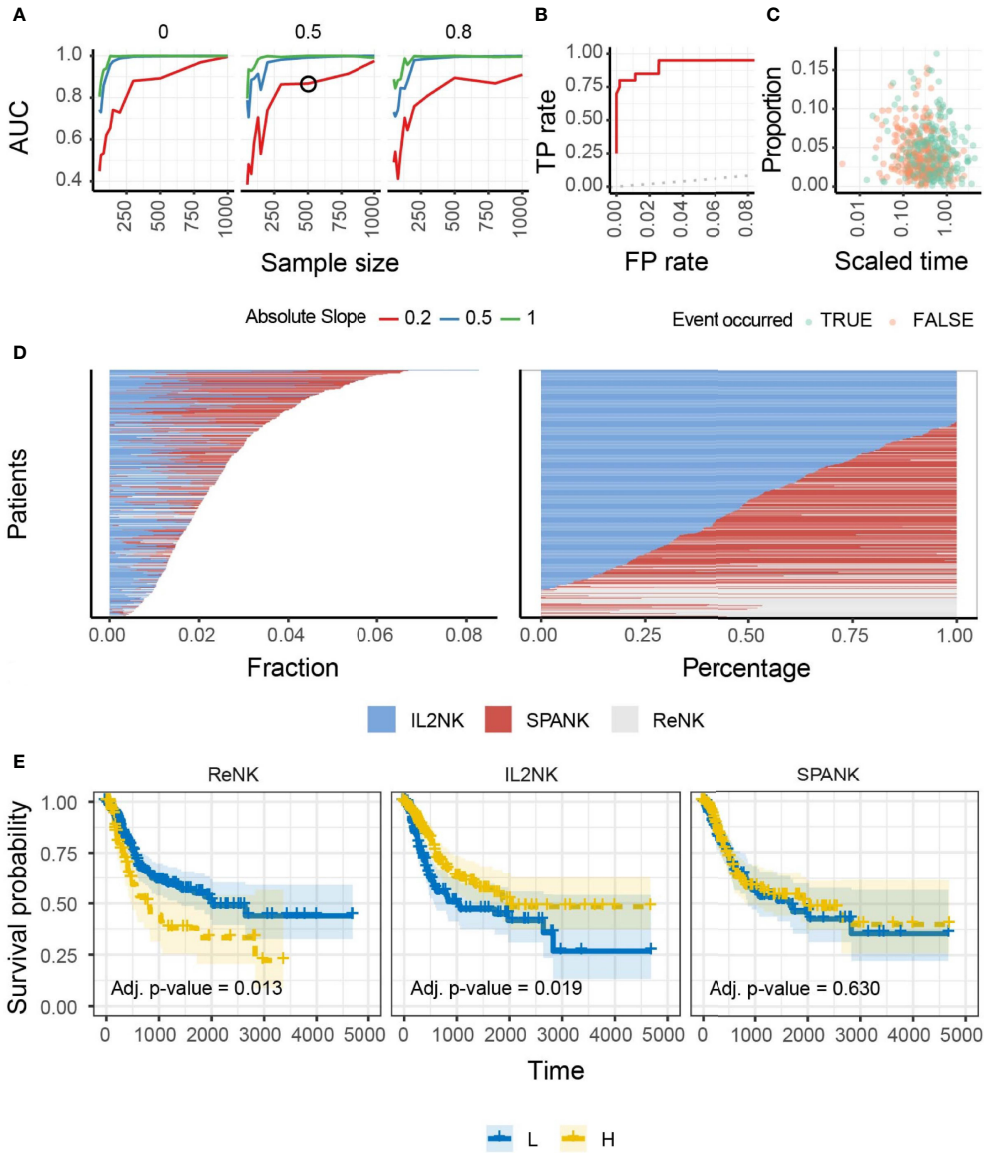

FIGURE 1 | Benchmark, overall abundance and survival associations of NK phenotypes in BLCA. **(A)** Accuracy of the inference of changes in proportion of IL-2-expanded NK cells from simulated mixtures. The detection of a significant proportional change when it exists in the simulation defined a true-positive. Data points represent the area under the curve (AUC) for mixtures created with a specific combination of sample size (*x*-axis), degree of change (slope; color-coded), and proportion of foreign cell type (facets; i.e., neurons, for which we do not include transcriptional profile in the training data). A simulation condition that represents the associations we detected in the TCGA database is circled. **(B)** Receiver operating characteristic (ROC) curve, measuring the accuracy (true-positive and false-positive) for the simulated mixture circled in **(A)**. A curve touching the top-left corner (0 false-positive and 1 true-positive rates) would represent the best achievable performance. A curve overlapping the dotted line (45°) would represent a random detection of proportional changes. **(C)** The underlying association between cell type and time-to-event (e.g., survival days) of the simulated dataset circled in **(A)**. Data points represent cell-type/sample pairs. **(D)** Abundance of NK cell phenotypes (fraction and percentage) for TCGA BLCA cohort; IL2NK is the most abundant NK cell phenotype in BLCA. **(E)** Kaplan–Meier survival curve for all three NK cell phenotypes for TCGA-BLCA; high tumor abundance of IL2NK, but not the ReNK or SPANK phenotypes, is associated with a favorable BLCA patient outcome (*x*-axis, days; *y*-axis, progression-free survival).

Since protumorigenic pathways associated with *PDGFD* expression were enriched in BLCA, we next determined the relationship between tumor expression of *PDGFD* or *PDGFRB* and BLCA patient prognosis (**Figure 3**). High tumor expression of *PDGFD* was associated with a poor BLCA prognosis compared with those BLCA patients with low tumor expression of *PDGFD* (**Figure 3A**). In contrast, tumor expression of *PDGFRB* did not influence BLCA patient prognosis (**Figure 3B**). Overall, these data show that a high tumor expression of *PDGFD* is associated with the activation of core cancer hallmarks and poor BLCA patient prognosis.

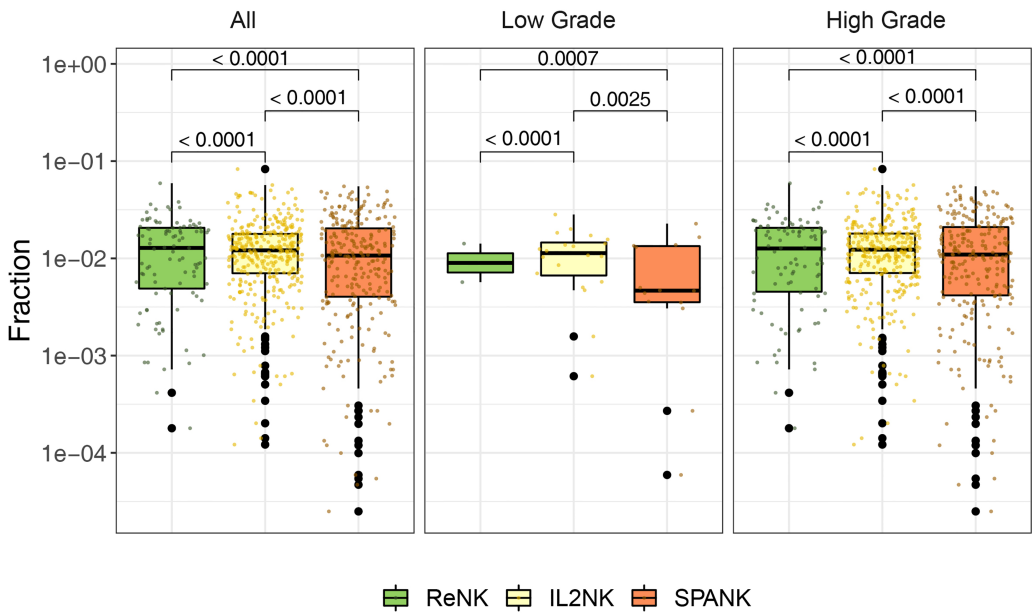

FIGURE 2 | Abundance of NK cell phenotypes in different clinical BLCA grades. Abundance of the ReNK, IL2NK, and SPANK NK cell phenotypes (log10 transformed fraction) for TCGA BLCA cohort partitioned into low and high clinical grades. IL2NK is the most abundant NK cell phenotype in low- and high-grade BLCA tumors, followed by the SPANK and then ReNK phenotypes. Wilcoxon signed-rank test was conducted to examine the differences between clinical grades. P-values were adjusted by Benjamini-Hochberg procedure.

Stratifying Tumors Based on *PDGFD* Expression Reveals the SPANK Phenotype Is Associated With a Poor BLCA Prognosis

In addition to the activation of protumorigenic pathways, PDGF-DD can evoke NK cell antitumor functions through binding to NKp44 and signaling *via* the associated DAP12 adaptor (38, 64, 100). In support of this, the DAP12 signaling pathway was strongly associated with *PDGFD* expression in BLCA tumors (**Supplementary Figures 5C, D**). However, the contribution of each NK cell phenotype in mitigating the detrimental effect of *PDGFD* expression on patient prognosis remained unclear (**Figure 3A**). We next determined the association between each NK cell phenotype and patient prognosis for BLCA tumors stratified for *PDGFD* expression. When BLCA tumors were stratified for high *PDGFD* expression, high tumor abundance of the ReNK was associated with poor prognosis, and neither the IL2NK nor SPANK phenotypes were associated with patient prognosis, underscoring the strong association between high BLCA tumor expression of *PDGFD* and poor patient prognosis (**Figure 4**). In contrast, when BLCA tumors were stratified for low *PDGFD* expression, a high tumor abundance of IL2NK was associated with improved prognosis, whereas the SPANK was associated with poor prognosis and to a lesser extent the ReNK (**Figure 4**). These results show that a high tumor abundance of IL2NK in BLCA tumors with low *PDGFD* expression is associated with a more favorable prognosis, whereas a high tumor abundance of the ReNK or SPANK phenotypes is associated with poor BLCA prognosis.

Critical Role for IL2NK-Associated NK Cell Receptors in BLCA Prognosis

NK cells express an array of activating and inhibitory cell surface receptors, but how these NK receptors function for effective antitumor immunity in different types of cancer remains unclear. Since the IL2NK phenotype was associated with improved prognosis, we next determined whether tumor expression of specific NK cell receptors was critical for BLCA patient prognosis (**Figure 5**). BLCA tumors with high expression of *KLRK1*, which encodes the activating NKG2D receptor, had a much improved prognosis compared with BLCA patients with low tumor expression of *KLRK1* (**Figure 5A**). Human NKG2D binds to a range of stress-inducible NKG2D-L, such as MICA, MICB, and ULBPs 1–6, and so we next determined patient prognosis for BLCA tumors stratified for *KLRK1* expression and each NKG2D-L, respectively (**Supplementary Figure 6**). When *KLRK1* expression was high in BLCA tumors, expression of each NKG2D-L did not influence patient prognosis (**Supplementary Figure 6**). However, when *KLRK1* expression was low in BLCA tumors, expression of *MICA* and *MICB* trended toward improved prognosis, whereas *ULBP1* expression trended toward poor prognosis (**Supplementary Figure 6**).

TNFRSF14, also known as herpes virus mediator of entry (HVEM), can induce lymphocyte activation or inhibition depending on *cis* or *trans* interactions with ligand. TNFRSF14 can bind to four possible ligands, TNFSF14, LTA, CD160, or BTLA. TNFRSF14 interactions with either TNFSF14, LTA, or CD160 can promote NK cell activation, whereas binding to BTLA can induce NK cell inhibition. We noted that high BLCA tumor expression of

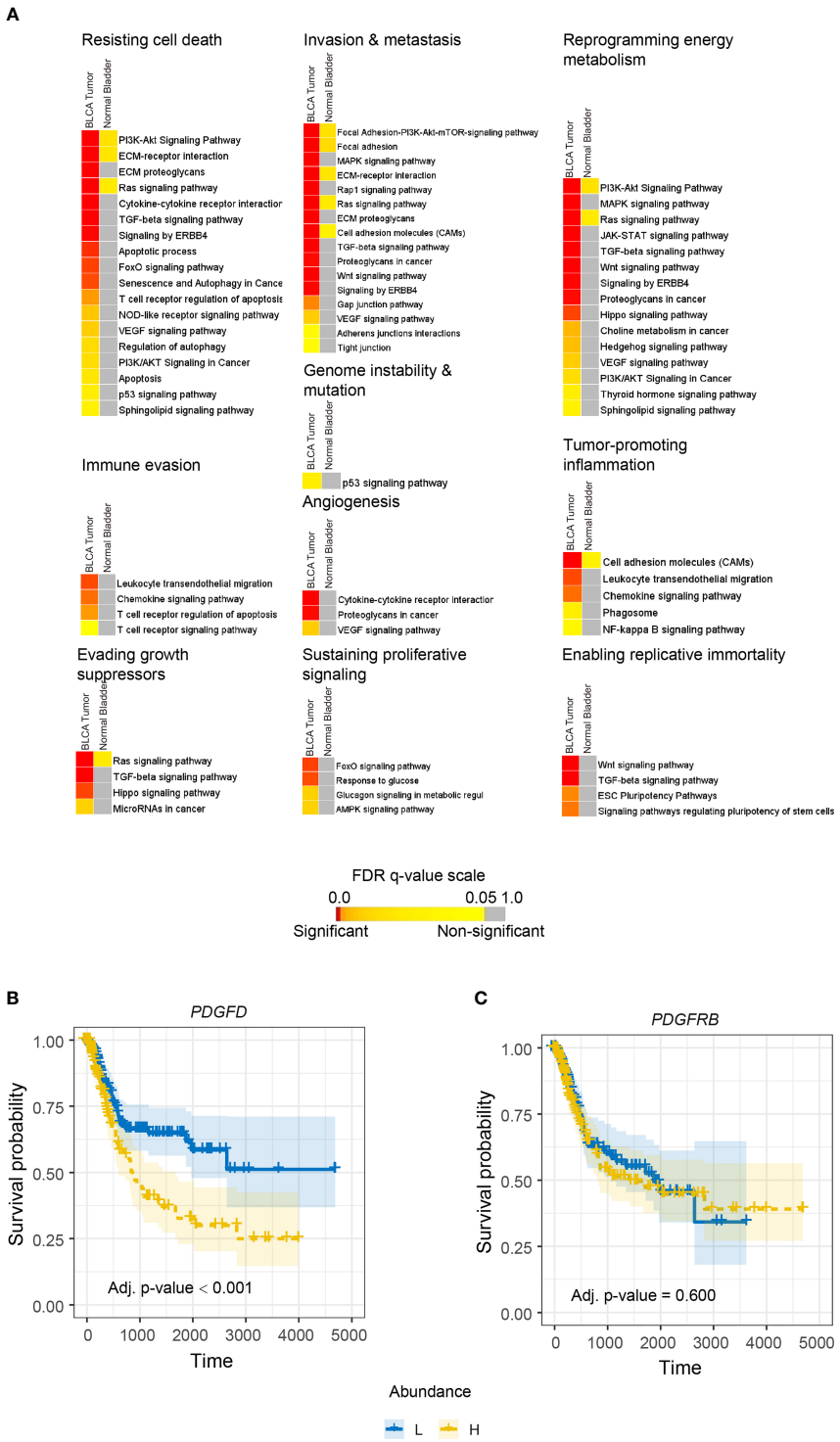

FIGURE 3 | *PDGFD* expression is associated with the activation of protumorigenic pathways and poor prognosis in BLCA. **(A)** Cancer hallmark signaling pathways associated with *PDGFD* expression in BLCA tumors and normal bladder tissue. Color-coded heatmaps represent the statistical significance of the functionally enriched pathways. Color toward red indicates the most significant pathways, while the yellow color represents less significant and gray color represents the non-significant events. Only selected significant terms were presented within the heatmaps. Kaplan–Meier survival curves constructed for **(B)** *PDGFD* or **(C)** *PDGFRB* expression in BLCA tumors. High tumor expression of *PDGFD* is associated with poor BLCA prognosis (*x*-axis, days; *y*-axis, progression-free survival).

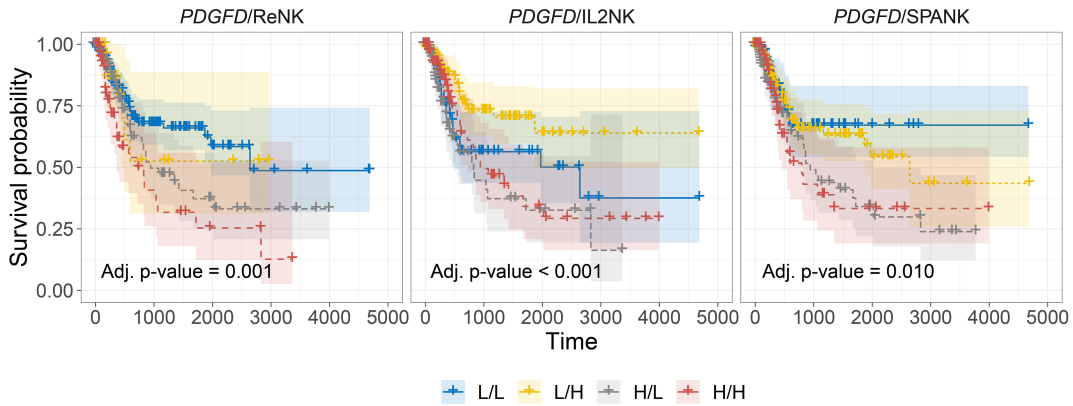

FIGURE 4 | Tumor abundance of IL2NK, but not the SPANK, counteracts the protumorigenic effects of *PDGFD* on BLCA patient prognosis. Combined BLCA patient survival analysis stratified for tumor expression (median split) of *PDGFD* and each NK cell phenotype, ReNK, IL2NK, and SPANK, respectively. KM curves display the survival of BLCA patients plotted in all four combinations for each stratum, respectively (L/L, L/H, H/L, and H/H). Low *PDGFD* expression and high IL2NK abundance in BLCA tumors are associated with improved prognosis, whereas low expression of *PDGFD* and abundance of the SPANK phenotype in BLCA tumors are associated with a poor prognosis (*x*-axis, days; *y*-axis, progression-free survival).

TNFRSF14 or *CD160* trended toward improved prognosis compared with either *TNFSF14*, *LTA*, or *BTLA* (**Supplementary Figure 7**). We next determined patient prognosis for BLCA tumors stratified for the expression of *TNFRSF14* and *CD160*. High expression of both *TNFRSF14* and *CD160* in BLCA tumors was associated with more favorable patient prognosis compared with all the other groups (**Figure 5B**). Finally, expressions of the *KLRK1*, *TNFRSF14*, and *CD160* NK cell receptor genes were all positively correlated with the IL2NK phenotype compared with the ReNK or SPANK (**Figure 5C**). These results show that high tumor expression of transcripts encoding the NK cell receptors KLRK1, TNFRSF14, and CD160 may be critical for antitumor immunity in BLCA because the expression of these receptors is associated with the IL2NK cell phenotype and a more favorable BLCA prognosis.

DISCUSSION

The clinical relevance of NK cells in cancer immune surveillance, particularly for solid tumors, remains unclear. We hypothesized that different NK cell phenotypes may be present in diverse cancer types and the abundance of these NK cell phenotypes may be associated with prognosis. We constructed transcriptional signatures representing ReNK, IL2NK, and SPANK and used a computational approach to determine the association between the abundance of these NK cell phenotypes in BLCA tumors and patient prognosis using the TCGA cohort. Using this approach, we found that a high tumor abundance of the IL2NK phenotype was associated with improved BLCA prognosis, but not the ReNK or SPANK.

PDGF-DD expression is dysregulated in several cancers including BLCA and activates several protumor pathways with adverse effects on prognosis (38, 97). Analysis of the expression of transcripts encoding PDGF-D and its receptor, PDGFRβ, showed that high expression of *PDGFD* in BLCA tumors was most strongly associated with poor prognosis. Since all three NK cell phenotypes were detected in BLCA tumors, we speculated that PDGF-DD-activated NK cells might counterbalance the effect of *PDGFD* expression on BLCA prognosis. Interestingly, when BLCA tumors were stratified for *PDGFD* expression and each respective NK cell phenotype, a high tumor abundance of the SPANK phenotype was associated with a poor BLCA prognosis. Conversely, a high abundance of the IL2NK phenotype in BLCA tumors was associated with improved prognosis. Since the NKp44 receptor for PDGF-DD is upregulated by IL-2-expanded NK cells, these results suggest a critical balance between activation *via* NKp44/PDGF-DD signaling and a clinically unfavorable prognosis *versus* maintaining a high proportion of the IL-2-expanded NK cell phenotype and a clinically favorable prognosis (**Figure 6**) (38). Moreover, the ReNK phenotype was associated with a poor BLCA prognosis, and it is entirely possible that failure of NK cells to become activated, e.g., by IL-2, is detrimental for BLCA patient survival (**Figure 6**). Interestingly, other cellular and tumor ligands have been reported to bind and regulate NKp44 signaling, such as Nidogen-1 (101), Syndecan-4 (102), a subset of HLA-DP molecules (103), a splice variant of the mixed lineage leukemia 5 (MLL5) gene (104), and proliferating cell nuclear antigen (PCNA) (105). It will be interesting to determine the expression of these latter gene products in the BLCA tumor microenvironment and the influence on the associations between the NK cell phenotypes that we describe and BLCA prognosis.

We speculated that if the IL2NK phenotype was critical for NK cell surveillance of BLCA tumors and improved prognosis, then NK cell receptors would also be associated with improved BLCA prognosis. In support of this hypothesis, high tumor expression of transcripts for the *KLRK1*, *TNFRSF14*, and *CD160* NK cell receptors was associated with improved BLCA prognosis, suggesting that the expression of these NK cell receptor gene products by IL-2-expanded NK cells may be critical for BLCA antitumor immunity. High tumor expression of *KLRK1*, more commonly known as NKG2D, was strongly associated with enhanced BLCA patient prognosis. KLRK1/NKG2D is an activating receptor expressed by NK cells and $CD8^+$ T cells and

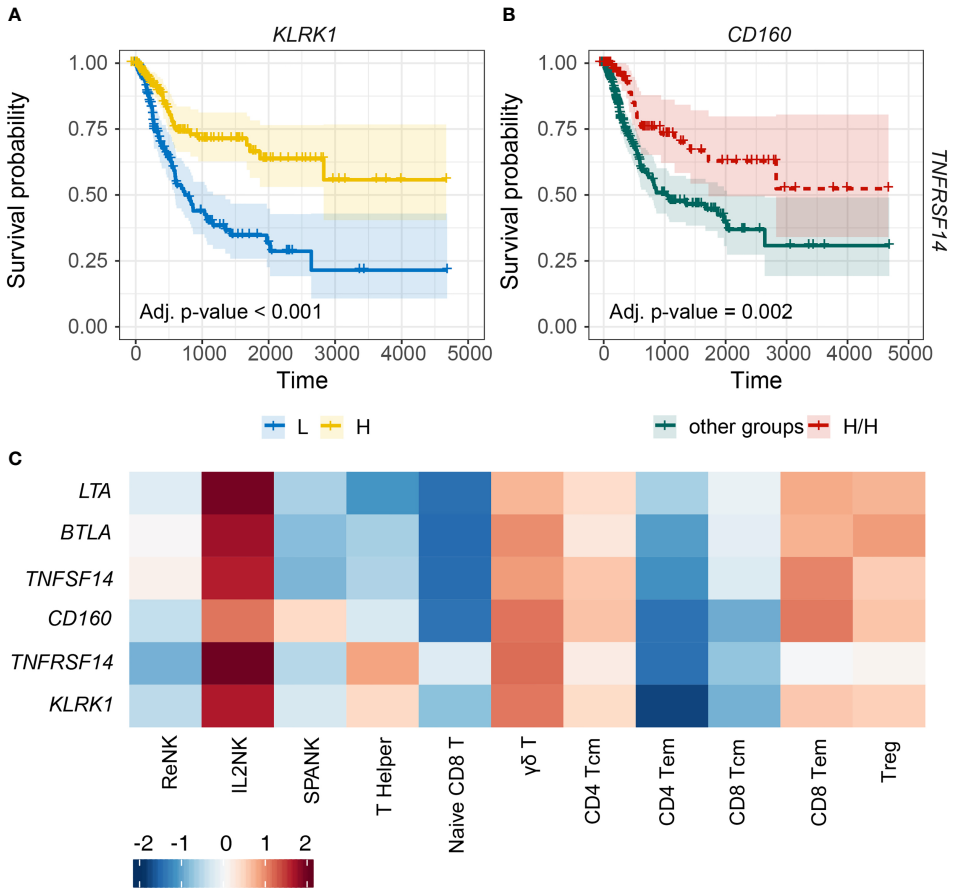

FIGURE 5 | Tumor expression of activating NK cell receptors is associated with the IL2NK phenotype and a more favorable BLCA prognosis. **(A)** KM plots displaying progression-free survival of BLCA patients stratified for tumor expression (median split) of *KLRK1* (*x*-axis, days; *y*-axis, progression-free survival). **(B)** Combined BLCA patient survival analysis stratified for tumor expression (median split) for *CD160* and *TNFRSF14*. KM curves display the survival of BLCA patients plotted for the highest strata of expression for *CD160* and *TNFRSF14* (H/H) compared with all other strata combinations for *CD160* and *TNFRSF14* combined. High tumor abundance of *CD160* and *TNFRSF14* is associated with improved BLCA prognosis (*x*-axis, days; *y*-axis, progression-free survival). **(C)** Heatmap displaying correlations between the expression of each NK cell receptor transcript (*y*-axis) and immune cell phenotype (x-axis), respectively (Tcm, central memory T cell; Tem, effector memory T cell; Treg, regulatory T cell).

recognizes a range of stress-inducible ligands, such as MICA, MICB, and the ULBP-binding proteins 1–6, collectively known as "NKG2D ligands" (NKG2D-L), that are expressed on malignant and virus-infected cells. Our data and recent *in vitro* studies (37) suggest that NKG2D is a key receptor for NK cell surveillance of BLCA tumor cells.

Like NKG2D, TNFRSF14 is also expressed by NK cells and T lymphocytes and has multiple ligands; TNFSF14, LTA, and CD160 activate TNFRSF14 signaling and NK cell antitumor functions, whereas BTLA inhibits TNFRSF14 signaling and downregulates NK cell antitumor functions. High tumor expression of *TNFRSF14* and *CD160*, but not *TNFSF14*, *LTA*, or *BTLA*, was associated with improved BLCA patient prognosis, suggesting that the TNFRSF14–CD160 interaction, like KLRK1, is a prominent pathway of NK cell tumor surveillance in BLCA. In support of this, the expressions of *TNFRSF14*, *CD160*, and *KLRK1* were all strongly positively correlated with the IL2NK phenotype compared with other immune cell phenotypes in BLCA tumors.

Overall, these data strongly suggest that the NKG2D/NKG2D-L and TNFRSF14/CD160 pathways play a prominent role in the immune surveillance of BLCA tumors by IL-2-expanded NK cells. Our results show that high tumor abundance of the IL2NK phenotype and transcripts for the *KLRK1*, *CD160*, and *TNFRSF14* receptors are associated with improved survival, whereas high tumor abundance of the SPANK and *PDGFD* transcripts is associated with poor survival. Collectively, these data may be optimized to improve BLCA patient prognosis and risk stratification in the clinic. Interestingly, TNFRSF14 is also expressed by many tumor cells including BLCA, and TNFRSF14 ligation can induce BLCA cell apoptosis (48). Consequently, how the TNFRSF14–CD160 interaction might occur between NK cells and tumor cells in the BLCA tumor microenvironment to promote antitumor immunity and favorably impact patient prognosis remains unclear. Such information may help determine if the TNFRSF14–CD160 and NKG2D–NKG2D-L pathways can cooperate for NK cell cytotoxicity of BLCA tumor cells and whether these immune

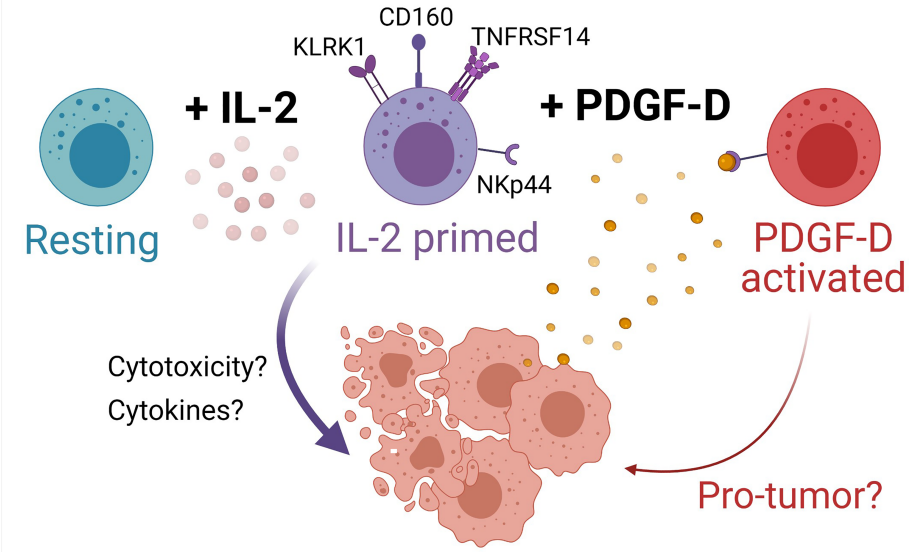

FIGURE 6 | Model for NK cell surveillance of BLCA. In contrast to resting NK cells (turquoise), IL-2 (pink circles) primes NK cells (purple cells) to express the activating receptors KLRK1 (also known as NKG2D), CD160, and TNFRSF14. Engagement of NKG2D promotes NK cell cytotoxicity of BLCA tumor cells (peach cells) expressing NKG2D ligands. BLCA tumor cells can also express TNFRSF14 and binding of CD160 on activated NK cells to TNFRSF14 on tumor cells promotes apoptosis of tumor cells, thus improving BLCA patient prognosis. The binding of TNFRSF14 to CD160 on neighboring NK cells may augment NK cell activation. IL-2-primed NK cells also express NKp44, whereas resting NK cells do not. BLCA tumor cells produce platelet-derived growth factor D (PDGF-DD, yellow circles) which induces cellular activation *via* NKp44 to generate a protumorigenic NK cell phenotype (red cells) that may be detrimental for patient prognosis. Created with BioRender.com.

surveillance pathways can be targeted for in BLCA patients, e.g., using recombinant approaches to enhance NKG2D recognition of NKG2D-L expressing BLCA cells (106) or blocking negative regulators of TNFRSF14 activation, such as BTLA (47).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YS, MK, and SM performed the analyses. YP, SM, and AB designed and directed the research. YS, MK, AS, and AB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724107/full#supplementary-material

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