INTRODUCTION

Whereas natural killer (NK) cells were discovered in the 1970s, it took another four decades to realize that NK cells are members of a family of innate lymphoid cells (ILCs), of which the majority lacks cytotoxic functions.\(^1\) NK cells are well-known for their capacity to kill virus-infected and tumour cells, and their expression of molecules involved in cytotoxicity is particularly pronounced in the population of CD56\(^{\text{dim}}\)CD16\(^{+}\) NK cells.\(^2\) Non-cytotoxic ILCs, the majority of which are tissue-resident,\(^1\) instead exert their effector functions predominantly in mucosal tissues, including the intestinal mucosa, through production of factors (cytokines, lipids and cell surface ligands/receptors) that regulate tissue integrity and immunity during both homeostasis and inflammation.\(^1\) ILCs are activated by the sensing of stress-related molecules within tissues (alarmins, cytokines and cell surface ligands/receptors), allowing for rapid local responses.\(^1\) The last few years have seen a rapid increase in reports of non-cytotoxic ILCs playing a role in cancer, including in CRC. ILC heterogeneity and plasticity have recently been extensively reviewed.\(^3\) In this review, we will provide an overview of ILC functions in the human intestinal mucosa and summarize the recent literature on the role of ILCs in CRC with a particular focus on data obtained from human studies. We will discuss the potential clinical implications of these ILC data in terms of improving anticancer treatments.
Whereas both ILCs and T cells are derived from a common lymphoid progenitor, human ILCs develop along a separate trajectory involving precursors expressing the T cell-inhibitory transcription factor Id2.4 Hence, ILCs lack many of the characteristic features of T cells including rearranged antigen-specific receptors. However, mature and differentiated ILCs and T cells both contribute to specialized effector programmes with ILCs described as an innate mirror image of the T cell family.1 In addition to NK cells, the family of ILCs is composed of so-called helper ILCs: ILC1, ILC2 and ILC3 that are analogous to T helper cells 1 (Th1), Th2 and Th17/Th22 cells, contributing to type 1, 2 and 3 immunity respectively (Figure 1).

Similar to NK cells and Th1 cells, ILC1 express the transcription factor (TF) Tbet and produce IFN-γ in response to IL-12 and IL-15. Several subtypes of ILC1 have been described in the human gut mucosa. CD127+ ILC1 that can be found throughout the human intestine5,6 express the TF Tbet but lack Eomes and cytotoxic activity. CD127+ ILC1 can be derived from ILC3 under the influence of IL-12,7,8 paralleling the ILC3-ILC1 plasticity that has been observed in the mouse gut.9 Another ILC1-like population identified in the intraepithelial compartment along the length of the human intestine5,6 is the intraepithelial ILC1 (ieILC1) expressing CD56, CD103 and NKP44.10 ieILC1 are accumulated in colitis in both humans and mice.10 Similar cells develop in il15ra-deficient mice, arguing for an ontogeny separate from conventional NK cells.10 However, ieILC1 lack expression of CD127, express Eomes and exert cytotoxicity10 showing that they are functionally closely related to NK cells. NK cells in the gut predominantly lack the high affinity IgG receptor CD16, which is highly expressed on circulating NK cells. Gut NK cells instead display a tissue-resident phenotype with high expression of CD69 and CD103.2 Since CD103 is a marker of intraepithelial localization in the gut, this may indicate that many of the gut NK cells in fact are CD103+ ieILC1, which also express CD69.10 TGF-β induces CD103 expression in ILCs,11 suggesting that such gut ieILC/NK cells are imprinted by TGF-β, which could also help explain their reduced expression of cytotoxic molecules including perforin, as compared to circulating NK cells.12 Recently, another population of cytotoxic CD127+CD94+ ILC1 that share features of both CD127+ ILC1 and CD94+ NK cells have been described to be accumulated in the human gut during inflammatory bowel disease (IBD).13 Whereas

**FIGURE 1** Innate lymphoid cells subsets and their T cell counterparts. Both ILCs and T cells differentiate from naive cells that are committed to their respective lineage but not yet activated or polarized. Differentiation to functionally mature subsets depends on the microenvironment and, in the case of T cells, activation by antigen-recognition. Once differentiated, the ILC subsets mirror that of their adaptive T cell counterparts with functions that are complementary but, in some cases, also redundant.
the exact lineage identity of such cells remains unclear, it was proposed that they stem from CD127+CD94+ ILC1. As such, they could represent the innate equivalent of cytotoxic CD4+ T cells, ie helper ILCs that can acquire cytotoxic functions, including expression of CD94 upon, eg IL-12 stimulation. Further work is required to understand the spectrum of cytotoxic and non-cytotoxic ILCs in the human gut and how this contributes to health and disease.

ILC2 represent a major subset in the mouse intestine, but is rare in the adult human gut,^5,14 although there is a slight increase in ILC2 frequency upon colonic inflammation.^6 Notably, ILC2 are frequent in the human foetal gut^14 and have been reported in the paediatric gut,15 raising the possibility that gut microbial colonization and/or competition with the adaptive immune system suppresses ILC2. Indeed, supporting the former hypothesis, ILC2 accumulate in antibiotic treated mice.16 In humans, the majority of ILC2 express the prostaglandin D2 (PGD2) receptor CRTH2 and rely on the TF GATA3 for production of the type 2 cytokines IL-4, IL-5 and IL-13.14 Cytokine production in ILC2 is seen in response to PGD2 and cytokines, and alarmins typically know to trigger type 2 reactions, IL-25, IL-33 and TSLP. Although human gut ILC2 is rare and therefore relatively uncharacterized, plasticity has been reported with ILCs in the gut that co-produce IL-13 and IFN-γ.17 Such plasticity has been thoroughly characterized in ILC2 obtained from blood, tonsils and airways showing a major role for IL-12 in inducing ILC2-to-ILC1 transdifferentiation (reviewed in 3). Similarly, in a type 3 immune-promoting environment, blood ILC2 have been described to take on features of ILC3, including production of IL-17A (reviewed in 3). Such ILC2-ILC3 plasticity remains, however, uncharacterized for human gut ILC2. Recently, ILC2-like cells lacking the conventional markers and surface receptors CRTH2 and/or CD127, potentially as a result of exposure to their corresponding ligands, have been described in human blood and lung tissue.22,23 However, the existence of such cells in the human gut, and if they contribute to the rarity of CRTH2+CD127+ ILC2 in this tissue, remains to be explored. Furthermore, in the quest for an innate equivalent of regulatory T cells, it was discovered that whereas there is little support for a distinct ‘ILCreg’ population,19 intestinal ILC2 can produce IL-10 upon exposure to factors such as IL-2 and IL-10 itself.19 IL-10-producing ILC2 can also be found in the airways where they suppress inflammation.20 However, it remains unclear whether such cells constitute a distinct population or if IL-10 production represent a regulatory effector state of ILC2.

The ILC3 compartment is composed of several subsets that all rely on RORγt for their development and function. Lymphoid-tissue inducer (LTI) cells are crucial for the formation of lymphoid structures through the interaction with mesenchymal stromal cells (MSCs) during mouse embryogenesis.21 LTI cells develop from precursors that are distinct from those generating other helper ILC subsets.22 Where as it is hard to formally prove the existence of LTIs in humans, RORγt+ ILC3 expressing NRPI that induce adhesion molecules on MSCs have been identified in human adult and foetal lymphoid structures including tertiary lymphoid structures in the lung of patients with chronic obstructive pulmonary disorder.23 NRPI+ ILC3 exist also in human gut tissue, but it is yet unclear whether such cells are confined to lymphoid structures. The dominant ILC3 population in the human gut express the natural cytokotoxicity receptor NKp446 and produce IL-22, which supports intestinal epithelial integrity in both mice24 and human in vitro cultures.25 However, ILC3 in mice and humans also produce IL-17 and GM-CSF, which was shown to cause inflammation in a mouse model of colitis.26-27 Furthermore, ILC3 can express MHCI molecules and thus present commensal bacteria antigens to CD4+ T cells, limiting IgA production by mucosal B cells and promoting microbiota tolerance during homeostasis in mice.28,29 This mechanism of tolerance seems disrupted in intestinal inflammation as ILC3 in the inflamed gut expresses reduced levels of MHCI molecules (HLA-DR in humans), reducing ILC3-induced CD4+ T cell tolerance to commensal bacteria and increasing commensal-driven colitis in mice.15 Whereas the role for HLA-DR+ ILC3 in human IBD remains unclear, several studies indicate that IBD is associated with ILC3-to-ILC1 plasticity,30 with a subsequent loss of the protective effects of ILC3. In fact, it has been suggested that ILC3 and ILC1 operate on a spectrum and that cells with features of both cell types, including cytokotoxic function, are found in tonsils31 and the inflamed gut tissue of IBD patients.30 Further work is required to dissect the diversity of cytotoxic and non-cytotoxic ILC3 and how that relates to the realm of ILC1 and NK cells.

3 | NK CELLS AND ILC1 IN CRC

Although the therapeutic options for CRC are increasing, it is still one of the most prevalent and deadly forms of cancer globally.32 The most common cause of CRC is chromosomal instability and mutations in oncogenes and/or tumour suppressor genes, but CRC can also be caused by microsatellite instability (MSI) due to impaired DNA mismatch repair pathways.32 CRC can elicit inflammation within the tumour and modulate adaptive and innate immune cells to favour its growth.32 However, this immune-reactivity can also be beneficial as MSI-positive CRC has been associated with an increased intratumoural immune cell infiltration and a better response to
checkpoint therapy, which has largely been ascribed to improved T cell-mediated tumour rejection. However, also ILCs, including NK cells, have been implicated in CRC immuno-surveillance.

Despite high T cell infiltration in some CRC tumours, NK cells are scarce in such tumours even if the levels of intratumoural effector cytokines (IFN-γ) and chemokines that recruit NK cells, including CCL3, CXCL10 or CXCL12, are elevated. However, NK cells and iILC1 cells represent 95% of tumour-infiltrating ILCs. The role of NK cells in cancer immuno-surveillance is well characterized, and NK cells have been described to eliminate tumours and metastases. The tumour participates in cancer immuno-surveillance via NK cells in two principal ways, namely downregulation of molecules that prevent NK cell reactivity (eg HLA class I via the so-called missing self hypothesis) or expression of molecules that activate NK cells (eg ligands of NKG2D). NK cells act against cancer cells by releasing cytotoxic molecules including perforin and granzymes in close proximity to the transformed cells. However, cancer cells can avoid triggering this cytotoxicity by expressing the MHC-like molecule HLA-E that binds the inhibitory NK cell receptor NKG2A, preventing degranulation of NK cells. Targeting the HLA-E/NKG2A inhibitory axis with an anti-NKG2A antibody represents a promising therapeutic strategy in CRC. In addition, peripheral blood NK cells from CRC patients have been reported to express lower levels of the natural cytotoxicity molecules NKP30, NKP44 and NKP46, and the NK cell-activating receptor NKG2D, decreasing NK cell-mediated cancer cell killing. Apart from their cytotoxic role, NK cells also produce cytokines and chemokines that regulate other immune cells such as recruiting dendritic cells (DCs) through secretion of factors such as CCL5, XCL1, XCL2 and IFN-γ, the latter enhancing DC-induced Th1 polarization.

As compared to NK cells, ILC1 are less cytotoxic but can also produce type 1 cytokines such as IFN-γ. IFN-γ induces modulatory effects on tumour immunity by enhancing antigen presentation, Th1 polarization, and having direct antiangiogenic and antiproliferative effects on the tumour. However, tumours can evade IFN-γ antitumour effects by losing their IFN-γ receptor or one of the components of the JAK/STAT signalling pathway or by activating an inhibitor of this pathway (ie SOCS proteins). This mechanism is also true in CRC tumours where genetic and epigenetic alterations targeting the IFN-γ pathway or the antigen-presenting machinery is found in the tumours, making these more prone to immune evasion and to developing resistance to immune-checkpoint blockade treatments. Notably, IFN-γ can also increase the expression of PD-L1 by tumour cells, impairing the recognition and cytotoxicity of cytotoxic T cells.

CD127+ ILC1 are found at increased proportions, along with high levels of IFN-γ, in the intestine of patients with IBD. Since IBD predisposes patients for an increased risk of developing CRC, these data support, but do not prove, a harmful role for IFN-γ or CD127+ ILC1, in CRC. Indeed, in a clinical trial with IFN-γ as a postoperative surgical adjuvant therapy for CRC, IFN-γ had a deleterious effect on time to relapse for the patients. Furthermore, analysis of blood of metastatic CRC patients treated with chemotherapy have shown an expansion of CD56+ ILC1-like cells that express the prototypical NK cell markers CD56 and NKG2A but lack killer-inhibitor receptors (KIR) and CD16 whilst expressing the non-cytotoxic receptor marker CD127. The accumulation of CD56+ ILC1-like cells in the blood was associated with a lower antitumour Th1 response and a lower progression-free survival, suggesting a deleterious role of this population in the metastatic CRC outcome. An ILC1-like CRC-specific subset expressing high levels of signalling lymphocytic activation marker 1 (SLAMF1), known to activate NK cells, was also found in CRC tumours by Qi et al. This study showed a higher SLAMF1 expression in CRC that was associated with a better prognosis, suggesting, in contrast to previous findings, a role for this ILC1-like subset in antitumour immunity. However, SLAMF1 expression was also found on ILC2. Hence, the contribution of distinct ILC subsets to the SLAMF1-associated effect in CRC remains unclear.

In summary, the role of NK cells and ILC1 in CRC is ambiguous as they can exert both antitumourigenic and protumourigenic effects on the tumour (Figure 2). Further work should be focussed on dissecting the spectrum of intratumoural NK cells and ILC1 and their contribution to CRC development, progression and metastasis. Understanding the crosstalk between ILC1/NK cells, cancer cells and stroma, and if this occurs in specialized tumour niches, also remains a topic for future investigations.

4 | ILC2 IN CRC

Whereas the role of ILC2 in protection against helminth infections and allergic diseases is undisputed, their role in cancer is only beginning to be deciphered. ILC2 have been shown to promote tumour immunity in hepatocellular carcinoma and melanoma. However, the role for ILC2 in CRC is more complex. ILC2 are present in the mouse gut at steady state, whereas they are scarce in the adult human intestine except during inflammation. Moreover, the frequency of IL-13+ ILC2 increases within colorectal tumours.
Recent findings state that intratumoural ILC2 might have an antitumour role. Indeed, higher expression of ILC2 signature genes, including GATA3 and KLRG1, in tumours was correlated with a better prognosis in CRC and ILC2-deficient mice have an increased tumour growth. It remains, however, unclear which factor(s) mediates this antitumour effect. Furthermore, it has been shown that the absence of ILC2 in tumours favours its growth and increases the spread of metastases in mice. Qi et al. have demonstrated the presence of ILC2 in human CRC tumour tissue. In addition, high expression of the ILC2-activating cytokine IL-33 within the tumour tissue was associated with a longer survival in CRC, suggesting that IL-33-responsive cells, including ILC2, might have a role in antitumour immunity in CRC. IL-33 expression has indeed been linked to an antitumourigenic role, and its production by tumour epithelial cells is increased during CRC. However, IL-33 can have protumourigenic
or antitumourigenic roles depending on the cancer type. On the one hand, IL-33 promotes antitumour responses by recruiting and activating CD8+ T and NK cells in the microenvironment in mouse melanoma and breast cancer cell lines. Supporting the antitumour function of IL-33, its loss in metastatic prostate and lung carcinomas has been identified as a potential strategy to escape immune barriers by the tumour.

However, in CRC, IL-33 seems to have a dual role, both contributing to tumour rejection and promoting tumour growth. The latter is achieved by enhancing cancer cell stemness by recruiting and stimulating macrophages to produce prostaglandin E2. IL-33 have also been shown to promote Treg function in the human intestine and in a colitis mouse model. Another mechanism for IL-33-mediated protumoural functions might be via peroxisome proliferator-activated receptor gamma (PPAR-γ), which is an actor of lipid metabolism and is selectively expressed in mouse and human ILC2, as demonstrated to play an important role in IL-33-induced ILC2 cytokine secretion and to promote tumour growth. Together with T cells, ILC2 can express the checkpoint inhibitor PD-1 in cancer, which is stimulated by PPAR-γ and could enhance antitumour responses to checkpoint therapy. Indeed, human ILC2 PD-1 deficiency has been shown to suppress tumour growth and the presence of human ILC2 PD-1high in engrafted CRC tumours promoted tumour progression in mice. In humans, PPAR-γ has recently been shown to induce PD-L1 in microsatellite stable patient-derived tumour organoids, which could improve the response to checkpoint blockade therapies.

In summary, the role of ILC2s in cancer is not clear and seems to involve antitumour and a protumour roles, which remains to be fully characterized (Figure 2). More specifically, further research on intratumoural ILC2 heterogeneity, including expression of PD-1 and PPAR-γ, could benefit the understanding of CRC development. In particular, PPAR-γ could constitute an interesting new therapeutic target given its expression is also shared by Th2, Tregs and epithelial cells.

### 5 | ILC3 IN CRC

An increasing number of studies currently point towards a role of ILC3 during CRC development by either modulating the gut microbiome or the gut microenvironment. In a recent paper, Goc et al showed that ILC3 was diminished, whereas ILC1 was increased in human CRC tumours compared with the paired healthy gut, possibly due to a plasticity of ILC3 towards ILC1. This was also confirmed by Qi et al who showed a reduction in ILC3 and increase in ILC1 in CRC tumour as compared to the healthy tissue. In addition to contributing to the immunoregulatory effects of ILC1 in CRC as described above, ILC3 directly impact tumour immunity in ways outlined below.

During gut inflammation, HLA-DR expression on ILC3 was found to be increased in CRC tumours by Goc et al and Rao et al. Mechanistically, it was shown that specifically removing MHCI+ ILC3 in mice favoured CRC tumour development, thus suggesting that MHCI+ ILC3 has a protective role in CRC. Supporting the role of a dialogue between ILC3 and T cells in reducing gut inflammation, a reduction in ILC3 and MHCI+ ILC3 in CRC tumours is associated with a specific microbiota driving anti-PD-1 immunotherapy resistance.

Recent data also support the role of ILC3 in regulating CRC immunity through the production of cytokines. The expression of the NCR NKP44 (or NKp46 in mice) on ILC3 separates two subsets of ILC3, NKP44+ ILC3 that secrete IL-22 and NKP44+ ILC3 that produce IL-17F. The principal source of intratumoural IL-22 is thought to be mainly NCR+ ILC3, as described in mice. In humans, ILC3 is also responsible for IL-22 production along with other cells such as conventional (Th22 cells) and unconventional T cells including MAIT cells and γδ T cells. High expression of IL-22 within CRC tumours of mice favours tumour development, inhibition of apoptosis by a STAT3-mediated mechanism and metastasis. Supporting this, IL-22 polymorphisms in humans are associated with an increased risk of developing CRC. IL-22BP, an antagonist of IL-22 produced by dendritic cells, was also described to be reduced in human colorectal tumours and inhibition or absence of IL-22BP favoured colorectal tumour development in mice. Moreover, tertiary lymphoid structures (TLS) are linked to less invasive CRC tumours. NKP44+ ILC3 can be found in TLS of CRC tumours where they express factors known to promote TLS formation, including LTA, LTB and TNF. Notably, NKP44+ ILC3 downregulates TLS formation-associated genes during CRC progression, fitting well with the observation that TLS numbers and their density are diminished in advanced stage tumours.

NCR+ ILC3 is found inside mucosal lymphoid structures in mice and the human intestine at steady state but can be dysregulated in humans through overexpression of IL-17A/F and IL-22 during inflammation such as Crohn’s disease and, in CRC, similar to T cells. IL-17A is a potent proinflammatory cytokine, which can increase tumourigenesis through direct and indirect mechanisms. IL-17A can directly favour tumour growth and immune evasion, and indirectly by the mobilization of myeloid-derived suppressor cells. It has been shown recently that anti-IL-17A antibody could enhance anti-PD-1 immunotherapy in CRC. Furthermore, the ILC3-activating cytokine IL-23 is increased in CRC tumours and favours tumour growth whilst promoting protumour IL-17 responses.
Finally, ILC3 can also contribute to tumour growth by generating IL-10 producing ILCs under the influence of TGF-β. However, the existence of such regulatory ILCs (ILCreg) is controversial, and further studies are required to understand the role of IL-10 producing ILCs in CRC.

In summary, ILC3 are dysregulated in CRC including a loss of capacity to regulate intestinal T cells and increased plasticity towards the ILC1 subset, increasing inflammation and promoting tumour growth in the cancer microenvironment in mice (Figure 2). However, we are still lacking data on the role of ILC3 and their interplay with other immune cells in the human CRC tumour environment. Such studies could be valuable for designing more effective and targeted therapies.

6 | CONCLUSIONS AND POTENTIAL CLINICAL IMPLICATIONS

The last decade has seen an improvement of CRC treatment and therefore patient survival. However, the clinical situation is far from satisfying, and despite screening programmes for high-risk patients, around 25% of cases are discovered at an advanced stage where effective treatment options are scarce. Currently, in addition to chemotherapies and surgery, CRC is not only treated with targeted drugs that interfere with tumour growth, but also immunotherapies mostly focussed on T cell-related immune checkpoints such as PD-1 and CTLA-4. An increasing body of literature highlights how innate lymphocytes, including NK cells and ILCs, also play a role in CRC and even mediate some of the effects of anti-PD-1 checkpoint therapy. Utilizing ILCs for treatment constitutes a promising strategy as exemplified by the recent development of chimeric antigen receptor (CAR)-engineered NK cells. However, major challenges still exist in how to harness the ambiguous ILC functions for anticancer treatment. One such issue is how to specifically amplify the antitumour functions of ILCs whilst preserving antitumour T cell functions. Another is how to optimize ILC adoptive transfer for efficient antitumour immunity. To address such challenges, we still need a better understanding of the roles of ILCs in CRC development, progression and metastasis.

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CONFLICT OF INTEREST

The authors declare no conflicting interests.

AUTHOR CONTRIBUTIONS

Both JM and AM contributed to the conceptualization, writing and preparation of the manuscript and conceptualization of figures. AM prepared the figures.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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