IN MEMORIAM OF PROFESSOR ALESSANDRO MORETTA

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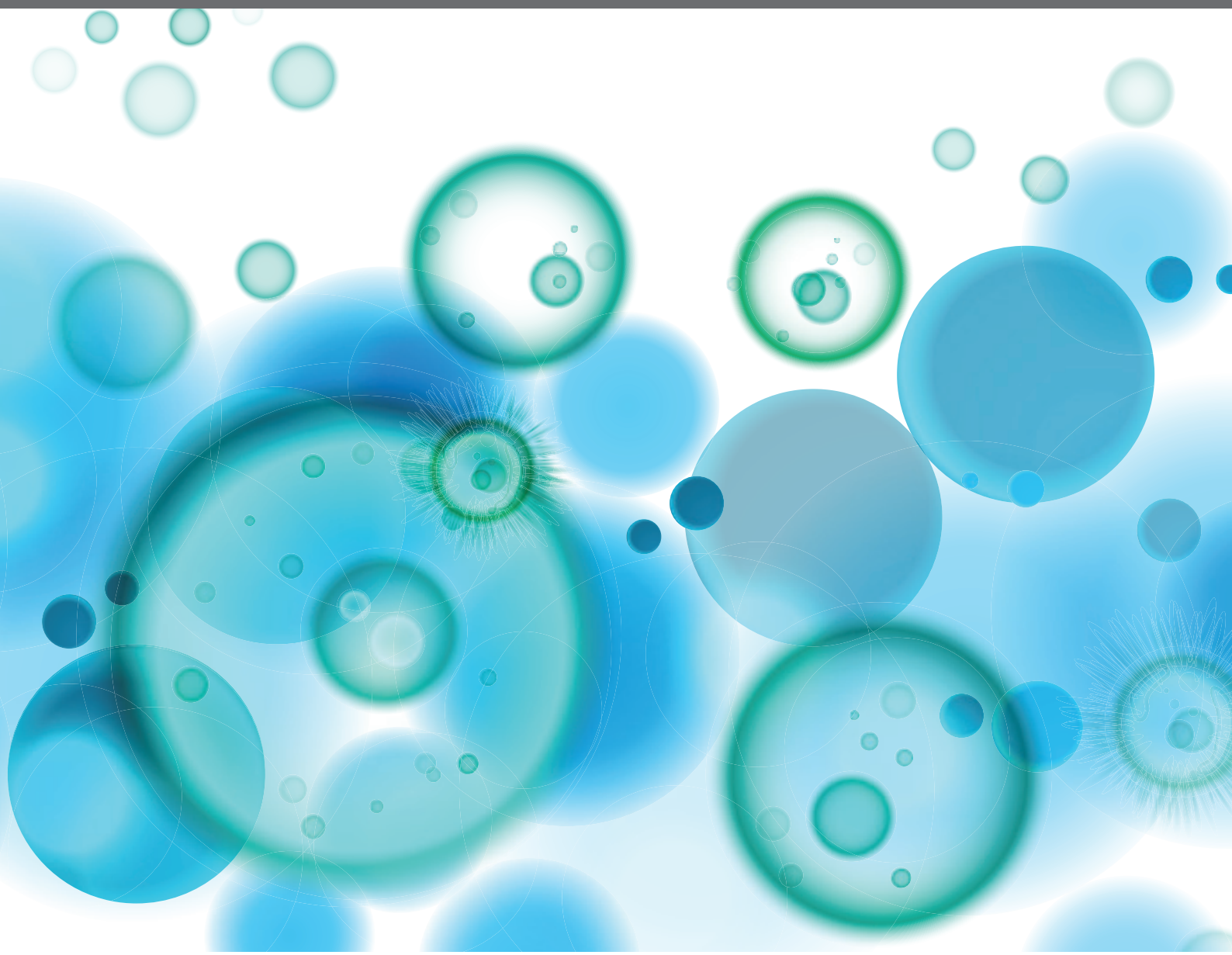

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IN MEMORIAM OF PROFESSOR ALESSANDRO MORETTA

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EDITORIAL
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Editorial: In Memoriam of Professor Alessandro Moretta

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Keywords: natural kill cell, transplanation, immune deficiency, infection - immunology, cancer biology

Editorial on the Research Topic

In Memoriam of Professor Alessandro Moretta

Prof. Alessandro Moretta was a major contributor to Frontiers in Immunology, particularly in the domain of NK cells and innate lymphocyte biology. We would like to honor his memory with a special tribute edition containing a collection of reviews covering the various periods of his scientific career, which was long, although sadly not as long as it might have been. The authors will try to connect his discoveries with the history of these cells to date. Their memories of these exciting times, full of enthusiasm, will be a useful resource for all readers. We would like the authors to share with the readers their discussions with him concerning the topic reviewed, and his view of the field, where possible.

These reviews will address the early years of discovery when Alessandro Moretta was a pioneer in the immune field, together with his brother Lorenzo and their collaborators, even before he began working on NK cells. By focusing on this period, we will highlight the tremendous enthusiasm surrounding these discoveries.

The activation of NK cells via the so-called "natural cytotoxicity receptors" (NCRs) 2B4, NTB-A and DNAM-1 will be highlighted by Vitale et al. and Pende et al. will address the discovery of NK-cell immunomodulation through the identification of killer cell Ig-like receptors (KIR) and the developments leading to its clinical use essentially in pediatric acute lymphoblastic leukemia.

Translational studies in the field of cancer will be presented by Pesce et al. for ovarian cancer and Leclerc et al., for lung carcinoma. Di Vito et al. will address the ontogeny of NK cells specifically post-allostem cell transplantation.

The primary immunodeficiency studies will be handled by Tabellini et al. as well as Zimmer for TAP transporter.

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We all three think that our colleagues have really presented important insights that would have delighted Alessandro.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We would like to thank all Alessandro's friends for this celebration.

Conflict of Interest: EV is the CSO of Innate Pharma. DO is the cofounder of Imcheck Therapeutics.

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OPINION
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A Tribute to Alessandro Moretta (1953–2018). Living Without Alessandro

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Alessandro Moretta was M.D. and Professor of Histology working for more than 30 years at the University of Genova (Italy) (**Figure 1**). For the majority of scientists, he is known as the godfather of human NK cells, although his achievements go far beyond this specific area. Many of us knew about his disease since 2010 and admired his courage during this awful period when he was still moving ahead, running his lab, and publishing outstanding articles. We were all happy that immunotherapy using anti-PD1 antibodies improved his very severe non-small cell lung carcinoma in 2014. Unfortunately, he passed away 7 February, 2018, and it is a terrible loss for the immunological community.

As a scientist, he was among the most productive colleagues with more than 500 articles and reviews since 1976. Despite the disease, he was still pursuing his objectives, namely to continue his research work as well as running a Ph.D. training program in order to promote immunology for scientists and doctors. His objective was to achieve his work and to retire at the age of 70 years.

Alessandro was both an M.D. and a scientist, and all his achievements since 1976 are along the same line: promoting high standard basic research and transferring the findings as soon as possible in patients' studies including cancer, viral infections, and immunodeficiencies. To use the classical terms, he was from the beginning both working from bench to bedside as well as to bedside to bench. His career has included two main periods after he obtained his M.D. in 1978: Lausanne with Jean Charles Cerrotini and Lorenzo his brother, and Genova when he came back to run his lab. All the periods throughout the 42-year period were highly successful.

During the Lausanne period, he mainly focused on the definitions of human T cells populations and the identification of cytotoxic T cell (CTL). This period was highly productive and provided critical informations on cytotoxic effector cells. He was among the first ones to use efficiently the novel technologies developed in the mid-1970s following Milstein and Kohler's discoveries, namely monoclonal antibodies (mAbs) and flow cytometers. With the help of these tools, he made an extensive delineation of cytotoxic T cells.

He also adapted T cell cloning by limiting dilution (LDA) to identify and characterize the phenotype and the function of T cell clones at the single cell level. This technology was instrumental to identify the cells possessing CTL function and cytokine production. Due to his strong translational appetite, he extended these findings to human tumor infiltrating lymphocytes as early as 1985. The discovery and a better understanding of cosignaling molecules led him to work on CD2 and Tp44 (now CD28) and their signaling capacities using the newly developed calcium flux and inositol phosphate assays. These were among the first studies investigating the signaling pathways activated and represented a first step toward pharmacological intervention. The potency of his single cell cloning technologies was extended to innate effectors namely a novel population that had just been described as γδ T cells in 1987.

Upon arrival in Genova, he dedicated his work to natural killer (NK) cells. This population had remained elusive after the initial seminal works performed in the mid-1970s both in humans and mice. He tackled this issue very efficiently as always, combining for the first time all the available

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FIGURE 1 | Prof. Alessandro Moretta 2014.

technologies to understand the biology of these cells in humans. He generated mAbs with a brilliant screening strategy based on the function of NK cell clones together with his expertise in LDA. A real tour de force. A large collection of mAbs specific for these cells were generated and characterized. To identify the molecular weight and structure of the molecules recognized, he devised within his laboratory, biochemical strategies based on immunoprecipitation. The final step that was implemented in his lab was the cloning of the genes coding for the molecules that his group had identified using mAbs. Altogether, these studies created a revolution in the field of human NK cell biology by identifying inhibitory receptors, activating receptors and co-receptors.

A first category of NK receptors with inhibitory function was identified with the discovery of p58 molecules that were later called killer cell immunoglobulin-like receptors (KIRs) in collaboration with his brother, Lorenzo Moretta (1). Rapidly, he discovered that the p58 molecules were receptors specific for allotypic determinants of HLA-C molecules. He also found an homologous receptor of 50 kDa named p50 that turned out to be activating counterparts of p58 receptors, primarily differing from p50 intracytoplasmic domains. These discoveries produced tools that are extensively used worldwide, such as the famous EB6 and GL183 mAb, and to prepare the next step of translational investigations of immunointervention. Additional receptors interacting with HLA-class I molecules were identified during this era, namely the heterodimer made of NKG2A and CD94.

Despite being extremely appealing, the missing-self hypothesis proposed by K. Karre and H. G. Ljunggren needed to be completed as the signals activating NK cells were unknown. Again, using his NK clones and mAbs, he made multiple novel discoveries in 3 years (between 1998 and 2000) i.e., the identification of three activating receptors named natural cytotoxicity receptors (NCR): Nkp44, Nkp46, and Nkp30. He also extended these studies to co-receptors and specifically identified the functions and ligands of DNAM-1 (CD226), namely PVR and nectin-2, as well as the role of the co-receptor 2B4.

In parallel, the identification of KIRs and their recognition of HLA-class I alleles opened new avenues in the field of hematopoietic stem cell transplantation, especially in haploidentical transplantation first *via* the pioneer work of A. Velardi team in acute myeloid leukemia (AML) as soon as 1999. With a great collaboration with F. Locatelli and L. Moretta, a project was launched and currently benefits to patients with pediatric acute lymphoblastic leukemias. Alessandro devised flow cytometry-based assays to identify the donors who were more appropriate to exert this GvL effect.

In 1999, Innate Pharma was launched in Marseille. A. Moretta was enthusiastic to be part of this project and to help organizing a program dedicated to the immunomodulation of NK cells *via* the blockade of the two inhibitory receptors KIRs and NKG2A receptors. Two clinical products were generated: Lirilumab targeting KIR and Monalizumab targeting NKG2A. These two products are currently used either in combination or alone in clinical trials in cancer patients. A first class of products targeting peripheral T cell lymphomas through the KIR receptor KIR3DL2 is also currently in Phase 1 clinical trial. He developed these mAbs with Innate Pharma and M. Bagot and A. Bensussan. In addition to these immunotherapeutic interventions, his discoveries have improved the understanding of NK-target interaction in patients. He found that the NCR were down regulated in AML patients at diagnosis and that this impaired NK killing in these patients (2002). These studies were extended to NK from patients with solid tumors where he identified dysfunction of NK cells in the microenvironment (TME) of breast, ovarian, and prostate cancers. Coming back to his appetite for bed to benchside approaches, he investigated NK cells in HIV-infected patients with A. Fauci. They found profound alterations of NK cells including the accumulation of subpopulations of CD56neg/low CD16+ as well as Siglec7 deficient NK cells. Altogether, these alterations mediated by cancer and infection further supported the view that NK cells were key players in the response against these pathological conditions. Since his curiosity was inextinguishable, he also decided to explore, although he was sick, novel research areas in the field of NK cell memory and regarding the regulation of NK cell functions in the TME. These projects were ongoing as he passed away, and manuscripts were about to be submitted. We are eager to find out what his discoveries were.

On top of being an outstanding researcher, Alessandro has always been an excellent teacher. For him, the transfer of information was among the missions he wanted to achieve and friendship was instrumental. He did this at multiple meetings as well as in his lab where he has trained several generations of scientists either in Lausanne or Genova who are either independent or should become soon like G. Pantaleo, M. Lopez- Botet, G. Ferlazzo, D. Mavilio, S. Sivori, R. Castrinoci, E. Marcenaro, C. Bottino and others.

Alessandro was among the researchers who were the most appreciated and liked in the immunological community. The reasons are multiple but to sum up briefly he was always smiling and laughing, debating in a very smart and gentleman like way, and characterized by his incredible generosity in sharing information and reagents. He was so keen exchanging through congresses held in Greece that he launched with a few friends dedicated to

innate immunity. The 15th edition of this meeting in June 2018 will be dedicated to honor Alessandro.

We have to give a special message to those who were with him during his last years including his wife Genny, and his brother Lorenzo who was always taking care of him, as well as his lab that was fully dedicated to him.

In addition to science, Alessandro was addicted to music (although not playing anymore guitar) and especially the songs of the 1970s. So, let us finish with "when the music is over" we have to go the "stairway to heaven" and his favorites, the famous Rolling Stones song "it's only rock and roll and I like it" translated by Alessandro to "it's only innate immunity and I like it" (2).

Ciao bello, noi manchi già.

AUTHOR CONTRIBUTIONS

EV and DO have equally contributed to this opinion regarding Prof. Alessandro Moretta.

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REVIEW
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Killer Ig-Like Receptors (KIRs): Their Role in NK Cell Modulation and Developments Leading to Their Clinical Exploitation

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Natural killer (NK) cells contribute to the first line of defense against viruses and to the control of tumor growth and metastasis spread. The discovery of HLA class I specific inhibitory receptors, primarily of killer Ig-like receptors (KIRs), and of activating receptors has been fundamental to unravel NK cell function and the molecular mechanisms of tumor cell killing. Stemmed from the seminal discoveries in early '90s, in which Alessandro Moretta was the major actor, an extraordinary amount of research on KIR specificity, genetics, polymorphism, and repertoire has followed. These basic notions on NK cells and their receptors have been successfully translated to clinical applications, primarily to the haploidentical hematopoietic stem cell transplantation to cure otherwise fatal leukemia in patients with no HLA compatible donors. The finding that NK cells may express the PD-1 inhibitory checkpoint, particularly in cancer patients, may allow understanding how anti-PD-1 therapy could function also in case of HLA class I^{neg} tumors, usually susceptible to NK-mediated killing. This, together with the synergy of therapeutic anti-checkpoint monoclonal antibodies, including those directed against NKG2A or KIRs, emerging in recent or ongoing studies, opened new solid perspectives in cancer therapy.

Keywords: HLA class I, killer immunoglobulin-like receptors, KIR ligands, inhibitory checkpoints, NK alloreactivity, NK cell education, polymorphism

INTRODUCTION

The groundbreaking discoveries made by Alessandro Moretta left a very big footprint in Immunology, and, more generally, in Medicine (1, 2). Indeed, also now that "the music is over" (3) the Alessandro's legacy will remain, witnessed by the many human lives saved thanks to his seminal studies and his continuous efforts to translate discoveries to the benefit of patients with leukemia or solid tumors.

Since the beginning of his scientific career, Alessandro focused his research on two different issues that revealed to be fundamental for his future major accomplishments: on one side, the generation of monoclonal antibodies (mAbs) to functional surface molecules expressed by human T cells, and on the other side, the development of a highly efficient T cell cloning technique allowing clonal growth of virtually 100% T cells (4). While these early studies provided important information on co-receptor molecules inducing accessory signals for T cell activation and on the frequency and subset distribution of T cells endowed with given functional capabilities, they offered an invaluable tool for the subsequent development of techniques suitable for efficient cloning of natural killer (NK) cells. In turn, NK cell clones were fundamental for the discovery of both killer immunoglobulin-like receptors (KIRs) and natural cytotoxicity receptors (NCRs), the two most important steps toward unraveling NK cell function.

General View on NK Cells

Notably, although NK cells were discovered in the mid '70s, the molecular mechanism(s) by which they discriminate between tumor and healthy cells remained obscure for long time. In late '80s, the "missing self-hypothesis," proposed by Karre and Ljunggren, inspired the subsequent seminal discoveries in mice and humans in early '90s (5). The "missing self-hypothesis" stemmed from the observation that NK cells could kill a lymphoma cell line that had lost major histocompatibility (MHC) class I surface molecules, while the original MHC class I^{pos} were resistant to lysis. A conceivable interpretation was that NK cells are able to sense the absence of "self" MHC class I molecules on target cells. Indeed, MHC class I specific inhibitory receptors were discovered in parallel in humans and mice (6, 7). Surprisingly, human and mice receptors were found to belong to distinct molecular families, namely the Ig-superfamily and the lectin family, respectively. The inhibitory signals generated by the engagement with their MHC class I ligands resulted in NK cell inactivation. The prototypes of human leukocytes (HLA) class I specific receptors were two highly homologous molecules (p58.1 and p58.2) (8, 9), expressed by partially overlapping NK cell subsets. They were shown to recognize allotypic determinants shared by the two main groups of HLA-C alleles. While KIRs are expressed at late stages of NK cell differentiation, more immature NK cells express another important inhibitory receptor, the CD94:NKG2A heterodimer, specific for HLA-E (10). CD94:NKG2A is the first HLA class I specific receptor to be expressed during NK cell differentiation. At a further differentiation stage, it may be co-expressed with KIR(s), while it is lost by the most mature NK cells which express KIR(s) only (11). Notably, the pool of mature NK cells expresses at least one inhibitory receptor for self-HLA class I (whether KIR or NKG2A), thus avoiding autoreactivity. The very few NK cells lacking self-reactive receptors are anergic. This status is acquired during NK cell maturation by a process referred to as NK cell "licensing" or "education" (12, 13). Although HLA class I specific inhibitory receptors were fitting with the "missing self-hypothesis," the understanding of the actual molecular mechanism(s) regulating NK cell function was incomplete, as the "on" signals inducing NK cell activation in the process of tumor cell lysis were unknown.

Again, the use of NK cell clones and the generation and selection of mAbs capable of modulating NK cell function allowed the identification of three novel activating receptors: NKp46 (14, 15), NKp44 (16, 17), and NKp30 (18), collectively named NCRs (19), playing a major role in tumor cell recognition and killing. In addition, Alessandro identified the function of other surface molecules acting as co-receptors, including p75/AIRM1, IRp60, 2B4, NTBA, and NKp80 (20–25), and could also determine the activating function of DNAM-1 (CD226) and its corresponding ligands (namely PVR and Nectin-2) (26). It became evident that NK cell activation is dependent on an array of receptors and co-receptors that interact with ligands overexpressed or expressed *de novo* on "stressed" cells and on tumor-transformed or virus-infected cells. While, in an autologous environment, healthy cells express HLA class I molecules that generate inhibitory signals via KIR or NKG2A, tumor- or virus-infected cells may display HLA down-regulation, allowing NK cell triggering via activating receptors and consequent target cell killing. In the case of viral infections that do not down-regulate HLA class I, the susceptibility to NK-mediated killing may be related to viral peptides that, upon binding to HLA molecules, could impair KIR engagement.

Altogether, these findings revealed that NK cell activation is under the control of inhibitory and activating receptors and their ligands on target cells, and thus receptor/ligand pairs could represent true checkpoints in the regulation of NK cell function (27). Notably, an important mechanism of tumor escape is the down-regulation of activating NK receptor expression, thus eluding the NK-mediated control of tumor growth and metastatic spread (28–30).

In humans, two main NK cell subsets were originally identified on the basis of the intensity of CD56 surface expression. The two subsets are differently distributed in blood and tissues: CD56^{dim} are largely predominant in peripheral blood (PB), while CD56^{bright} are much more abundant in tissues. CD56^{bright} NK cells are relatively immature, express NKG2A and not KIR, are poorly cytolytic, secrete cytokines (primarily IFN-γ and TNF-α), and undergo intensive proliferation in response to IL-2 or IL-15. In contrast, CD56^{dim} NK cells express NKG2A and/or KIR, are mature, display a strong cytolytic activity and cytokine secretion capability rapidly upon activation. Remarkably, on the basis of the surface expression of NKG2A and/or KIR, and other markers, CD56^{dim} NK cells could be further subdivided in different subsets representative of distinct differentiation stages characterized by the progressive decrease of the proliferative capacity, paralleled by an increase of cytolytic activity (11, 31). The most mature, terminally differentiated, NK cells are KIR^{pos} CD57^{pos} CD16^{bright} and may express the HLA-E specific activating receptor NKG2C. As recently revealed (also with the Alessandro's contribution), NKG2C^{pos} cells undergo expansion in CMV infections, displaying adaptive features and memory-like function (32–35).

During the last decade, cells belonging to the innate lymphoid cells (ILCs) were identified. They share with NK cells a common ID2^{pos} lymphoid precursor. Absent or infrequent in PB of healthy

individuals, they reside primarily in mucosal tissues, skin, and lymphoid organs (e.g., tonsils), where they participate to innate defense against pathogens and to tissue repair/regeneration (36–38). They are referred to as "helper" ILC, being non-cytolytic and producing typical sets of cytokines. While they will not be further discussed here, it is noteworthy that an important subset of ILC3 (the NCR^{pos} ILC3) is characterized by the expression of NCR, the activating receptors originally described and characterized by Alessandro.

NK cells can migrate from blood to tissues or lymphoid organs. Their traffic is regulated by chemokines and their corresponding receptors, addressing different NK subsets to specific compartments or inflammatory sites. In addition, since $CD34^{pos}$ precursors, capable of differentiating toward NK cells, have been detected in tissues including liver (39), tonsils (40), thymus (41), and decidua (42), it is likely that some of the tissue resident NK cells may undergo differentiation from these precursors and, under the influence of specific tissue microenvironment, acquire unique functional properties.

While NK cells mediate a strong anti-tumor activity, their effectiveness may be greatly compromised by the suppressive microenvironment of different tumors. Suppression is mediated by a number of mechanisms, including release of soluble factors by tumor cells and by cells present in the microenvironment that have been attracted and/or "conditioned" by tumor cells. These cells include M2 macrophages, myeloid-derived suppressor cells (MDSC), T-reg and stromal cells (30). In addition, hypoxia, frequently occurring in tumor lesions, also contributes to the inhibition of immune effector cells. In the case of NK cells, the principal effect is the down-regulation of the activating NK receptors, thus rendering NK cells "disarmed" and unable to recognize specific ligands on tumor cells. Another related tumor-induced mechanism playing an important role in tumor escape is the expression of inhibitory checkpoints, primarily PD-1, in NK (and T) cells, and of the corresponding ligands in tumor cells (PD-L1 and PD-L2). The PD-1/PD-L1 interaction has been shown to induce NK cell inactivation (43). Notably, PD-1 expression in NK cells has been reported and functionally analyzed by Alessandro and coworkers (44). PD-1 will be briefly discussed, in the frame of novel immune-based strategies that appear very promising in tumor therapy. The important role of NK cells and their receptors in defense against tumors and leukemia is also witnessed by the great success achieved in the T-depleted, haploidentical hematopoietic stem cell transplantation (haplo-HSCT) setting to cure high-risk leukemia. The unthinkable benefit of this therapeutic approach is primarily related to the graft-vs.-leukemia effect of donor NK cells, arising from grafted stem cells and/or infused with the graft.

Thus, in less than 3 decades, a cell of the innate immunity that was substantially underscored (45) became an important tool to successfully treat otherwise incurable leukemia. Many of these major therapeutic achievements are based on seminal Alessandro's discoveries illustrated in this review.

While the main focus of our applied researches has been the involvement of KIRs in defense against tumors and leukemia, it is important to underline that KIRs and their polymorphisms have been shown to play a relevant role also in inflammation, infection, autoimmune diseases, and reproduction (46–48).

MILESTONES IN THE DISCOVERY OF HLA SPECIFIC RECEPTORS IN EARLY '90S

In late '80s, Alessandro Moretta came to Genoa from Lausanne with a "very special cell line": the hybridoma producing GL183 mAb. Through this mAb he identified a novel 58-kD surface molecule expressed on a subset of NK cells and capable of modulating NK cell cytolytic function (8). The paper describing this molecule was published in 1990 and this was the beginning of a new exciting era that led to the discovery of various HLA class I specific receptors, using the same approach based on mice immunization with NK cell clones and on the hybridoma technology. Soon thereafter, by immunizing mice with a $GL183^{neg}$ NK cell clone, EB6 mAb was selected for its ability to react with a molecule distinct from GL183 but sharing most of its characteristics, as revealed by phenotypic, biochemical, and functional studies (9). Notably, the reactivity of the two mAbs allowed the identification of four NK cell subsets that could be extremely variable in terms of relative percentages among different individuals. Both mAbs could inhibit the cytolytic activity of selected NK clones (expressing either GL183 or EB6) in redirected killing assays, using the $Fc\gamma R^{pos}$ murine P815 target cell line. Interestingly, a striking correlation existed between the $EB6^{pos}$ $GL183^{neg}$ phenotype of NK cell clones and their ability to lyse normal allogeneic cells, such as PHA-blasts, in a previously defined type of alloreactivity (i.e., stimulator/responder combination in mixed lymphocyte reaction, MLR), termed "1 anti-A" or "group 1" (49–51). In the same years the seminal study by Ljunggren and Karre (an elaboration of the Karre's PhD thesis in early '80s) was published, formulating the "missing self" hypothesis (5). In humans, p58 molecules appeared to fit with this proposal, rendering NK cells capable of self/non-self discrimination. Then a close relationship could be determined between the expression of EB6- and GL183-reactive molecules, termed p58.1 and p58.2, respectively, and their specificity for different HLA-C alleles (52–54). Evidence was provided that HLA-C*03 and HLA-C*04 conferred selective protection from $GL183^{pos}$ and from $EB6^{pos}$ NK clones respectively (52, 55). These two specificities were extended to a series of HLA-C alleles related to HLA-C*03, characterized by Ser^{77}-Asn^{80}, and those related to HLA-C*04, characterized by Asn^{77}-Lys^{80} (56). These two distinct groups of HLA-C alleles encompass virtually all the expressed HLA-C molecules. Thus, $EB6^{pos}$ $GL183^{neg}$ "group 1" NK clones could lyse allogeneic cells expressing only HLA-C alleles with Ser^{77}-Asn^{80} motif, while $EB6^{neg}$ $GL183^{pos}$ "group 2" NK clones were alloreactive against target cells expressing only HLA-C alleles with Asn^{77}-Lys^{80} motif. Importantly, treatment of NK clones with mAbs masking p58 molecules resulted in the killing of HLA-C protected cells, showing that this inhibitory receptor/ligand interaction was responsible for the blocking of target cell lysis (55). Consistent with these notions, NK clones co-expressing p58.1 and p58.2 molecules were inhibited by the interaction

with any allogeneic target cells, thus being unable of any type of alloreactivity (57). In the same years, NKB1 was described as a 70 kDa glycoprotein, whose expression was detected by DX9 mAb, and DX9^{pos} NK clones recognized a group of HLA-B alleles carrying the Bw4 public epitope (58, 59). Thus, analysis of NK cell clones, derived not only from different donors but also from single donors, allowed the detection of the great NK cell heterogeneity with extremely variable patterns of expression and co-expression of the various receptors with different HLA specificities. In 1992, studying in different donors the NK cell repertoire related to the ability to lyse allogeneic cells, five distinct allospecificities were described (60). While "group 1" and "group 2" had been previously defined, "group 3" could be later explained by the expression of p70 alone (detected by Z27 mAb), and "group 5" by the expression of both p58.1 and p70 (57). These data supported the notion that each HLA class I specific receptor could be physically and functionally independent (57, 61). Another important piece of history was added by the evidence that EB6 and GL183 mAb could also recognize activating receptors, named p50.1 and p50.2, respectively, according to their molecular mass (62). Subsequently, another activating receptor, termed p50.3, was identified by three PAX mAbs and was not stained by either GL183 or EB6 mAb (63). Differently from p58 molecules, found in all individuals, p50 receptors could be detected only in some donors. A crucial step was represented by the identification of the cDNAs coding for these receptors, demonstrating that they belong to the same molecular family (64). The p58 and p50 molecules were highly homologous in their extracellular regions formed by 2 Ig-like domains, while major differences existed in their transmembrane and cytoplasmic portions. Notably, while in p58 the transmembrane region included only non-polar residues, in p50 it contained the charged amino acid Lys. Moreover, whereas p58 displayed a 76–84 amino acid cytoplasmic tail containing two YxxL motifs ($D/Ex_8D/ExxYxxLx_{26}YxxL$), later referred to as immunoreceptor tyrosine-based inhibitory motifs (ITIMs), p50 was characterized by a shorter tail (39 amino acids) lacking YxxL (64–66). The cDNA encoding the Bw4 specific receptors, named NKB1/p70, was also identified, revealing that its extracellular region was composed by 3 Ig-like domains and that its cytoplasmic tail included ITIMs (65, 67). All the genes coding for these receptors were mapped on chromosome 19q13.4. Concomitantly, cloning of CD94-encoding gene revealed that CD94 is a type II protein of the C-type lectin superfamily and that this gene is located on chromosome 12 (68). NK cell clones expressing CD94^{bright} phenotype did not lyse cell lines transfected with different HLA class I alleles, and their cytotoxicity could be restored by mAb-mediated masking of CD94 or HLA class I (69). Moreover, a functional ambivalence of CD94-associated surface antigens was described (see below) (70). Finally, an additional inhibitory NK receptor of the Ig-like superfamily was identified to be specific for HLA-A*03 and -A*011 allotypes (71, 72). In our laboratory the production of Q66 mAb allowed the characterization of this receptor, which appeared to be a disulphide-linked dimer and was termed p140. Importantly, KIRs (and NKG2A) were also detected in a subset of CD8^{pos} αβ T cells (73) in which they could interfere with TCR-mediated cell activation. These T cells were found to represent oligoclonally or monoclonally expanded cell populations (74). Subsequent studies revealed that such KIR^{pos} CD8^{pos} cells were HLA-E restricted (75, 76).

All these data, published between 1990 and 1996, represent true milestones for our present knowledge of the HLA specific receptors. Alessandro pioneered these studies with the discovery of p58 receptors and gave a fundamental contribution to most of the other major findings. Tracking all the rational steps leading to these discoveries in a seminal Alessandro's review, published in Annual Review in Immunology in 1996, has been for us both moving and impressive (7). Indeed, his contributions still represent a complete and modern insight on NK cell biology.

KIR NOMENCLATURE

Different research groups started to work on the NK cell recognition of HLA class I molecules, therefore receptors and encoding cDNAs were named differently, so that the need for a common nomenclature arose. Thus, in late '90s, the acronym KIR (killer-cell inhibitory receptor) was proposed to indicate the members of this novel family (77). Then, following the experimental evidences that some KIR receptors were able to transduce activating signals, it was suggested to preserve the KIR acronym simply changing the meaning of "I" into "immunoglobulin-like." The KIR nomenclature was designed in order to reflect the structure and the function of the encoded molecules, as well as the nucleotide sequence similarity among the different *KIR* family members. Thus, the first 2 digits following KIR correspond to the number of the extracellular domains (2D and 3D), while the third digit provides information on the length of the cytoplasmic tail (L or S) and consequently reveals the protein function (inhibitory or activating, respectively). Moreover, the two pseudogenes are indicated with the letter "P" (namely, *KIR2DP1* and *KIR3DP1*) (78). The establishment of a centralized, publicly accessible KIR database (IPD-KIR), collecting all the officially recognized nucleotide and protein sequences, has been of great help to all the immunologists involved in the study of *KIR* gene polymorphism (79). Temporally, the first KIR Nomenclature report coincided with the first release of the IPD-KIR database. Finally, like other immune cell surface proteins, a CD number has been assigned to KIR proteins (namely the CD158 series).

Reconnecting with what has been described in the previous paragraph, it is possible to attribute the KIR nomenclature to the molecules with the "old" denomination. Thus, p58.1 and p50.1 correspond to KIR2DL1 and KIR2DS1, p58.2 and p50.2 to KIR2DL2/L3 and KIR2DS2, p50.3 to KIR2DS4, NKB1/p70 to KIR3DL1, and p140/NKAT4 to KIR3DL2.

KIR GENES DISPLAY AN EXTRAORDINARY LEVEL OF POLYMORPHISM

KIR gene family is characterized by an extraordinary high degree of diversity, which arises from variability in *KIR* gene content, due to differences in both

presence/absence (P/A) of *KIR* genes and *KIR* gene copy number, and from allelic polymorphism (80–89). Several strategies have been developed to analyze *KIR* repertoires, including approaches able to analyze P/A of the different *KIR* genes, *KIR* gene copy number variations, and, more recently, a "whole" *KIR* allele typing by next generation sequencing (NGS) approach (87, 90–100) underlining the huge interest of the immunologists in the KIR field.

KIR Haplotypes

As previously mentioned, *KIR* gene family has been mapped on chromosome 19q13.4 where *KIR* genes, each spanning ∼10–16 kb, are tightly arranged in a head-to-tail orientation. This family consists of 13 genes (*KIR2DL1*, *KIR2DL2/L3*, *KIR2DL4*, *KIR2DL5A*, *KIR2DL5B*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1/S1*, *KIR3DL2*, *KIR3DL3*) and 2 pseudogenes (*KIR2DP1*, and *KIR3DP1*) (82, 101, 102). With few exceptions, *KIR* haplotypes can be divided in two regions, termed centromeric (Cen) and telomeric (Tel), by the presence of a recombination hot spot (86, 87, 89). Four genes, named framework genes (i.e., *KIR3DL3*, *KIR3DP1*, *KIR2DL4*, and *KIR3DL2*), mark the beginning and the end of these two regions. Based on the different number and kind of *KIR* genes present, three different centromeric regions (referred to as Cen-A, Cen-B1, and Cen-B2) and two different telomeric regions (namely Tel-A and Tel-B) have been identified (**Figure 1A**). Despite the high level of *KIR* gene content variability described, two main groups of *KIR* haplotypes, termed as "A" and "B," have been identified (46, 80, 87, 102–106). The A haplotypes have a fixed gene content and are composed by Cen-A and Tel-A regions (**Figures 1A,B**). They include inhibitory *KIR* genes coding for receptors recognizing HLA-C (*KIR2DL1* and *KIR2DL3*), HLA-B (*KIR3DL1*), HLA-A (*KIR3DL2*), and HLA-G (*KIR2DL4*) molecules and only one activating *KIR* (*KIR2DS4*) that, in some A haplotypes, does not code for a surface receptor (102). The remaining *KIR* haplotypes are collectively referred to as B haplotypes, and comprise haplotypes carrying Cen-A/Tel-B, Cen-B/Tel-A, and Cen-B/Tel-B combinations (**Figure 1B**). They are characterized by the presence of at least one of the following *KIR* genes: *KIR2DS2*, *KIR2DL2*, *KIR2DL5B*, *KIR2DS3*, *KIR3DS1*, *KIR2DL5A*, *KIR2DS5*, and *KIR2DS1*. Consequently, *KIR* A haplotypes differ from each other predominantly on the basis of *KIR* allelic polymorphism, while *KIR* B haplotypes mainly by their gene content. Notably, although with different frequencies, A and B haplotypes are maintained within all human populations, strongly suggesting that they have distinct and complementary functions and that they are under balancing selection (46, 107, 108). Moreover, the peculiar structure of *KIR* locus, including the presence of several genes sharing exon/intron organization, having a high degree of homology, and displaying the same orientation, facilitates non-reciprocal recombination that promotes deletion or duplication of *KIR* genes (i.e., formation of truncated or extended haplotypes), as well as the generation of hybrid *KIR* genes (i.e., *KIR* alleles including exons derived by different *KIR* genes) (**Figure 1C**) (86, 87, 109–113).

KIR Allelic Polymorphism

As previously reported, the first *KIR* cDNA sequences have been cloned in mid-90s. The great interest in the *KIR* field led to the identification, at the present, of ∼1,000 alleles (https://www.ebi.ac.uk/ipd/kir/). This extremely high level of polymorphism, second only to that of *HLA* genes, arose from studies including different populations and relatively simple methodologies. Several allelic polymorphisms have been reported to impact KIR protein expression and function. Indeed, the presence of particular amino acid residues determining protein misfolding and resulting in the lack of surface receptor expression has been identified among KIR3DL1, KIR2DL1, and KIR2DL2 allotypes (95, 114–117). Moreover, *KIR* alleles characterized by a termination codon causing the premature end of the protein (namely *KIR* Null alleles) have been reported. Additionally, some *KIR2DL4* and *KIR2DS4* alleles are characterized by nucleotide deletions that, causing frameshift, lead to truncated polypeptides (102, 118, 119). Notably, different clades of alleles coding for surface receptors characterized by a low or high surface expression have been detected in *KIR3DL1* and *KIR2DL1* genes (95, 120, 121). Polymorphisms affecting KIR function may also cause differences in ligand affinity or in signal transduction. In this regard, it is well-established that KIR2DL1 allotypes coded by Cen-A regions are stronger receptors than those coded by Cen-B1 regions, and KIR2DL2 allotypes (coded by Cen-B regions) have a higher ligand affinity than KIR2DL3 allotypes (coded by Cen-A regions) (116, 122). In addition, the amino acid polymorphisms of KIR3DL1, the KIR receptor displaying the highest level of allelic variation, have been shown to impact not only its surface expression but also its affinity for HLA class I Bw4 bearing allotypes (123–126). Different amino acidic variations relevant for KIR signal transduction have been also reported in the exons coding for the transmembrane and cytoplasmic regions. In particular, the lack of charged residues, important for its association with signal-transducing polypeptides, has been described for a KIR2DS2 allele (127). In *KIR2DL1*, polymorphisms in the exons of the cytoplasmic tail, either causing the premature termination of translation at the end of the transmembrane domain or affecting the strength of inhibitory signal, have been reported (128, 129).

Thus, the extraordinary plasticity of the *KIR* gene family and the variability due to both *KIR* gene content and allelic polymorphism make unlikely that unrelated individuals share the same *KIR* genotype.

KIR/KIR-LIGAND PAIRS

As mentioned above, the HLA class I ligands recognized by KIR2DL1, KIR2DL2/L3, KIR3DL1, and KIR3DL2 have been identified almost conjointly with the characterization of the receptors. Thereafter, the resolution of the crystal structures of KIRs in complex with their ligands, the development of several tools including soluble KIRs, KIR tetramers, HLA class I tetramers folded with selected peptides, and retrovirally transduced human cell lines (i.e., NKL, Jurkat) expressing only one KIR, allowed to refine KIR/KIR-ligand interactions (122, 130–134).

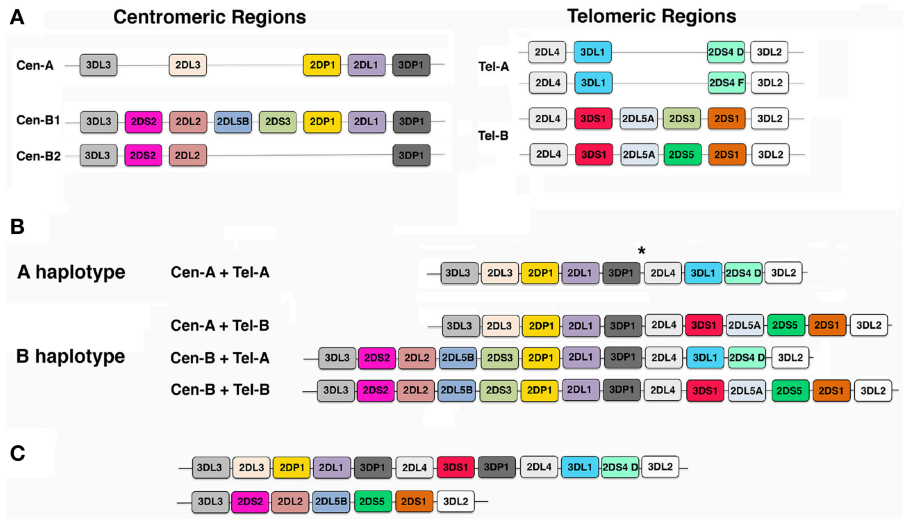

FIGURE 1 | Gene organization of *KIR* locus. **(A)** A cartoon representation of the most frequent centromeric (Cen) and telomeric (Tel) regions detected in Caucasians. **(B)** Schematic pictures of *KIR* gene order in the A haplotypes and in 3 representative B haplotypes. * indicates the hot spot of recombination. **(C)** Examples of an extended and a truncated haplotype. Each colored box represents a *KIR* gene; for simplicity *KIR* gene names are reported in the boxes without the *KIR* acronym.

Inhibitory KIRs

In **Figure 2,** the molecular structure of the inhibitory KIRs (iKIRs) and of their known cellular ligands is summarized. Using KIR2DL1-Fc (i.e., chimeric molecules comprising the extracellular portion of the receptor linked to the Fc region of human IgG1) in multiplex HLA class I binding assays, it has been possible to demonstrate that KIR2DL1 binds specifically, and with high avidity, to all HLA-C C2 but not HLA-C C1 or any HLA-A or HLA-B allotypes. The only exception is represented by KIR2DL1*022, a peculiar KIR2DL1 receptor carrying Lys^{44} (instead of Met^{44}) that switches its ligand specificity to HLA-C C1 (129). By the use of KIR2DL1-Fc and plasmon resonance, it has been shown that several KIR2DL1 molecules recognize distinct HLA-C C2 allotypes with different avidities (the highest for HLA-C*15:02 and the lowest for HLA-C*04:01). As reported in early studies, and as expected on the basis of the homology in their extracellular domains, KIR2DL2 and KIR2DL3 bind a similar set of HLA-C ligands. Again, using KIR-Fc molecules it has been confirmed that KIR2DL2 and KIR2DL3 allotypes recognize, at high affinity, HLA-C C1 bearing epitope, and it has been demonstrated their ability to bind to two HLA-B allotypes (i.e., HLA-B*46:01 and HLA-B*73:01) that, being originated by crossing-over between HLA-C and HLA-B alleles, conserve the C1 epitope. Moreover, KIR2DL2 and KIR2DL3 display the capability to bind HLA-C C2 allotypes, even though with low affinity. Thus, in contrast to the simplicity of KIR2DL1 ligand recognition, a more complicated situation governs KIR2DL2 and KIR2DL3 ligand interactions (116, 122). The only known exception is represented by KIR2DL3*005 that, differing from other KIR2DL3 receptors, displays a HLA-C binding ability similar to that observed for KIR2DL2 allelic products (135). Increasing evidences indicate that both KIR2DL2/L3 and KIR2DL1, in addition to their ability to discriminate between C1 and C2 epitopes, bind numerous peptide/HLA-C combinations retaining a degree of peptide selectivity (136). Generally, KIR2DL2 and KIR2DL3 are characterized by a greater selectivity for peptide than KIR2DL1, and this ability seems to be particularly relevant for the recognition of the low affinity HLA-C C2 allotypes. Notably, the capability of KIR2DL1 to recognize HLA-C*08:02 (i.e., a HLA-C C1 allotype) presenting a restricted number of peptides has been recently reported (137). Taken together these studies underline how NK cells are able to sense, not only the down-regulation of HLA class I, but also alterations in HLA presented peptidome that may occur during viral infections or malignant transformations.

Between the two D0-D2 receptors, KIR2DL4 is the only one for which the ligand has been characterized. Differently from other iKIR2D receptors, KIR2DL4 binds to HLA-G, a non-classical HLA class I molecule that, in healthy cells, is restricted to the trophoblast cells invading the maternal decidua during early pregnancy. This receptor contains only one ITIM in the long cytoplasmic tail and a charged residue in the transmembrane region allowing its association with the γ chain of FcεR. These features render this receptor a peculiar member of the KIR family displaying an unusual hybrid structure and sharing characteristics with both inhibitory and activating KIRs. Functional studies revealed that KIR2DL4 is characterized by a weak inhibitory potential and that its engagement with soluble ligand results in strong cytokine release in the absence of killing (138, 139).

It is well-established that KIR3DL1 binds not only to HLA-B but also to some HLA-A Bw4 bearing allotypes. Indeed, it has been reported that among $Bw4^{pos}$ HLA-A allotypes, HLA-A*24:02, -A*32:01, -A*23:01, but not HLA-A*25:01, are able not only to inhibit but also to educate $KIR3DL1^{pos}$ NK cells (140). Among the five residues of α1 helix defining the Bw4 motif,

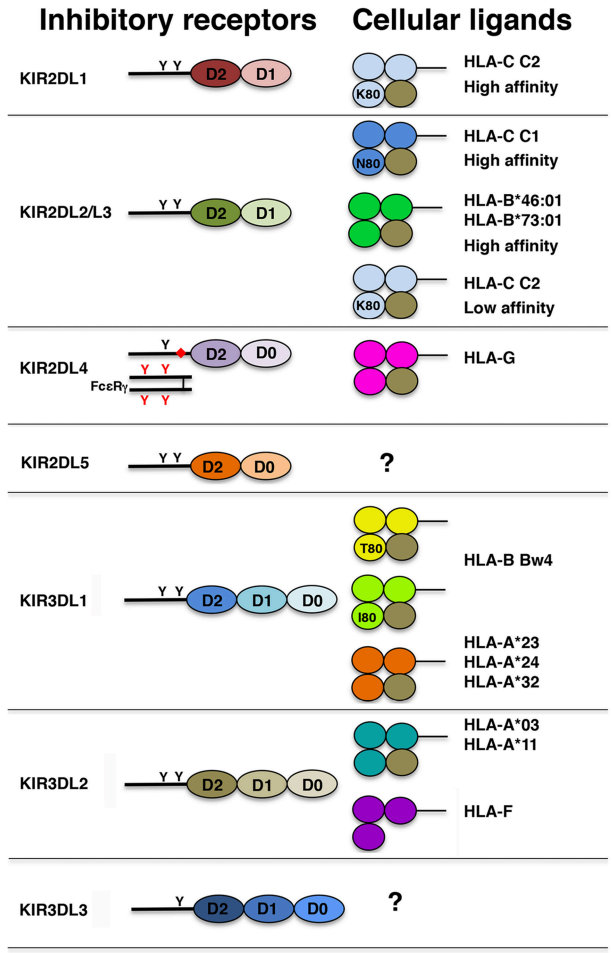

FIGURE 2 | Schematic representation of the structure of iKIRs and their HLA class I ligands. Each Ig domain is depicted as an oval and, according to its structural features, labeled as D0, D1, and D2. Presence of ITIMs in KIR cytoplasmic tails and ITAMs in FcεR γ chain is indicated with black and red Y, respectively. The charged residue in KIR2DL4 transmembrane region is reported as a red diamond. Each domain of HLA class I molecules is represented as a colored circle and β2m as a brown circle. In HLA-C and HLA-B molecules, K80, N80, T80, and I80 indicate the presence, at residue 80, of lysine, asparagine, threonine, and isoleucine, respectively.

the Ile/Thr dimorphism at residue 80 has been proposed as a marker of KIR3DL1 ligand affinity. Recent studies clearly indicate that, although many high affinity ligands possess Ile^{80} (Bw4 I^{80}), KIR3DL1/HLA $Bw4^{pos}$ allotype interactions are very complex and strongly dependent on the avidity and surface expression of both receptor and ligand. Indeed, functional analyses provided clear evidences that both KIR3DL1 polymorphism and ligand variability strongly affect the avidity of KIR/KIR-L combinations sharply influencing NK cell function (123, 126, 141). As previously mentioned, KIR3DL2 allotypes bind two HLA-A allotypes (HLA-A*03 and -A*11) (71, 72). Functional analyses revealed that this receptor is characterized by low inhibitory capacity and its ligand interaction is highly dependent on the HLA-A bound peptide (132). Recently, it has been demonstrated that KIR3DL2 also recognizes the non-classical HLA class I molecule HLA-F at open conformation (142). Another relevant function of this iKIR is represented by its ability to function as sensor for pathogen-associated molecular patterns. Indeed, KIR3DL2 has been shown to bind CpG oligodeoxynucleotides (ODNs), and its D0 domain is primarily involved in ODN recognition (143). Remarkably, interaction between KIR3DL2 and ODN did not result in the delivery of inhibitory signals but in a sharp down-modulation of its surface expression, and in the induction of cytokine release underlining the antimicrobial role of NK cells in the course of infection.

Activating KIRs

From a structural point of view, in addition to a short cytoplasmic tail lacking ITIMs, a common feature of activating KIR (aKIRs) is the presence, in their trasmembrane region, of a charged residue (Lys) that allows their association with the signaling adaptor protein KARAP/DAP12 containing immunoreceptor tyrosine-based activating motifs (ITAMs) (144, 145). With the exception of KIR2DS1, the aKIR ligands remained elusive for a long time. Indeed, although the extracellular domains of some aKIRs display a high sequence homology with those of some iKIRs (namely, KIR2DS1-KIR2DL1, KIR2DS2-KIR2DL2, and KIR3DS1-KIR3DL1 pairs), it has been difficult to demonstrate aKIR/HLA class I interactions. **Figure 3** shows the different aKIR/ligand pairs identified so far.

The best characterized aKIR is KIR2DS1. Functional analyses using *in vitro* expanded $KIR2DS1^{pos}$ NK cell clones, as well as KIR2DS1 soluble receptor and KIR2DS1 tetramer binding analyses, clearly demonstrated that this aKIR recognizes HLA-C C2 allotypes, although with an affinity lower than that of KIR2DL1 (i.e., its inhibitory counterpart) (62, 129, 146–149). Moreover, using both site-directed mutagenesis approaches and soluble receptors, it has been established that the residue at position 70 (Lys^{70} or Arg^{70}) sharply affects the ligand affinity of KIR2DS1 molecules (146). Notably, a recent paper reported that modulation of HLA-C by certain strains of HCMV is required for a potent KIR2DS1-mediated NK cell activation. This strongly suggests a role of this aKIR in viral recognition (150). The HLA class I ligands recognized by KIR2DS2 have been identified only recently, and they include HLA-C C1 allotypes and HLA-A*11:01 (133, 147, 151). Remarkably, this aKIR/KIR-L interaction is highly peptide-dependent. In particular, the capability of KIR2DS2 to directly recognize peptides from HCV helicase presented by HLA-C*01:02 (an HLA-C C1 allotype) may explain, at least in part, the protective effect of this aKIR in chronic hepatitis C infection (152). In addition, it has been also reported that KIR2DS2 recognizes, on several human carcinoma cell lines, a still undefined, β2m-independent cell surface protein (153). Correlative studies reported that HIV-infected individuals carrying both KIR3DS1 and HLA-B Bw4 I^{80} display slower progression to AIDS and lower viral load. While these data could suggest KIR3DS1 interaction with HLA-B Bw4 I^{80} allotypes, the KIR3DS1 ligands have remained indefinite until recently, and they are represented by the open conformation of the non-classical HLA-F molecule and by the HLA-B*51, an HLA-B Bw4 I^{80} allotype (154–156).

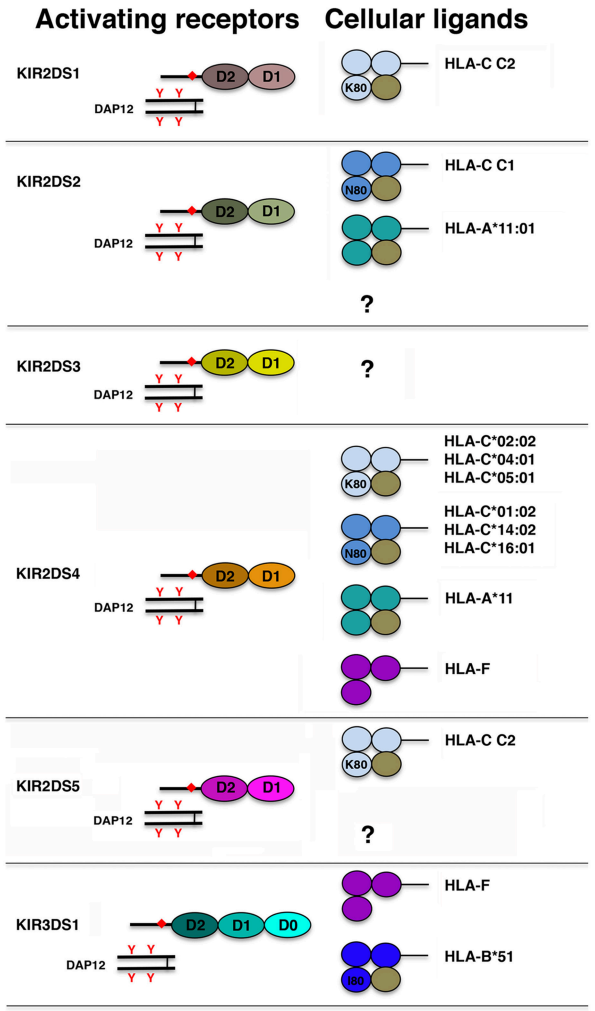

FIGURE 3 | Schematic representation of the structure of aKIR and their HLA class I ligand. Each Ig domain is depicted as an oval and, according to its structural features, labeled as D0, D1, and D2. Presence of a charged residue in the KIR transmembrane region and of ITAMs in DAP12 cytoplasmic tail is indicated with orange diamond and orange Y, respectively. Each domain of HLA class I molecules is represented as a colored circle and β2m as a brown circle. In HLA-C and HLA-B molecules, K80, N80, and I80 indicate the presence, at residue 80, of lysine, asparagine, and isoleucine, respectively.

KIR2DS3, KIR2DS4, and KIR2DS5 have no inhibitory counterparts. Most likely because KIR2DS3 does not seem to be expressed at the cell surface (157), it is still a receptor with unknown function and unknown ligand. On the contrary, the majority of KIR2DS4 and KIR2DS5 alleles code for surface activating receptors (63, 158). KIR2DS4 binds a minority of HLA-C allotypes carrying either C1 or C2 epitopes as well as HLA-A*11 (159). Recently, a highly peptide dependent ligand recognition has been reported also for KIR2DS4. This aKIR has been shown to recognize HLA-C*05:01 presenting a peptide derived from the highly conserved bacterial recombinase A, thus providing evidence that it is involved in defense against bacterial infections (160).

As previously reported, KIR2DS5 can be located either in the centromeric half of some KIR haplotypes (i.e., Cen-B1 region) or in the telomeric half (namely Tel-B region). With the only exception of KIR2DS5*005, KIR2DS5 centromeric alleles differ from the telomeric ones. Notably, four out of five KIR2DS5 centromeric alleles but only two out of six KIR2DS5 telomeric alleles code for receptors recognizing several HLA-C C2 molecules (161).

NK CELL EDUCATION AND RECEPTOR REPERTOIRE

During their development, NK cells go through a process termed "education," involving the interaction between inhibitory NK receptor (iNKR) and self HLA class I molecules. Although different models have been proposed, a general rule for education is that the strength of inhibitory interaction(s) dictates the efficiency of NK effector function (12, 13, 162, 163). A prominent role is played by the highly diversified system represented by the combinations between inhibitory KIR with their KIR-L (iKIR/KIR-L pairs). Another relevant iNKR is represented by the CD94:NKG2A heterodimer, a type II protein belonging to the C-type lectin superfamily, which recognizes the non-classical HLA class I molecule HLA-E (10). Since HLA-E binds peptides cleaved from the leader sequences (from −22 to −14 residues) of HLA-A, -B, or -C, it has been generally considered as a sensor of the overall amount of HLA class I molecules expressed on the cell surface. Recently, the Met/Thr (M/T) dimorphism at position −21 of the leader sequence of HLA-B (i.e., −21M and −21T) has been described to strongly impact the CD94:NKG2A/HLA-E interaction (164). Indeed, only a minority of HLA-B allotypes having −21M can supply HLA-E binding peptides, while the majority carrying −21T do not. Accordingly, individuals with −21M HLA-B could show higher HLA-E expression and more efficient NKG2A^{pos} NK cells. The activating counterpart of CD94:NKG2A is represented by CD94:NKG2C heterodimer, which binds HLA-E as well, although with lower affinity.

Extremely variegated NK cell receptor repertoires can be observed among different individuals (7, 60, 80, 164–167). This diversity is primarily due to the high polymorphism of *KIR* and *HLA* class I genes, which segregate independently, leading to diverse compound genotypes (46). Another important feature of the repertoire is the clonal distribution of KIR and CD94:NKG2A. During education NK cells must engage at least one iNKR with cognate HLA class I to become fully functional, otherwise, they will be hypo-responsive. This process ensures that each NK cell produced in the body maintains tolerance to self. The hypothesis that NKR expression represents the result of a stochastic event, in which the various encoding genes are independently regulated, had been already discussed in Alessandro's review (7). From studies of NK cells at the clonal level, it was clear that each NK cell could express either one or more iNKR specific for self HLA (self-iNKR) as well as additional iNKR specific for non-self HLA. The presence of NK cells expressing a single self-iNKR confers an advantage to the host, since it allows to sense the loss or peptide-induced alteration

of even a single HLA class I allotype, and thus to kill the damaged cell, through the mechanism of "missing self-recognition."

While a strong interaction between iKIR and its KIR-L positively impacts NK cell education, the behavior of aKIR is opposite and induces down-regulation of NK cell responsiveness in the presence of a strong cognate ligand. This phenomenon has been described for KIR2DS1 and KIR3DS1. Thus, KIR2DS1^{pos} NK cells are anergic in HLA-C C2/C2 individuals, while they are educated in C1/Cx ones (168). Similarly, KIR3DS1-mediated positive recognition of HLA-B*51 (Bw4 I^{80}) could be detected in NK cell clones derived from Bw4 I^{80} negative donors, but not in those from Bw4 I^{80} positive donors (156).

Although genetics of *KIR* and *HLA* has the major impact on the receptor repertoire of the circulating NK cell pool, also environmental factors may play an important role. In this regard, the phenotypic analysis of NK cells derived from twins, who are genetically identical for *KIR* and *HLA* genes, has been particularly relevant (169). Indeed, twins' NK cell populations are similar but not identical to each other, especially in adult age. In this context, viral infections may impact the NK cell phenotype. In particular, HCMV shapes the receptor repertoire, driving to adaptive NK cell differentiation through epigenetic alterations (170–173). Thus, the expanded NK cell subset is characterized by the expression of CD94:NKG2C, mainly co-expressing KIR2DL specific for self HLA-C allotypes, and CD57, a marker of terminally differentiated stage (32, 33). Recent data revealed that the expansion and differentiation of adaptive NKG2C^{pos} NK cells could be determined by differentially recognition of distinct HCMV strains encoding variable UL40 peptides (174).

Nowadays, new technologies allow the identification of distinct subsets by the detection of multiple molecules in the whole NK cell population, even freshly derived from blood. Multi-parametric flow cytometry makes it possible the simultaneous use of numerous mAbs, detecting the pattern of different subpopulations carrying one or another iKIR, aKIR, CD94:NKG2A, and CD94:NKG2C. **Table 1** describes the reactivity of some anti-KIR mAb, that can be used in combination to dissect various iKIR and aKIR. Moreover, mass cytometry by time-of-flight (CyTOF) technology allows the concomitant assessment of more than 30 parameters, revealing that at least 30.000 and even more distinct PB NK phenotypes can be detected in each individual (169).

NK cell education in pregnancy or in disease and the particular KIR/HLA combinations associated with either a protective role or a detrimental effect in placentation, infectious diseases, autoimmune and inflammatory diseases, and in cancer have recently been reviewed (46–48, 136, 175). Our group studied NK cell education in disease, particularly focusing on leukemia patients receiving HSCT (see below) or patients with X-linked lymphoproliferative disease 1 (XLP1). XLP1 is a primary immunodeficiency caused by mutations in *SH2D1A*, the gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP). Seminal studies coordinated by Alessandro provided evidence that, in the absence of SAP, 2B4 and NTB-A (belonging to SLAM family) co-receptors associate with protein tyrosine phosphatases thus delivering inhibitory instead of activating signals (22, 24, 176). This specific immune dysfunction in XLP1 patients causes the inability of NK cells to kill EBV-infected B cells (B-EBV), over-expressing their ligands (i.e., CD48 and NTB-A), with dramatic clinical consequences. Following this knowledge, we further analyzed the NK cell receptor repertoire of these patients, showing that substantial proportions of NK cells lacking any inhibitory receptor specific for self-HLA (self-NKR^{neg}) are present and are fully functional, indicating that inhibitory 2B4 participates to NK cell education (177). Remarkably, self-NKR^{neg} NK cells can efficiently kill CD48^{neg} target cells, such as mature DC, with consequently defective antigen presentation, further exacerbating the immune defect of these patients.

TABLE 1 | Some monoclonal antibodies recognizing KIR.

Clone	Specificity	Isotype	Source[§]
143211	KIR2DL1/S5	IgG1	R&D
EB6B	KIR2DL1/S1, KIR2DL3*005	IgG1	Our lab, Beckman Coulter
11PB6	KIR2DL1/S1, KIR2DL3*005	IgG1	Our lab, Miltenyi Biotec
HP-3E4	KIR2DL1/S1/S4	IgM	BD Biosciences
HP-MA4	KIR2DL1/S1/S3/S5	IgG2b	Biolegend, eBiosciences
GL183	KIR2DL2/L3/S2	IgG1	Our lab, Beckman Coulter
Y249	KIR2DL2/L3/S2	IgM	Our lab
CH-L	KIR2DL2/L3/S2	IgG2b	BD Biosciences
DX27	KIR2DL2/L3/S2	IgG2a	Biolegend, Miltenyi Biotec
ECM-41	KIR2DL3 (not *005 and *015)	IgM	Our lab
180701	KIR2DL3 (not *005 and *015)	IgG2a	R&D
1F12	KIR2DL3/S2	IgG2b	C. Retière ^
FES172	KIR2DS4	IgG2a	Our lab, Beckman Coulter
PAX180	KIR2DS4	IgG1	Our lab
UP-R1	KIR2DL5	IgG1	Biolegend
DF200	KIR2DL1/L2/L3/S1/S2/S5	IgG1	Our lab
NKVSF1	Pan KIR2D	IgG1	Miltenyi Biotec
Z27	KIR3DL1/S1	IgG1	Our lab, Beckman Coulter
DX9	KIR3DL1	IgG1	Miltenyi Biotec
Q66	KIR3DL2	IgM	Our lab
AZ158	KIR3DL1/S1/L2	IgG2a	Our lab

[§] *The names of the vendor and/or the lab of production is indicated. "Our lab" means the laboratories directed by A. Moretta, L. Moretta and M.C. Mingari.*
^ *C. Retiére: Etablissement Français du Sang-Pays de la Loire, Nantes, France.*

INHIBITORY CHECKPOINTS IN NK CELLS AND THEIR TARGETING IN TUMOR THERAPY

As previously reported, the HLA class I specific inhibitory receptors are constitutively expressed by NK cells and finely regulate their function preventing NK-mediated damage to healthy tissues and allowing the elimination of tumors displaying defective HLA class I expression. Additional inhibitory checkpoints are inducible and play a wider physiological role in immune cell homeostasis. On the other hand, in tumors, they may severely compromise NK-mediated responses and promote tumor growth and metastasis. In particular, CTLA-4 and PD-1 are major regulators of immune responses and

contribute to the maintenance of peripheral T cell tolerance. PD-1, a member of the Ig-superfamily, was first identified in T lymphocytes (178) and, more recently, in NK cells (44). In cancer, upon binding to its ligands (PD-L1 and/or PD-L2) expressed on tumor cells, PD-1 may impair the antitumor cytotoxicity, thus favoring tumor immune escape. Human PD-1^{bright} NK cells have been detected in patients with CMV infections and tumors (44, 179). For example, in ovarian carcinoma patients, a sizable fraction of PD-1^{pos} NK cells can be detected in PB, while larger percentages are present in the ascitic fluid of the same patients. Although PD-1 mRNA and protein are detectable at the cytoplasmic level, the mechanisms leading to its surface expression in human NK cells have not been defined yet. The detection of PD-1^{pos} in NK cells associated to tumors suggests that signals delivered by the tumor or its microenvironment may be involved in its surface expression. Besides PD-1, several other inhibitory checkpoints expressed by NK cells have been detected, and have been recently reviewed (180). Briefly, CD96 and TIGIT belong to the same family of the activating receptor DNAM-1 (their common ligands, CD155 and CD112, are nectin-like molecules). TIGIT was found to be up-regulated in tumor-associated NK cells in colorectal cancer. KLRG1 is expressed by NK cells upon activation (as well as by other lymphoid and myeloid cell types). LAG-3 marks exhausted T cells infiltrating tumors, while its possible modulatory effect on NK cells has not been defined yet. TIM-3 binds primarily to Galectin-9 but also to other tumor-associated antigens. A recently identified inhibitory receptor which is likely to play an important role in controlling NK cell activation, proliferation and function is IL-1R8, a member of IL-1 receptor family and a component of the human IL-37 receptor. Mice lacking IL1R8 do not develop carcinogen-induced hepatocarcinoma (181). Thus, it is conceivable that IL-1R8 blocking may unleash NK cells and promote efficient antitumor responses.

PD-1/PD-L1 and Their Targeting in the Therapy of Cancer

As mentioned above, PD-1 may impair T cell responses against tumor cells. Importantly, immunotherapy with mAbs able to disrupt the PD-1/PD-L1 interaction proved to be highly effective, allowing unthinkable achievements in the cure of a fraction of tumor patients. In this context, the number of mAbs specific for PD-1 or PD-L1 approved for cancer therapy is growing fast and the spectrum of tumors for which the use of such agents is recommended includes non-small lung cancer, melanoma, urothelial cancers, kidney tumors, Hodgkin lymphoma, colorectal cancer, and hepatocellular carcinoma. While the use of such checkpoint inhibitors represents a real revolution in cancer treatment, in a still too large fraction of patients these agents are ineffective. Accordingly, a major goal is to predict the patient response to treatment, to avoid possible side effects and useless high costs of the therapy. The expression of PD-L1 on tumor cells undoubtedly represents a valuable biomarker. However, its predictive value is still unsatisfactory because of various limitations, including inter- and intra-tumor heterogeneous PD-L1 expression, use of PD-1/PD-L1 specific mAbs displaying a substantially different reactivity (182), different diagnostic material (biopsies vs. surgical specimens). Patient selection criteria are being standardized, for example, by defining the optimal number of biopsies that give results comparable to the whole tumor section, considered as the gold standard (183, 184). Given these limitations, research in progress is aimed at identifying additional checkpoints possibly to be used alone or in combination with PD-1/PD-L1 or CTLA-4.

Therapeutic Use of Anti-KIR or Anti-NKG2A mAbs

After the launch of Innate-Pharma in Marseille, Alessandro's antibodies and his expertize were fundamental for the design of a program focalized on NK cells harnessing the blockade of inhibitory KIRs and NKG2A. Thus, three clinical grade mAbs were produced: Lirilumab (also called IPH2101, formerly 1-7F9) targeting KIRs, IPH4102 selectively recognizing KIR3DL2, and Monalizumab targeting NKG2A. These reagents are currently used in clinical trials to treat cancer patients. Thus, Lirilumab, a first-in-class humanized IgG4 mAb directed against a common epitope shared by KIR2D, disrupts the KIR/KIR-L interaction by rendering NK cells "alloreactive" and allows killing of tumor target cells. This antibody has been used in a phase I trial in association with Lenalidomide, for NK stimulation, to treat Multiple Myeloma. This combined treatment resulted to be safe, tolerable, and associated with signs of clinical efficacy (185). Because KIR3DL2 is highly expressed on cutaneous T cell lymphomas, including mycosis fungoides and Sézary syndrome (186), IPH4102 has been developed for the treatment of these diseases. Phase I trials provided evidence that the treatment is well-tolerated and efficacious. These encouraging results would support the large-scale clinical trials (187).

Recent studies identified NKG2A as an important checkpoint leading to remarkable antitumor effects in animal models (188–190). Encouraging results have also been obtained in a preliminary clinical trial in highly aggressive head and neck cancers (188). Notably, NKG2A, constitutively expressed by NK cells, can be induced also in T cells upon cytokine or antigen-induced activation (191, 192). Indeed, previous *in vitro* studies showed that mAb-mediated masking of NKG2A or KIRs, in both polyclonal and clonal NK or T cell populations, induced killing of HLA class I^{pos} tumor target cells (149, 188, 193–195). Vivier group also showed that the antitumor effects of anti-NKG2A mAb could be potentiated by the combined use of other therapeutic mAbs (188). Based on the expression of inhibitory checkpoint ligands on tumor cells, three therapeutic approaches have been proposed: (1) HLA-E^{pos} tumors lacking the expression of other ligands for inhibitory checkpoints or tumor antigens: blocking of NKG2A could unleash both NK- and T-cell-mediated antitumor responses in a murine model; (2) tumors expressing both HLA-E and PD-L1: by the combined blocking of NKG2A and PD-1/PD-L1 axis, not only enhancement of NK and T cell cytotoxicity but also induction of T cell proliferation and establishment of T cell memory were detected; (3) tumors

expressing HLA-E and specific tumor antigens (e.g., EGF-R): blockade of NKG2A increased the efficacy of anti-EGF-R by allowing a CD16-mediated efficient ADCC by mature, highly cytotoxic NK cells.

KIR AND HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRASPLANTATION

The discovery of both HLA class I specific inhibitory receptors and NK cell alloreactivity has been exploited, after a relatively short time interval, for the treatment of acute, high-risk leukemia in the haplo-HSCT setting. HSCT is the life-saving therapy for such leukemia with adverse molecular or cytogenetic characteristics, or with poor response to chemotherapy, or relapsing. However, an HLA 10/10 allelic matched donor, whether sibling or unrelated, is available only for two out of three patients, and this proportion can be even lower for patients belonging to certain ethnic groups. Thus, haplo-HSCT has been developed in the attempt to rescue these patients (196). In such transplantation setting, donor derived NK cells are defined as "alloreactive" when they express, as inhibitory receptor, exclusively KIR(s) specific for self-HLA class I allele(s) (KIR-L), allowing the elimination of patient cells missing that particular KIR-L. A seminal study by Ruggeri et al. (197) reported a 5-year survival probability of ∼60% in adult AML patients in case of donor NK alloreactivity (KIR/KIR-L mismatch in graft vs. host direction), while survival was <5% in its absence. In 2002, Alessandro and some authors of the present contribution started a fruitful collaboration with Franco Locatelli, first at the University of Pavia and subsequently at the Ospedale Pediatrico Bambino Gesù in Roma (149, 198). In these studies, patients were represented by children and young adults (1–20 years) with acute high-risk ALL or AML. Notably, in the haplo-HSCT setting, most pediatric patients find an available donor, represented by one parent.

KIR-Based Donor Selection

In view of the major relevance of NK alloreactivity, a great attention was paid by our group to the selection of the best possible donor, based primarily on the "perfect mismatch" (199) and on the size of alloreactive NK cell subset. To this end, a reliable method was developed to define the presence and the frequency of such subpopulation in potential donors (149). This is based on combined genotypic and phenotypic analyses to assess (i) the presence in the donor of KIR-L(s) absent in the recipient, (ii) donor's *KIR* genotype to evaluate the presence of KIR(s) specific for the mismatched KIR-L(s), and (iii) identification of the alloreactive subset using appropriate anti-KIR and anti-NKG2A mAb combinations (198). Leukemia patients, after the conditioning regimen, receive very high numbers ("megadoses") of highly purified T-depleted $CD34^{pos}$ cells, isolated either from bone marrow (BM) or PB. Isolation from PB is preceded by donor treatment with G-CSF in order to "mobilize" $CD34^{pos}$ cells from BM. Notably, different from AML, adult high-risk ALL were poorly responding to haplo-HSCT even in the presence of donor NK alloreactivity. Thus, it was somewhat surprising to find opposite results in pediatric patients. Indeed, in case of NK alloreactivity, the survival rate was ∼70% in ALL and ∼40% in AML, while in the absence of NK alloreactivity, the percentages were ∼35 and ∼20%, respectively. Of note, in the transplantation setting with "pure $CD34^{pos}$" cells, most deaths due to leukemia relapses or transplant-related mortality occurred early, within few weeks/months after transplantation. As mentioned above, NK cells, during their differentiation from HSC, first express CD94:NKG2A, while KIR are acquired only at later stages. Accordingly, the generation of alloreactive, mature NK cells requires a relatively long time interval (6–8 weeks). Thus, for several weeks, the absence of mature (alloreactive) NK cells together with the absence of adaptive immunity may be critical for the control of leukemia relapses and infections. The survival rate of leukemia patients receiving a $CD34^{pos}$ haplo-HSC could be considered satisfactory in view of the extremely poor prognosis of patients not receiving transplantation. However, the delayed appearance of alloreactive NK cells, conceivably explaining the occurrence of early relapses and infections, suggested, in 2010, the application of a new graft manipulation based on the depletion of αβ T lymphocytes and $CD19^{pos}$ B lymphocytes. Thanks to this novel approach, the graft infused contains, besides HSC, other cell types, including intermediate precursors ($CD34^{neg}$), various myeloid cell types, mature NK cells, and γδ T lymphocytes. Notably, both NK and γδ T lymphocytes may exert a potent anti-leukemia activity and contribute to the control of infections. Accordingly, patients could immediately benefit of high numbers ($30–40 \times 10^6$/kg) of donor mature NK cells and γδ T cells ($3–5 \times 10^6$/kg). The clinical outcome in 80 children was excellent with an overall survival of ∼70% not only in ALL, but also in AML (200). Surprisingly, NK alloreactivity did not appear to play a relevant role in the clinical outcome. The marked improvement of the survival rate in the absence of alloreactivity may be due, at least in part, to the additional selection criteria progressively applied to identify the most suitable among the available donors. These include the following positive characteristics: (i) *KIR* B/X genotype, (ii) HLA-$C1^{pos}$/$KIR2DS1^{pos}$ for HLA-$C2^{pos}$ recipients, (iii) high absolute cell numbers of NK and γδT lymphocytes, and (iv) high expression of NKp46 and presence of NKG2C (198, 200). The presence of mature NK and γδ T from the beginning of the transplantation and their persistence in the recipient as efficient immune cells, due to the lack of GvHD prophylaxis, can explain the successful clinical results. In immunocompromised patients undergoing allogeneic HSCT, HCMV infection/reactivation frequently occurs (201). Several studies documented a role of HCMV in promoting rapid NK cell maturation and in the selective expansion of $NKG2C^{pos}$ adaptive NK cells (34, 202, 203). This HCMV related effect on NK cell repertoire was also observed in pediatric patients receiving αβ T/B cell-depleted haplo-HSCT (204). Remarkably, a study revealed that, in $NKG2C^{neg/neg}$ umbilical cord blood donors, HCMV infection promoted a maturation and expansion of NK cells expressing aKIRs (205). These findings suggest that, in the absence of NKG2C, aKIRs may sense HCMV

infection and promote selective expansion of mature aKIR^{pos} NK cells, underscoring the concept that aKIRs may sense microbial infections.

Taken together, the clinical results in haplo-HSCT indicate that cells belonging to innate immunity play a crucial role in the cure of patients with otherwise lethal leukemia and provide a solid background to further improve the survival rate and to implement novel NK-based therapies (e.g., CAR-NK). In this context, helper ILC, primarily ILC-3, may provide a tool to further improve early innate defense against pathogens after HSCT. Notably, it has been shown that ILC-3 development may be favored by given sources of HSC for transplantation (206).

CONCLUDING REMARKS

There is little doubt that major advances in immunology and the exploitation of fundamental discoveries offered invaluable tools for the therapy of cancer. Indeed, the recent years witnessed unprecedented successes in the cure of different, high aggressive tumors and leukemia. In this context, NK cells and their receptors were shown to play an unexpected role. Thus, the discovery of KIRs provided the molecular basis for haplo-HSCT, which allowed the cure of patients with acute, high-risk leukemia for whom no HLA-compatible donors were available. These data also provided the first evidence that not only T and B lymphocytes, but also cells of the innate immunity may be exploited for tumor therapy. While checkpoint inhibitors targeting PD-1 or CTLA-4 represent the most effective tools to successfully treat a fraction of otherwise incurable cancer patients, their combined use with therapeutic antibodies blocking NKG2A and KIR may further extend the benefit to a large proportion of patients. Thus, the Alessandro's legacy, besides unraveling the molecular mechanisms of NK cell function, also extends to thousands of leukemia patients recovered from their otherwise fatal disease.

The continuous interest and evolution on KIR research is also witnessed by the organization of a KIR Workshop taking place every year and a half, allowing to share the most recent advances on KIRs in health and disease, with a fruitful exchange of ideas and knowledge in a friendly atmosphere. Last October, the KIR Workshop 2018 has been organized by our group, and Alessandro had begun to collaborate with us on its organization and realization. This meeting was dedicated to Alessandro.

AUTHOR CONTRIBUTIONS

LM, DP, and MF wrote, referenced the review, and prepared the figures. All authors contributed with their relevant published studies, cited in this review, and critically revised the manuscript.

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frontiers in Immunology

MINI REVIEW
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PD/1-PD-Ls Checkpoint: Insight on the Potential Role of NK Cells

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The identification of inhibitory NK cell receptors specific for HLA-I molecules (KIRs and NKG2A) provided the molecular basis for clarifying the mechanism by which NK cells kill transformed cells while sparing normal cells. The direct interactions between inhibitory NK cell receptors and their HLA-I ligands enable NK cells to distinguish healthy from transformed cells, which frequently show an altered expression of HLA-I molecules. Indeed, NK cells can kill cancer cells that have lost, or under express, HLA-I molecules, but not cells maintaining their expression. In this last case, it is possible to use anti-KIR or anti-NKG2A monoclonal antibodies to block the inhibitory signals generated by these receptors and to restore the anti-tumor NK cell activity. These treatments fall within the context of the new immunotherapeutic strategies known as "immune checkpoint blockade." These antibodies are currently used in clinical trials in the treatment of both hematological and solid tumors. However, a more complex scenario has recently emerged. For example, NK cells can also express additional immune checkpoints, including PD-1, that was originally described on T lymphocytes, and whose ligands (PD-Ls) are usually overexpressed on tumor cells. Thus, it appears that the activation of NK cells and their potentially harmful effector functions are under the control of different immune checkpoints and their simultaneous expression could provide additional levels of suppression to anti-tumor NK cell responses. This review is focused on PD-1 immune checkpoint in NK cells, its potential role in immunosuppression, and the therapeutic strategies to recover NK cell cytotoxicity and anti-tumor effect.

Keywords: NK cells, PD-1, PD-L, KIR, NKG2A, immune checkpoint, immune checkpoint blockade, immunotherapy

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INTRODUCTION

The immune system defends our body against foreign microbes/antigens, while simultaneously preventing self-reactivity. To this aim, immune cells are regulated by a balance between inhibitory and activating receptors/co-receptors expressed on their surface, which, upon interactions with their ligands, deliver negative, or positive signals that dictate the outcome of immune responses.

Receptors delivering inhibitory signals function as immune checkpoints and play a more general role in maintaining peripheral tolerance and preventing autoimmunity (1, 2).

However, immune-regulation displays also its negative tradeoffs. Thus, local tolerance in the tumor microenvironment represents a common survival strategy, exploited by different tumors to escape elimination by the immune system (3).

Immune checkpoint blockade is based on the use of monoclonal antibodies (mAbs) directed against inhibitory checkpoints expressed by immune cells (or their ligands expressed by tumor cells). The mAb-mediated disruption of these receptor/ligand interactions may revert the functional inhibition of these cells and restore an effective anti-tumor cytotoxic activity, possibly leading to durable tumor regression.

The best-known immune checkpoints are represented by CTLA-4 and the axis including PD-1 and its ligands PD-Ls (PD-L1/PD-L2). Both CTLA-4 and PD-1 were originally identified in T lymphocytes, while PD-Ls may be expressed on different tumor cells. Since the discovery of these immune checkpoints, the innovative cancer immunotherapeutic approach was focused on the restoration of T cell-mediated specific responses to tumor antigens.

Anti-CTLA-4 mAbs, such as ipilimumab, were the first of this class of immunotherapeutics that became available in clinical practice achieving the US FDA approval for the treatment of metastatic melanoma (4, 5).

The first PD-1/PD-Ls disrupting agent approved for the treatment of solid tumors was the anti-PD-1 nivolumab; subsequently, several PD-1/PD-Ls blockers have been introduced in clinical practice and many others are currently under investigations across different solid and hematologic malignancies, including non-small cell lung cancer (NSCLC), melanoma, head&neck cancer, renal cell carcinoma and urothelial carcinoma, and high-grade Hodgkin's lymphoma. In addition, various combination therapies have recently been explored in clinical trials, and some combination therapies are being introduced in clinical practice, with particular reference to NSCLC, including combinations of nivolumab and ipilimumab, or platinum-based chemotherapy in association with the anti-PD-1 pembrolizumab, or a combination of platinum-based chemotherapy plus the anti-PD-L1 atezolizumab and the anti-angiogenic bevacizumab. It is possible that combination therapies may earn a progressively prominent role in clinical practice in the upcoming years (6, 7). More recently, maintenance with the anti-PD-L1 agent durvalumab after chemo-radiation for unresectable, locally advanced NSCLC, resulted in a significant improvement in terms of progression-free survival and overall survival (8). While, on one hand, immunotherapy with immune checkpoint inhibitors is characterized by impressive results across various solid and hematologic tumors, on the other hand, the great variability of response among patients (indeed a still large fraction of patients fails to respond), suggests that the complex biology of immune checkpoint pathways has not yet been fully understood. Notably, the documented expression of PD-L1 on tumors might influence the clinical decision of treating patients with immune checkpoint inhibitors in specific settings; indeed, strong expression of PD-L1 ($\geq$50%) is required for prescribing pembrolizumab as first-line treatment for advanced NSCLC; similarly, a positive expression of PD-L1 (at least 1%) is necessary for the administration of pembrolizumab in second and further lines and for the administration of durvalumab after chemo-radiation with curative intent for locally advanced NSCLC. However, the available tumor samples are frequently represented by biopsies that may yield inadequate information on the actual PD-L1 expression, eventually representing a potential issue in the management of NSCLC (9). Furthermore, the predictive role of PD-L1 expression has been questioned by additional clinical data; in first place, the outcomes of PD-1/PD-L1 inhibitors in other malignancies apart from NSCLC do not appear to be influenced by PD-L1 expression. Furthermore, in NSCLC, single-agent treatments with nivolumab and atezolizumab for previously treated disease were effective regardless of PD-L1 expression. Similarly, the outcomes achieved by combination regimens involving multiple immune checkpoint blocking agents, such as ipilimumab plus nivolumab in solid tumors, including renal cell cancer (RCC), NSCLC, and melanoma, or involving platinum-based chemotherapy plus a PD-1 or PD-L1 inhibitor in NSCLC were not influenced by PD-L1 expression (10–13). Taken together, these data suggest that other factors, apart from the mere percentage of tumor cells expressing PD-L1, must play a relevant role in immune checkpoint blockade (14).

In this context, independent research groups published data on the contributions of host cells in the PD-1/PD-L1 blockade mediated cancer immunotherapy in patients with PD-L1-negative tumors responding to this blockade therapy (15–18).

Indeed, besides tumor cells, various types of host cells also constitutively express PD-L1, or can upregulate its expression upon stimulation with inflammatory cytokines, including IFN-gamma. These observations imply that PD-L1 from tumor and/or host compartment works in concert to dampen the anti-tumor immune response (19). In addition to the PD-1/PD-L1 axis, also the PD-1/PD-L2 interaction may play an important role in evading anti-tumor immunity, suggesting that PD-1/PD-L2 blockade must be considered for optimal immunotherapy in PD-L2-expressing cancers, such as RCC and NSCLC (20, 21).

The Multifaceted Nature of NK Cells

NK cells are classified as lymphocytes on the basis of their origin from the common lymphoid progenitor cell in the bone marrow. However, different from T and B lymphocytes, they do not express antigen-specific cell surface receptors encoded by rearranging genes. Thus, NK cells are considered to be players of innate immune defenses, and, in particular, represent cytotoxic innate lymphoid cells (ILCs) (22, 23). They were originally identified over 40 years ago (24, 25), on the basis of their ability to kill tumor/virus-infected cells in the absence of prior activation. Later, NK cells have been recognized as immunoregulatory cells, secreting pro-inflammatory cytokines, and many chemokines, and expressing different receptors for both cytokines and chemokines. This means that NK cells may recruit and may be recruited to inflammatory sites where they can colocalize with other immune cells, including dendritic cells with which NK cells can cooperate (22, 26, 27). These different cell-to-cell interactions endow NK cells with regulatory function affecting both the quality and the strength of adaptive immune defenses (23, 28).

Several lines of evidence indicate that NK cells or their receptors play a critical role in immunosurveillance of spontaneous tumors and in preventing tumor metastases. In addition, it has been shown that impairment in NK cytotoxic

activity is associated with a higher cancer risk (29). Indeed, tumors have evolved mechanisms to escape NK cell control. This knowledge prompted efforts to exploit NK cell functions for better management of cancer patients.

The Discovery of NK Cell Immune Checkpoints

Similar to T cells, NK cells express surface receptors that can be targeted in checkpoint blockade strategies (30, 31).

The first NK cell immune checkpoints were identified by Alessandro Moretta in 1990 with the discovery of p58 molecules that were later called killer cell immunoglobulin-like receptors (KIRs). He demonstrated that KIRs were specific for allotypic determinants of HLA-I molecules. He also greatly contributed to the identification of additional receptors interacting with HLA-I molecules including the HLA-E specific CD94/NKG2A heterodimer (32–34). These findings represented a true revolution in the field of human NK cell biology and opened new avenues in the field of NK cell-based immunotherapeutic approaches.

Initially, NK cell-based immunotherapy had mainly been exploited to treat hematological malignancies and relied either on the adoptive transfer of NK cells or on NK cells generated from transplanted hematopoietic stem cell to treat high-risk leukemia (35, 36).

Recent approaches have been based on mAb-mediated blockade of specific NK cell immune checkpoints (37).

In 1999, Alessandro Moretta, together with Eric Vivier, Hervé Brailly, Marc Bonneville, Jean-Jacques Fournié and Francois Romagné, founded Innate Pharma, the first Biotech Company that aimed to target NK cells in innovative anti-tumor immunotherapeutic strategies (https://www.innate-pharma.com/en/profile/founders). Starting from mAbs generated in Alessandro's lab, directed against the HLA-I specific inhibitory receptors, the first two immune checkpoint inhibitors were generated: lirilumab targeting pan-KIR2D and monalizumab targeting NKG2A. These therapeutic antibodies, with the capacity to disrupt the interactions between KIR or NKG2A and HLA-I, are expected to unleash the anti-tumor NK cell cytotoxic activity mimicking "missing-self" response. Both lirilumab and monalizumab have been shown to be safe with limited side effects upon prolonged treatments in phase I clinical trials (38). These agents are currently undergoing phase I/II clinical trials across a range of hematologic and solid tumors in monotherapy or in combination with other agents, including rituximab (an anti-CD20 mAb), and other forms of immune checkpoint blockade (39–41).

In addition to KIR and NKG2A, other inhibitory checkpoints may be expressed on NK cells. They include the T-cell Ig and ITIM domain (TIGIT), CD96 (TACTILE), LAG-3 and TIM-3.

TIGIT and CD96 are co-inhibitory receptors expressed on both T and NK cells and compete with the activating receptor DNAM-1 for binding to PVR (CD155) and Nectin-2 (CD112) (42). TIGIT expression has been reported as upregulated in tumor-associated NK cells in different malignancies (43). Thus, it has been hypothesized that TIGIT could play a role in carcinogenesis due to its ability to inhibit NK cell cytotoxicity. A recent study provided evidence that TIGIT blockade may induce anti-tumor immune activity in preclinical models, and its combination with PD-1/PD-L1 inhibitors is being explored (44). Pre-clinical data also showed that blockade of CD96 alone or in combination with anti-PD-1 or anti-CTLA-4 or doxorubicin promotes NK cell activity (in terms of IFN-γ release) and a better control of tumor progression (45, 46).

LAG-3 is a negative costimulatory receptor homologous to CD4 and expressed on activated T and NK cells (47). High-affinity ligands for LAG-3 are HLA-II molecules that are mainly expressed by antigen presenting cells, but also by some cancer cells. Despite its inhibitory activity has been defined only in T cells, this immune checkpoint is currently considered as a good target for immunotherapy because of its potential to activate both T and NK cells. Indeed, LAG-3 mAbs are currently in pre-clinical development in association with standard chemotherapy (NCT02614833) and in combination with anti-PD-1 therapy (NCT02676869, NCT01968109).

TIM-3 is a checkpoint receptor that binds several ligands including galectin-9 (Gal-9) (48), phosphatidylserine on apoptotic cells (49), high mobility group box 1 (HMGB1) (50), and CEA-related cell adhesion molecule-1(CEACAM1) (51). TIM-3 is expressed on both adaptive and innate immune cells (52, 53). The engagement of this inhibitory checkpoint on NK cells may have different and opposite effects (53). These divergent functions are likely associated with the existence of multiple and different TIM-3 ligands. Blockade of TIM-3 could restore T-cell effector function in preclinical models and result in increased NK cytotoxicity (54).

The Identification of the PD-1$^+$ NK Cell Subset

The effect of the PD-1/PD-L1 blockade has been usually attributed to the restoration of cytotoxic T lymphocyte activity, and killing of tumor cells expressing HLA-I molecules. However, a partial or complete loss of HLA-I expression is one of the most frequent mechanisms of tumor escape from the host's immune system in different human tumor types. In this context, it is important to remember the "missing self" hypothesis postulated by Karre in 1986 (55), and formally proven in humans by Alessandro Moretta (56–59). The "missing self" hypothesis stated that absence, or reduced expression, of *self*-HLA-I molecules may be sufficient to render a target cell susceptible to killing by NK cells. Thus, NK cells recognize tumors that avoid T cell-mediated killing through abnormal or absent HLA-I expression. This is clinically relevant for patients with tumors displaying low levels of HLA-I at diagnosis and suggests the potential of NK cell-based adoptive immunotherapy.

Along this line, we were convinced that even NK cells could contribute to the clinical benefit of immunotherapeutic strategies targeting PD-1/PD-L1 axis, but to confirm our hypothesis we first had to demonstrate that even NK cells could express PD-1 (**Figure 1**).

Initially, we tested different mAbs to check the best reagent to analyze this receptor on NK cells. We selected a reagent generated

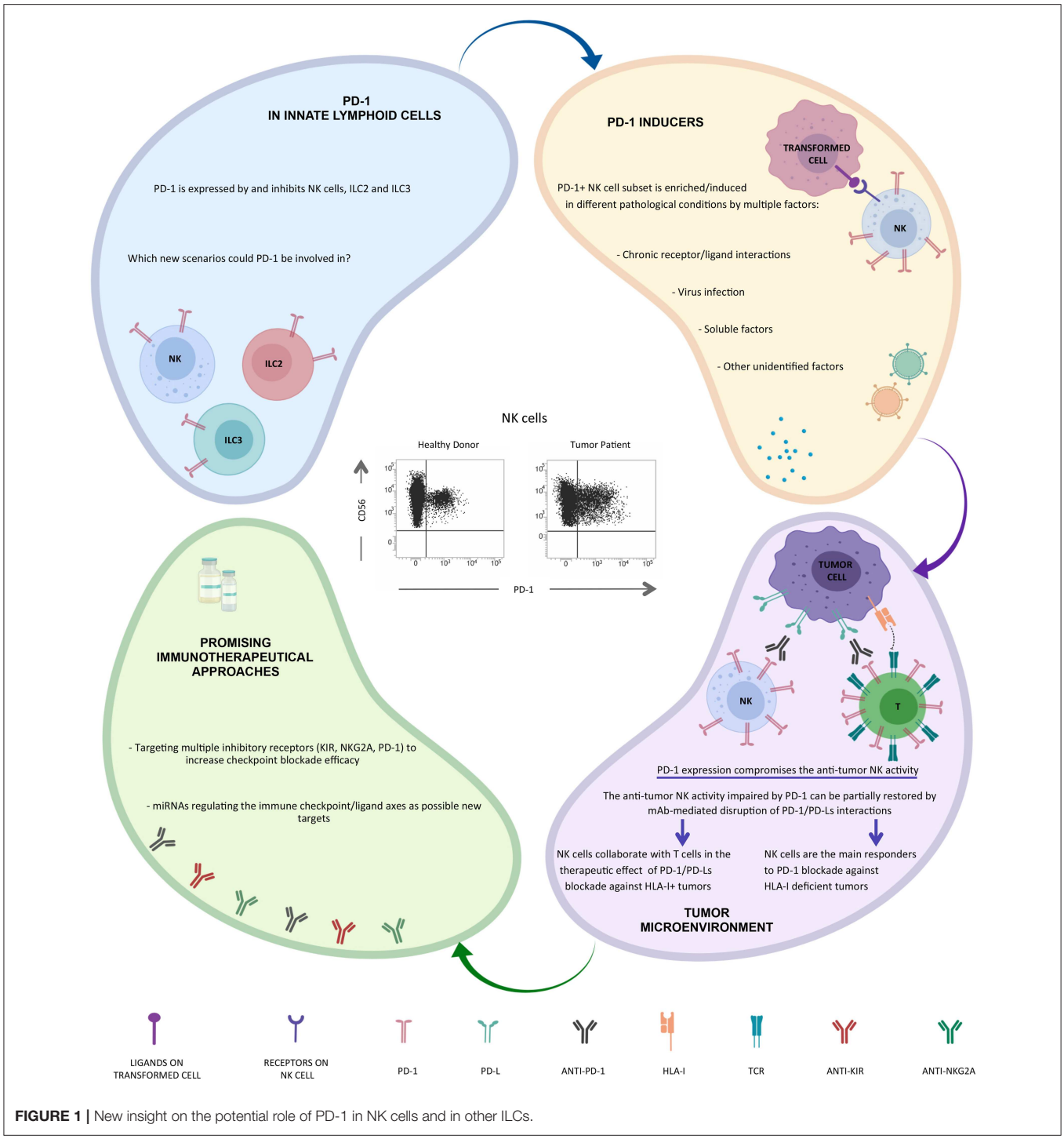

FIGURE 1 | New insight on the potential role of PD-1 in NK cells and in other ILCs.

by our friend and international scientist Daniel Olive, as the most performing among all the tested mAbs (60, 61). Regarding the biological samples, we started the analysis of healthy donors, and thanks to the high number of healthy donors analyzed and also to the excellent reagent used, we were able to identify a subset of NK cells expressing high level of PD-1 in 25% of the donors analyzed (62). In order to verify what we were observing, we increased the cases and the controls; this allowed us not only to confirm this expression but also to characterize this new PD-1^{+} NK cell subset. PD-1 expression was confined to CD56^{dim} NK cells and, if present, to CD56^{neg} NK cells, whereas the CD56^{bright} subset was consistently PD-1^{neg}. By comparing the PD-1^{+} and PD-1^{neg} NK cells derived from the same healthy donor, we found that the PD-1^{+} subset was confined to fully mature NK cells. Indeed,

these PD-1$^+$ NK cells were homogeneously characterized by the CD56^{dim}KIR$^+$LIR-1$^+$NKG2A^{neg}CD57$^+$NCR^{dim} phenotype.

The fact that only one fourth of the individuals analyzed were characterized by a PD-1$^+$ NK cell subset could be the result of given acute or chronic infection affecting only part of the population. Further analysis revealed that a direct correlation between HCMV infection and presence of a PD-1$^+$ NK cell subset could be established. Indeed, the totality of PD-1$^+$ individuals was seropositive for HCMV. Furthermore, as expected, the PD-1$^+$ donors showed a reconfiguration of the NK cell receptor repertoire, typically induced by HCMV infection (62, 63). Interestingly, different PD-1 mRNA splicing isoforms and a cytoplasmic pool of PD-1 protein are detectable in virtually all NK cells analyzed (mainly CD56^{dim}), thus indicating a possible rapid recruitment of this molecule on cell surface in response to precise, yet undefined, stimuli (64).

Once the presence of PD-1 on healthy donor NK cells was demonstrated, we moved to cancer patients. Given our previous expertise in ovarian cancer (65), we decided to focus our attention on NK cells derived from this kind of tumor patients. Our analyses showed that PD-1+ NK cells were detectable in the peripheral blood (PB) of the majority of these patients. More importantly, this NK cell subset was further increased in the tumor microenvironment, thus suggesting a possible accumulation/induction of this subset in this compartment. Again, the PD-1+ NK cells were confined to the CD56^{dim} NK subset, although our ongoing analyzes indicate that the features of the tumor-associated PD-1$^+$ NK cell subset are different from those of the subset present in healthy donors (manuscript in preparation).

As the interest on PD-1$^+$ NK cells is now turned on, several papers have been recently published, confirming the presence of the PD-1$^+$ NK cell subset in tumor patients. In particular higher proportions of PD-1$^+$ NK cells can be also detected on PB NK cells from multiple myeloma or Kaposi sarcoma patients and on PB and tumor-associated NK cells in head&neck cancer patients (41, 66). *In vitro*, PD-1 expression may be induced on NK cell surface upon persistent stimulation by tumor cells expressing ligands for activating NK receptors (66). In addition, virus infection (e.g., HCMV) (62) and/or soluble factors released in the tumor microenvironment (including endogenous glucocorticoids) may be involved in PD-1 induction (67, 68) (**Figure 1**).

This phenotype correlates with an impaired NK cell activity (cytotoxicity, proliferation, and cytokine production) against PD-L^{pos} tumor cells that can be partially restored by mAb-mediated disruption of PD-1/PD-L interaction (41, 62, 69) (**Figure 1**).

This is an important detail because we know how *in vivo* the use of anti-PD-1 or anti-PD-L mAbs may generate beneficial effects toward the anti-tumor response mediated by T lymphocytes, but evidently also from NK cells.

Therefore, when we talk about tumor and NK cells we should not consider the recognition of HLA by the main inhibitory checkpoints expressed by NK cells, i.e., KIR or NKG2A, as the only system that plays a fundamental role in the control of tumor transformation, but we should also consider a possible participation of PD-1 in this system. In fact, simultaneous expression of different inhibitory checkpoints could provide multiple levels of suppression to anti-tumor responses of NK cells.

Now, several data suggest that NK cells are potential PD-1 blockade responders and that NK cell removal abrogates the anti-tumor efficacy of this immunotherapy (69).

Furthermore, PD-1 expression on NK cells may correlates with poor prognosis in different type of cancers (70). These findings strongly suggest a possible role for NK cells in immunotherapeutic strategies targeting the PD-1/PD-L1 axis particularly against HLA-I deficient tumor cells, but, interestinlgy, NK responses were still important for controlling cancer development also in cancer models in which CD8$^+$ T cells played a substantial role (69) (**Figure 1**).

Thus, the analysis of expression/coexpression and function of inhibitory checkpoints is extremely important in order to design innovative immunotherapeutic strategies.

In this context, clinical trials are presently undergoing in which anti-NKG2A (monalizumab) or anti-KIR (lirilumab) antibodies are used as a combotherapy with anti PD-1 (nivolumab) for various type of solid tumors in order to obtain a complete reconstitution of anti-tumor NK cell citolytic activity (71).

These innovative approaches have a particular relevance especially if we think that tumor infiltrating T cells may express PD-1 but also KIR and/or NKG2A. Thus, the combined blockade of different checkpoints may simultaneously activate both innate and adaptive immune responses.

Interestingly, recent data indicate that PD-1 is also expressed by and may regulate both ILC2s and ILC3s, and that mAb-mediated blocking of PD-1 restored their effector functions. Since ILCs play a critical role in different inflammatory conditions, including tumors, these cells may represent interesting targets for immunotherapy (52, 72, 73) (**Figure 1**). Novel immunotherapeutic approaches could be based on the use of microRNA. In this context, it has been recently shown that the hsa-miR-146a-5p may negatively regulate the surface expression of certain KIRs by mimicking a "missing self" condition and, as a consequence, by improving the NK cell mediated cytotoxicity (74). Moreover, recent studies have provided novel evidence that miR-148a-3p and miR-873 negatively regulate tumor cell PD-L1 expression (75, 76). Thus, these regulatory miRNA/targets axes might serve as an additional tool in tumor therapy.

CONCLUDING REMARKS

Tumor development often induces a suppressive microenvironment hampering cytotoxic lymphocytes effector-functions thus promoting tumor progression. T and NK cells result powerless just when we need them more. One of the main escape mechanisms by which tumor turn off our defense is the exploitment of immune checkpoints pathway. Restoring and harnessing immune cells to cure cancer represents

an attractive challenge for scientists. In the 90s, Alessandro Moretta discovered the first NK cell immune checkpoints: KIRs and NKG2A. Soon after, Innate Pharma generated the first two therapeutic immune checkpoint inhibitors: lirilumab, targeting KIR, and monalizumab, targeting NKG2A. This was the beginning of a revolution. In the same years, Tasuku Honjo and Jim Allison discovered that the reactivation of the immune system by blocking two major immune checkpoints, CTLA-4 and PD-1, could represent a practice-changing approach in oncology.

Honjo and Allison received the Nobel Prize in Medicine 2018 "for their discovery of cancer therapy by inhibition of negative immune regulation." During his speech at the Nobel Banquet, Honjo stated: "As a result, Jim and I have experienced many occasions that have made us feel well-rewarded, such as meeting cancer patients who say their lives were saved by our therapies." He also added: "Jim and I both know that the development of our discovery is just beginning. We encourage many more scientists to join us in our efforts to keep improving cancer immunotherapy."

Alessandro realized that immunotherapy with anti-PD-1 mAbs can be also useful for reactivating NK cells against the tumor in particular in the case of T-mediated tumor resistance (HLA-I^{neg} tumor cells). This intuition was confirmed by the identification of the PD-1 receptor on a subset of NK cells by his group. This subset is increased in the tumor microenvironment, as also shown by different research groups around the world (41, 62, 66).

The very recent discovery that PD-1 is also expressed on other groups of ILCs, including ILC2s and ILC3s, opens up new scenarios (52, 72, 73).

Once again Alessandro Moretta gave a fundamental contribution to the field of NK cell biology and to the clinical use of NK cell immune checkpoints. Initially, with the discovery of the first inhibitory checkpoints, then with the discovery of the various NK cell activating receptors (77), here not discussed, and finally, with the identification of the PD-1 immune checkpoint expression on NK cells. This latest discovery by Alessandro was certainly one of his most important contributions, and we collaborators, but above all friends, know what the discovery of this molecule on his beloved NK cells and its clinical implications has meant to him. Still today, all Alessandro's discoveries represent important bases for understanding how to best use NK cells in cancer therapies (**Figure 1**).

Thank you Alessandro for allowing us in your life, for supporting of us when we needed it most, for making us all always feel special and, most of all, for teaching us how to move forward with courage, simplicity and dignity.

Thank you Alessandro for having taught us how to do science, day by day, with scientific strictness, together with modesty. Thank you for having instilled in us your curiosity, and infinite passion for Science. Being afraid doesn't make any sense.

We miss you every moment in our life!

AUTHOR CONTRIBUTIONS

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

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REVIEW
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An Historical Overview: The Discovery of How NK Cells Can Kill Enemies, Recruit Defense Troops, and More

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Natural killer (NK) cells were originally defined as effector lymphocytes of innate immunity characterized by the unique ability of killing tumor and virally infected cells without any prior priming and expansion of specific clones. The "missing-self" theory, proposed by Klas Karre, the seminal discovery of the first prototypic HLA class I-specific inhibitory receptors, and, later, of the Natural Cytotoxicity Receptors (NCRs) by Alessandro Moretta, provided the bases to understand the puzzling behavior of NK cells. Actually, those discoveries proved crucial also for many of the achievements that, along the years, have contributed to the modern view of these cells. Indeed, NK cells, besides killing susceptible targets, are now known to functionally interact with different immune cells, sense pathogens using TLR, adapt their responses to the local environment, and, even, mount a sort of immunological memory. In this review, we will specifically focus on the main activating NK receptors and on their crucial role in the ever-increasing number of functions assigned to NK cells and other innate lymphoid cells (ILCs).

Keywords: human natural killer cells, innate immunity, natural cytotoxicity receptors, Toll-like receptors, activating NK receptors

INTRODUCTION

When Alessandro Moretta was appointed as Professor of Histology at the University of Genoa and started to set up a new lab and recruit people, including most of the authors of this review, the knowledge of how NK cells could exert their activity against tumors and viruses was very limited. The "missing-self" hypothesis had just been proposed by Karre and Ljunggren (1), but there was no idea on the molecular mechanisms by which NK cells could spare the "good" cells and kill the "bad" ones. Within <10 years, Moretta's lab generated a large number of monoclonal antibodies (mAbs) that allowed the identification and characterization of many key receptors, including,

among many others, the first-discovered Killer Ig-like receptors (KIRs) (2–4) and the Natural Cytotoxicity Receptors (NCRs) (5). These discoveries provided the mechanistic explanation of the "missing-self" theory. Indeed, they showed that NK cells could kill target cells by integrating signals from activating and inhibitory receptors, by recognizing ligands on tumor or virus-infected cells and by sensing changes in HLA class I expression (6–9).

Later studies indicated that NK cells, besides "killing the enemies," could also "incite the defense troops" by interacting with Dendritic Cells (DCs) to induce and polarize the adaptive immune response (10–12). A relevant role for given NK receptors newly identified by the Moretta's group, together with certain Toll-like receptors (TLRs), was found also in this context (13–16). This field was then further investigated, revealing the quite large net of interactions that NK cells can undertake with innate (granulocytes and macrophages) and adaptive immune cells, and even stromal and tumor cells (17–24).

After this early era of major discoveries, studies on NK cells increased exponentially, revealing an extraordinarily complex world, which now comprises a number of circulating or specialized tissue-resident NK cell subsets (25). Some studies also showed that NK cells can adapt their function to environmental changes or even maintain memory of certain viral infections (26–30). Moreover, many of the ligands for the activating NK receptors have now been identified and demonstrated to be variably expressed by tumor or virus-infected cells (31, 32). Much information have been added to the mechanisms that regulate the availability and function of NK cells within tumor tissues giving hints on the possible use of NK cells in the therapy of solid tumors (33–38). Finally, the extensive studies of the KIR repertoire and the "old" data on NK/DC interaction have posed the basis for a reliable exploitation of NK cells in hematopoietic stem cell transplantation (HSCT) to cure hematologic malignancies (39–42), while the new findings on the immune checkpoints regulating T and NK cell functions have reinforced the idea of blocking HLA class I-specific NK receptors to unleash the NK cell anti-tumor potential. In this context, human/humanized anti-KIR or anti-NKG2A mAbs or combinations of mAbs blocking NKG2A and the PD-1/PD-L axis are tested in animal models and clinics (33, 43–48).

Alessandro Moretta, who has continued his work on NK cells with immutable enthusiasm all over his life, also contributed to these latter advances in the field with many key data, spanning from the tumor escape mechanisms acting on the activating receptor expression, to the characterization of the memory-like NK cell subset, the role of activating KIRs, and the role of immune checkpoints on NK cells in tumor patients. Nevertheless, it is indubitable that the identification of the first KIRs (which will be treated in a review aside) and of many NK activating receptors represents his real landmark discovery and legacy to Science. Indeed, the characterization of these receptors impressed an acceleration of the initial research and, still now, represents the basis for many new findings on NK cells and beyond (**Figure 1**).

The association of different NCR splice variants with tumor tissues or with non-pathological decidua tissues, the role of NKp30, NKp46, and NKp80 in the NK-mediated cross-talk with DCs, granulocytes, or monocytes, and the definition of NKp46 and NKp44 as markers of non-cytotoxic ILCs, are only some of the indications for the involvement of these receptors in near future studies on NK cell-based therapies against cancer, for long-standing investigations on the maternal-fetal tolerance, and, more extensively, on tissue homeostasis.

NATURAL CYTOTOXICITY RECEPTORS

Only few years after the identification of the first KIRs and of CD94/NKG2A, three non-HLA class I-specific activating receptors (namely NKp46, NKp30, and NKp44) were discovered in Alessandro Moretta's lab. These receptors, together with NKG2D, turned to be crucial for the recognition of both tumor and virus-infected cells (5, 49, 50). They were first characterized for their functional features (i.e., their ability to induce NK cell cytolytic activity and cytokine release) (51–54) and then also at the molecular level, when the cDNAs coding for these receptors were isolated (53, 55, 56) and the crystallographic structures were solved (57–60). NKp46, NKp30, and NKp44 were all selectively expressed on NK cells (although their expression was differently induced during activation) and revealed, since the initial studies, to be the main receptors responsible for the so-called "natural cytotoxicity" of NK cells. Thus, based on these findings, these receptors were collectively termed as Natural Cytotoxicity Receptors (NCRs), although neither the protein structure, nor the gene location gave indications for their belonging to a receptor family. Their discovery paved the way to a huge number of studies aimed at elucidating their function in both physiological and pathological conditions and characterizing the NCR/NCR ligand (NCR-L) interactions. As mentioned above, NCR expression was initially thought to be confined to NK cells, and NKp46 is still being considered a reliable NK cell-associated marker, both in humans and in mice (61, 62). Soon thereafter it became clear that these receptors could also be expressed in other immune cell types (63), extending their role to additional biological processes. For example, the characterization of the heterogeneous family of Innate Lymphoid Cells (ILCs) (25, 64, 65) revealed that NKp44 is also expressed by IFN-γ-producing intraepithelial ILC1 and by a subset of ILC3 present at the epithelial/mucosal surfaces, in tonsils, and in decidua tissue (66–71). Notably, NKp44^{pos} ILC3 display a unique cytokine pattern, being able to produce IL-22 following cytokine stimulation (68). In these cells, NKp44 triggering induces TNF-α production and activates a pro-inflammatory program (72), suggesting that NKp44 could play a role in the pathogenesis of different immune-mediated disease, including psoriasis (73). In addition, NCR^{pos} (NKp44^{pos}) ILC3 have also been detected in the lymphoid infiltrate of non-small cell lung cancer, and have been found to release pro-inflammatory cytokines following interaction with tumor cells and tumor-associated fibroblasts (34, 67, 74). NKp46 expression has been detected in CD4^{pos} T lymphocytes derived from patients with Sézary syndrome, an aggressive form of cutaneous T-cell lymphoma (CTCL) (75). Notably, in these cells, NKp46 can act as an inhibitory co-receptor able to decrease CD3-mediated proliferation of Sézary

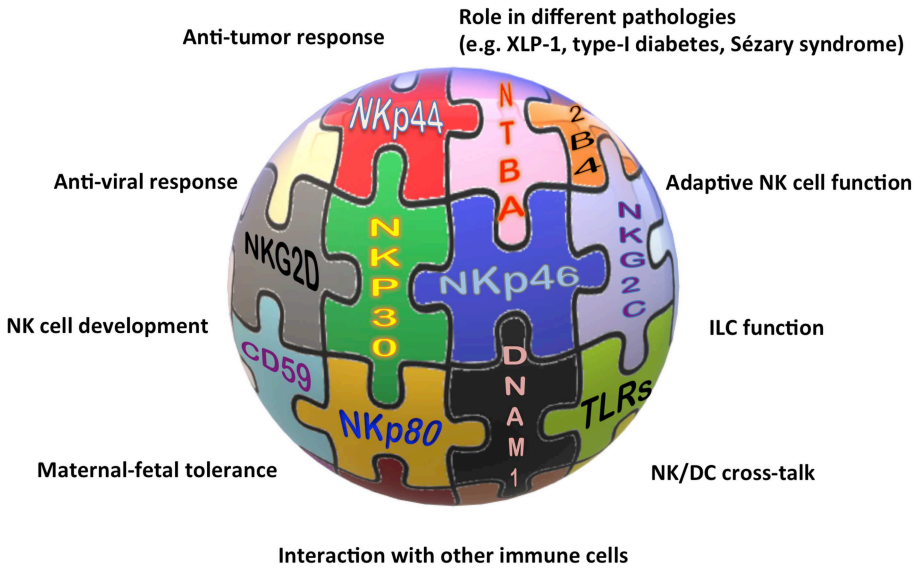

FIGURE 1 | The "activating" solution of the NK cell puzzle. Different activating receptors collaborate to induce NK cell triggering in healthy and pathological conditions.

cells, and has been proposed as an additional diagnostic marker, besides KIR3DL2, for the detection of these malignant cells (76).

One of the most investigated issues about NCRs is the characterization of their ligands. Although the landscape of NCR ligands is still incomplete, a common emerging theme is the multiplicity and heterogeneity of NCR/NCR-L interactions (31, 77–80). Most NCR ligands have been shown to activate NK cell function, while others dampen NK cell activation or act as "decoy ligands" when released in soluble form (81–85). The panel of cellular NCR-Ls currently includes surface glycoproteins, nuclear proteins that can be displayed at the cell surface, soluble molecules that can be either secreted, enzymatically shed, or conveyed through extracellular vesicles (82, 85–92). The expanding knowledge of NCR-Ls has opened the possibility of targeting NCR/NCR-L interactions in the context of cancer immunotherapy strategies. In addition, it has allowed the identification of several mechanisms of tumor escape related to the interaction between NK cells and malignant cells in the tumor microenvironment (22, 93–101). Finally, the importance of NK cell activity, and of NCRs in particular, in the therapeutic effect and outcome of oncolytic virotherapy has now being appreciated (102–104). NCR-Ls are also being studied as possible biomarkers in a variety of pathological conditions. Thus, a soluble form of B7-H6 (sB7-H6), an NKp30 ligand, has been demonstrated in the peritoneal fluid of ovarian cancer patients and in patients with metastatic gastrointestinal stromal tumor (GIST), neuroblastoma, or hepatocellular carcinoma (HCC) (83, 84, 105, 106). The presence of soluble BAG6/BAT3 (another NKp30-L) in the plasma of chronic lymphocytic leukemia patients was found to correlate with advanced disease stages (81). Along this line, high levels of soluble Nidogen-1, an NKp44 ligand, have been detected in the sera of patients with ovarian or lung cancer (107, 108).

Regarding the possibility of exploiting NCRs in anti-tumor approaches, it must be considered that NKp46 and NKp30 expression is down-regulated in NK cells derived from patients with different types of both hematological and non-hematological cancers (93, 109–116). This downmodulation leads to the impairment of NK cell anti-tumor potential and consequently to the need to develop strategies aimed at restoring NCR function (i.e., the use of cytokines, immunomodulatory drugs, anti-cancer drugs, or anti-KIR mAbs) (117–120). In addition, tumor cells themselves can become more resistant to NK cell-mediated attack by down-regulating NCR-Ls or releasing them in a soluble form (decoy ligands).

The role of NCRs stretches beyond cancer. B7-H6 is also involved in the inflammatory response: its expression is induced on monocytes following exposure to pro-inflammatory cytokines or TLR ligands, and high levels of sB7-H6 are found in the serum of patients with sepsis induced by Gram-negative bacteria (121). NK cells, in general, have been studied in different autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and type I diabetes (TID) (122–124). Focusing on NCRs, NKp46 has been shown to play a role in the pathogenesis of TID and in the destruction of normal pancreatic β cells (125), suggesting the possibility to target this receptor through specific anti-NKp46 mAbs (126).

A few years after the NCR discovery, the existence of different splice variants of these receptors was revealed (32, 127). Thus, three alternatively spliced NKp30 isoforms were identified, characterized by distinct intracellular regions and different functional capabilities. In GIST patients the prevalence of NKp30c isoform has been associated to decreased NK cell functionality and to reduced survival (128). Along this line, a similar pattern of NKp30 isoform expression has been detected in HCC patients (106). Notably, NKp30c isoform and sB7-H6 have

been studied in metastatic GIST patients, revealing their possible use as predictive biomarkers of disease progression and response to imatinib mesylate treatment (105). NKp44 splice variants have been studied in different neoplastic disorders, and in particular in acute myeloid leukemia patients, indicating a correlation between the prevalence of the ITIM-bearing inhibitory NKp44-1 isoform and poor survival (129). The induction of NKp44-1 expression has been also observed in decidua NK cells, driven by cytokines released in the decidua microenvironment, and could play a role in promoting tolerance toward the fetus (127, 130).

Among the NCRs, NKp44 is the main receptor involved in the interplay between NK cells and trophoblast cells during pregnancy (131, 132), and is expressed also by a subset of ILC3 and by IFN-γ-producing ILC1-like cells found in the decidua (133). Decidua NK cells represent a peculiar NK cell subset, characterized by NKp44 expression, poor cytotoxic activity, and contributing to decidua development, vascularization, and tissue building/remodeling (134–136). Notably, in these cells, NKp44 triggering has been shown to induce IP10, IL-8, and VEGF release (132, 137).

ACTIVATING CO-RECEPTORS

Alessandro Moretta gave fundamental contributions also to the identification and/or characterization of other surface receptors, including 2B4 (138–140), NTBA (141, 142), CD59 (143), and NKp80 (144), that play a complementary or a synergistic role with NCRs in inducing NK cell activation. Some of these molecules received great interest because of their involvement in NK cell function and development. 2B4 (145, 146) and NTBA (142), belonging to the signaling lymphocyte activation molecule (SLAM) family, have been shown to act as co-receptors, able to potentiate NK cell cytotoxic activity induced by the main triggering receptors, including NKp46 (140, 141). While 2B4 receptor recognizes CD48 (146, 147), NTBA is involved in homophilic interactions (142). Notably, 2B4 and NTBA dysfunction was described to be associated with a severe form of immunodeficiency, the X-linked lymphoproliferative syndrome type 1 (XLP-1), caused by mutations in *SH2D1A*, the gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) (148). Interestingly, in the absence of SAP, the 2B4 and NTB-A co-receptors associate with the protein tyrosine phosphatases thus delivering inhibitory, instead of activating, signals (141, 149–151). This immune dysfunction is mainly responsible for the NK cell inability to kill EBV-infected B cells (B-EBV) that express CD48, resulting in extremely severe clinical consequences. A rapid diagnostic flowchart for XLP1, based on a 2B4-specific functional assay, combined with intra-cytoplasmic SAP staining, has been proposed (152). Moreover, the abnormal 2B4 function also influences 2B4 cross-talk with other NK receptors. Indeed, inhibitory 2B4 molecule selectively blocks ITAM-dependent activating receptors, namely NCR and CD16, while it affects neither NKG2D nor DNAM-1, which do not transduce through ITAM (152). This finding explains the selective inability, shown by NK cells, to kill B-EBV cells, which highly express CD48 and are mainly recognized by NCRs. In addition, in the NK cell repertoire of XLP-1 patients, NK cells lacking any self HLA class I-specific inhibitory receptor are highly represented and fully functional, indicating that the inhibitory 2B4 participates to NK cell education (153). Interestingly, a similar role for 2B4 has been described also in particular non-pathological processes. Indeed, at early stages of NK cell differentiation, when HLA class I-specific inhibitory receptors are not yet expressed, the delivery of inhibitory signals by 2B4, as a consequence of the late SAP expression, renders self-tolerant immature NK cells that otherwise would be autoreactive (154). Another peculiar situation is represented by decidua NK cells, in which 2B4 functions as an inhibitory receptor due to the absence or very low levels of SAP expression (155).

CD59 has been found to associate to NKp46 and NKp30 receptors and to enhance NK cell-mediated cytotoxic activity (143).

NKp80 molecule was initially described as a co-receptor, expressed by all NK cells, and able to cooperate with triggering receptors in the induction of natural cytotoxicity (144). Later, NKp80 was found to recognize the Activation-Induced C-type Lectin (AICL), a myeloid-specific activating receptor expressed by monocytes, macrophages, and granulocytes (156). NKp80-AICL interaction results in the secretion of pro-inflammatory cytokines from both cell types. In addition, it has been shown to participate in the NK cell-mediated elimination of malignant myeloid cells (156). NKp80 also plays an important role in the process of NK cell development. Indeed, it marks functionally mature NK cells developing in secondary lymphoid tissues (SLT). In particular, on the basis of NKp80 expression, two distinct subsets of SLT stage 4 cells can be distinguished: an $NKp80^{neg}$ population with both NK- and ILC3-associated features and an $NKp80^{pos}$ population with features similar to PB $CD56^{bright}$ NK cells (157).

Among the surface molecules behaving as co-receptors in the activation of NK cell functions, a major role is assigned to DNAX Accessory Molecule (DNAM-1 or CD226), an adhesion molecule displaying activating function, expressed not only by all NK cells but also by T lymphocytes and monocytes (158). Alessandro Moretta's group gave an important contribution in this field with the identification of two different DNAM-1 ligands, namely PVR and Nectin-2, belonging to the Nectin family (159). These molecules are widely expressed on a variety of both hematological and solid tumors (160, 161), representing suitable targets for immunotherapeutic approaches (162). The role of DNAM-1 ligands in tumor cell recognition and killing by NK cells is actually more complex, since, besides DNAM-1, also the inhibitory receptors CD96 and TIGIT can recognize PVR or PVR and Nectin-2, respectively (163, 164). Accordingly, TIGIT and CD96 have been proposed as immune checkpoints, and are becoming appealing targets for the development of antibodies to be used in combination with other immune checkpoint inhibitors with the aim of unleashing both T and NK cell cytotoxic potential against tumors (165, 166).

ROLE OF NK CELLS IN IMMUNE REGULATION

NK-DC Crosstalk

In the late '90s, it was becoming evident that innate immune cells do not act in isolation but potentiate their efficiency by interacting with each other, resulting even in the regulation of adaptive immune response. In 2001 Ralph Steinman (eventually a Nobel Laureate for the discovery of dendritic cells) visited our laboratories in Genoa and that occasion represented a starting point for a fruitful collaboration aimed at investigating the cross-talk occurring between human DCs and NK cells. As always, Prof. Moretta's insights were pivotal in all the studies carried out in that period, identifying which receptors and which subsets of these two innate immune components participate in this interaction, how this last one influences immune responses and to which extent similar stimuli (e.g., TLR ligands) are integrated by DCs and NK cells during innate immunity.

Until then, DCs were known for their critical role in initiating immune responses and priming antigen-specific T cell response (167), acting as sentinels in peripheral tissues, continuously sampling the environment. The dogma also foresaw that upon activation by danger signals, they up-regulated chemokine receptors and co-stimulatory molecules, which allowed them to migrate into lymph nodes and to efficiently induce T cell responses (167). Thus, the idea that DCs could also act as early activators of innate lymphocytes and, in turn, receive activating signals by activated NK cells, was ground-breaking in the field of innate immunity (14).

One of the relevant outcomes of NK/DC interaction is the so called "editing" of DCs, a term coined by Prof. Moretta to indicate the ability of NK cells to eliminate DCs in immature stage, and therefore *bona fide* tolerogenic DCs, while sparing activated/mature DCs able to efficiently induce the subsequent adaptive immune response in secondary lymphoid organs (12, 168, 169). The protective mechanisms of mature DCs was identified in the up-regulation of HLA class I molecules, especially of the non-classical HLA-E (170), occurring upon activation of DCs by either danger signals or NK cells themselves. At the same time, also the activating receptors involved in DC recognition by NK cells were identified (12, 171). The relevance of NKp30 receptor in NK/DC cross-talk was not limited to the mechanisms of killing of immature DCs but extended to the maturation process of DCs upon interaction with NK cells (172).

Remarkably, this cytolytic DC editing by NK cells was identified as a NK-mediated capability of dampening the graft-vs.-host disease in bone marrow transplantation (40) and graft rejection in solid organ transplantation (173, 174). It is noteworthy that, in case of improved skin graft rejection, NK cells were found to home to lymph nodes where they killed allogeneic DCs in a perforin-dependent manner (174).

Interestingly, and consistent with their concomitant role during the early phase of immune responses, NK cells and DCs are often able to sense similar stimuli in parallel. It was reported by Moretta's group that TLR engagement not only activates immature DCs but also renders NK cells more prone to receive triggering signals from pathogen-associated molecules, thus exerting a regulatory control on the early steps of innate immune responses against infectious agents (16), as more specifically addressed in the next paragraph.

All these studies on DC/NK interactions indicate a critical role for NK cells in the initiation and regulation of immune responses and provide a strong rationale for a combined targeting of NK cells and DCs in novel immunotherapeutic strategies, harnessing this cellular cross-talk in the treatment of patients with cancer and chronic infections resistant to conventional therapies.

Alessandro Moretta's contribution to the knowledge on the molecular basis of these cellular interactions paved the way to clinical interventions exploiting DC/NK cell cooperation. As a matter of fact, NK cell activation by DCs is particularly efficient, since DCs promote both effector functions and survival/proliferation of NK cells (169). As a whole, these basic discoveries, largely achieved under Prof. Moretta's guidance, revealed a particular translational relevance. For instance, in the field of haplo-HSCT, a beneficial role of NK cells in mediating graft-vs.-leukemia effects and in preventing GvHD was highlighted. The support provided by DCs for the proliferation/survival of NK cells is relevant also for establishing more efficient protocols for *ex vivo* NK cell expansion, given that NK cell-based immunotherapies are currently being reconsidered in both post-transplant hematological settings and in immunotherapy strategies for advanced solid tumors (41, 119, 175–180).

Finally, DCs activated by NK cells are better inducers of the anti-tumor CTL response, at least *in vitro*, as compared with the standard mature DCs currently employed in DC-based clinical trials (181) and could therefore be considered in immunization strategies for the development of next-generation vaccines (182, 183).

Expression and Function of TLRs on Human NK Cells

Another field of research in which Prof. Moretta undoubtedly gave important contributions is the expression and function of TLRs in human NK cells. Indeed, in 2004 his group provided a solid experimental evidence that pathogen-associated products, known to strongly activate DCs and other innate immune cells, can also act on TLRs expressed by NK cells, inducing their activation both in terms of increased cytotoxicity and cytokine release (16). Alessandro Moretta and coworkers not only described the effect of TLR ligands on NK cell function, but also analyzed the role of TLR in the NK/DC crosstalk. This led to the concept of "NK cell-mediated editing of DCs," the "quality control" process by which NK cells select DCs that are suited for T cell priming. The capability of TLR agonists of potentiating NK cell function was further defined in subsequent studies (184–193). Thus, in 2010 a peculiar cooperation between TLR9 and KIR3DL2 in inducing triggering of NK cell function upon treatment with CpG-ODN (TLR9 ligand) was described (194, 195). This study revealed that KIR3DL2 can bind CpG-ODNs at the NK cell surface and shuttle them to endosomes

where TLR9 is localized, thus resulting in sharp down-regulation of KIR3DL2 surface expression and in TLR9-mediated induction of cytokine release. Moreover, it was demonstrated that the KIR Ig-domain involved in the direct recognition of CpG-ODN is represented by D0. Since this domain was hypothesized to be expressed by the putative ancestral mammalian KIR, these data suggested that, originally, certain KIRs could exert a function different from recognition of HLA class I molecules. Moreover, this newly defined functional capability of KIR3DL2 provided an important clue to understand the driving forces that led to the conservation of the KIR3DL2-encoding gene in all haplotypes, despite the low frequency, in the human population, of HLA-A*03 or -A*11 alleles (i.e., the ligands of KIR3DL2). Furthermore, in the Sézary Syndrome, in which KIR3DL2 represents a specific marker for the assessment of circulating tumor burden and for patient follow-up (76), CpG-ODN has been shown to promote not only the internalization of KIR3DL2 receptor but also the generation of apoptotic signals (196). Thus, CpG-ODN may exert a direct anti-tumor effect on Sézary cells through binding to KIR3DL2. In this context, a good clinical response without major side effects was observed upon class-B CpG-ODN subcutaneous administration in CTCL patients (197). CpG-DNA and other TLR agonists have been also explored as adjuvants for immunotherapy. Indeed, many clinical trials based on the use of CpG-ODNs as immunotherapeutic agents revealed that CpG-ODNs can promote Th1 immune responses and may be used in combination with chemotherapy to induce potent anti-tumor immune responses with relevant clinical benefits (186, 198, 199).

NK Cell Subsets in Anti-virus Responses

Besides cancer and other diseases, NCRs also contribute to the NK cell-mediated control of viral infections through the recognition of virus-infected cells. Indeed, the first characterized NCR-Ls were of viral origin, namely influenza virus hemagglutinins (200, 201). Later on, additional viral ligands were identified and, in most cases, they were shown to induce NK cell activation following NCR engagement (31, 78, 202). It is of note, however, that some viral NCR-Ls can inhibit NCR functions, representing a possible immune evasion strategy (203). It has been very recently demonstrated in mouse that NK cells may play a regulatory role during acute and

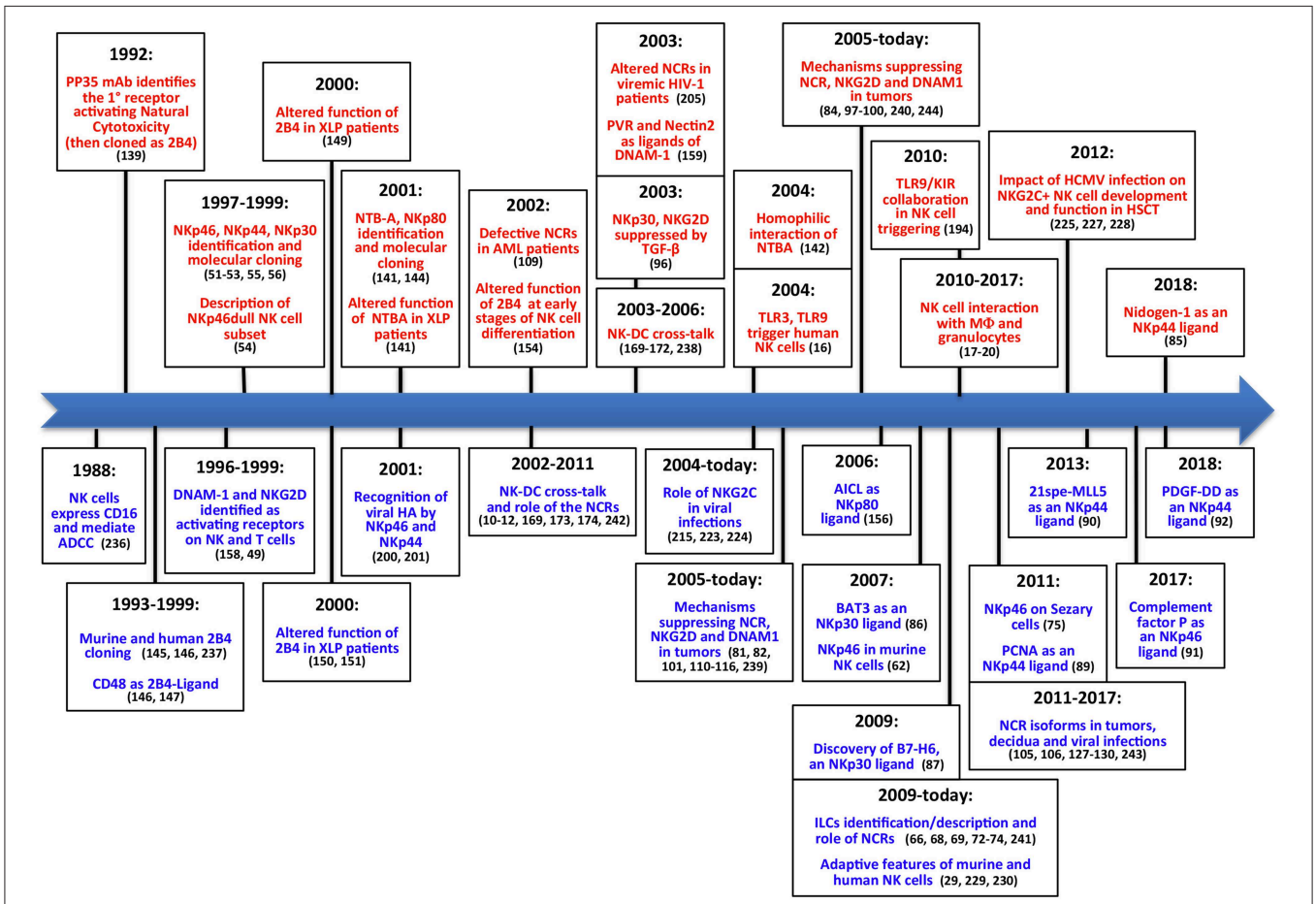

FIGURE 2 | The main steps concerning activating NK receptors/coreceptors. The timeline illustrates the main discoveries concerning NK cell activating receptors during a timespan of about 30 years. Contributions deriving from Alessandro Moretta's research group are indicated in red **(upper part)**, while contributions obtained by other groups are shown in blue **(lower part)**.

chronic lymphocytic choriomeningitis virus (LCMV) infection through the NKp46-mediated killing of LCMV-specific CD8 T cells (204).

In recent years Prof. Moretta and his co-workers gave major contributions to broaden our knowledge on NK cell diversity and functional specialization. This occurred primarily thanks to studies focused on NK cell-mediated responses to virus infections. Fundamental results came from the characterization of NK cells in patients chronically infected by HIV that revealed a deep functional impairment of NK cells likely determining their scarce capacity to efficiently control this virus. In this context, the relevance of NCR contribution to the course of HIV infection became clear when their reduced expression on NK cells in viraemic HIV-infected patients was demonstrated (205, 206). The NCR role in anti-viral response was also supported by the demonstration that NKp46 and NKp30 inducibility exerted a protective role in HIV-infected patients with excellent control not only of virus replication but, more importantly, also of retroviral reservoir (207, 208). Outside the HIV field, the study of NCR expression on NK cells similarly provided compelling evidence of their involvement in the response to acute HCV infection (209), and in HCV eradication in treated chronic carriers (210, 211). Interestingly, in chronically infected HIV patients the accumulation of a dysfunctional NK cell subset, virtually absent in healthy subjects, characterized by an aberrant $CD56^{neg}$ $CD16^{bright}$ surface signature (205, 212, 213) and defective DC editing was observed (214). This unusual population has been subsequently identified in several other pathological conditions including viral infections and immune deficiencies, in which these cells are responsible for an altered response to a chronic immune activation (215–219).

Besides HIV, a fundamental role in shaping NK cell repertoire and function has been described for CMV infection (220–222). Based on the pioneering studies by M. Lopez-Botet who first described the imprinting exerted by CMV on NK cells (223, 224), Alessandro Moretta contributed to identify CMV infection as a key driving force promoting the differentiation of functionally and phenotypically skewed NK cells with several studies conducted in HSCT recipients (225–228). In this setting, CMV infection/reactivation could induce not just NK cell maturation toward highly differentiated stages (characterized by the expression of CD94/NKG2C or activating KIRs), but also the unexpected acquisition of immunological memory. Indeed, NK cells maturing in CMV-reactivating patients share features with adaptive immune cells, such as long-term persistence, virus-induced clonal expansion, and epigenetic modifications (227, 229–234).

This anti-paradigmatic concept of memory or adaptive NK cells, to which Prof. Moretta contributed, holds important translational promise as this NK cell population characterized by longevity and superior ADCC ability, represents a potential tool for novel immunotherapeutic anti-cancer strategies, namely antibody-based tumor immunotherapies and generation of long-living anti-tumor CAR-NK cells (179, 235).

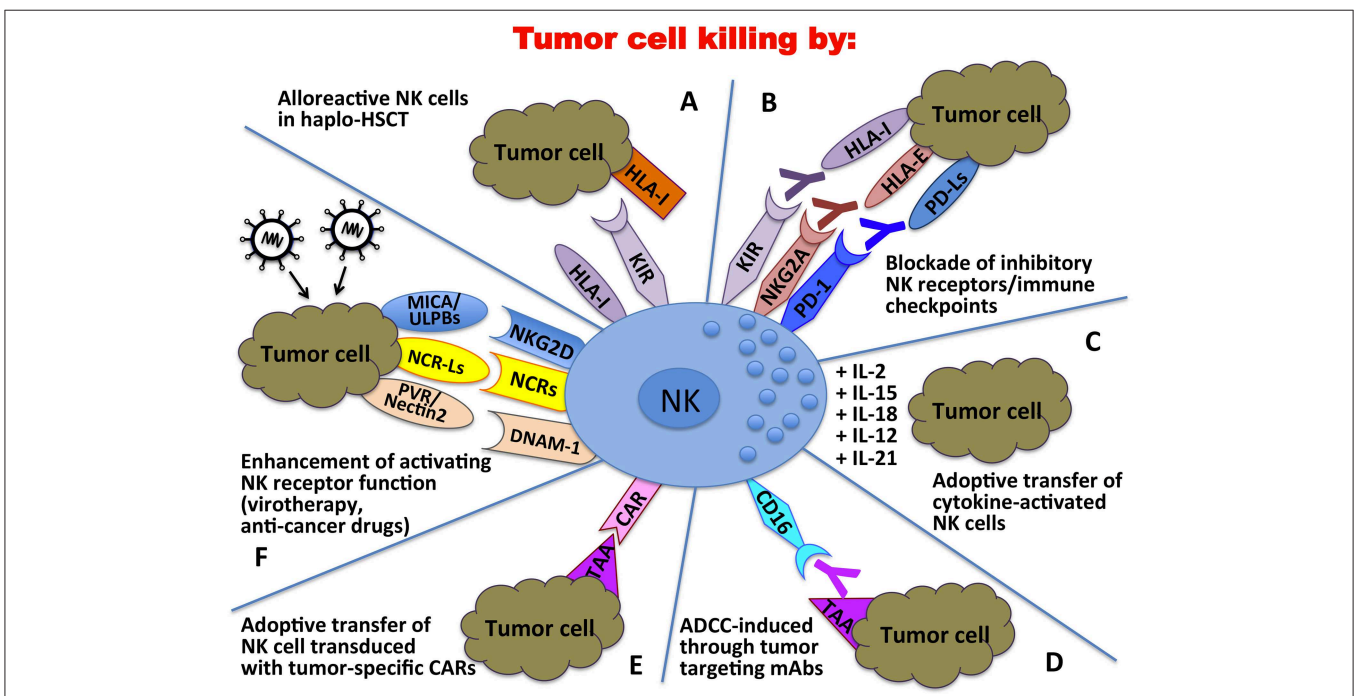

FIGURE 3 | Clinical applications of NK cells in the immunotherapy against tumors. In haplo-HSCT, alloreactive NK cells can kill residual leukemic cells **(A)**; mAbs directed against immune checkpoints can unleash/restore NK cell anti-tumor activity **(B)**; tumor cell killing can be enhanced by adoptive transfer of cytokine-activated NK cells **(C)** or NK cells transduced with tumor-specific Chimeric Antigen Receptors (CARs) **(E)**; tumor targeting mAbs can induce NK cell-mediated ADCC **(D)**; activating NK receptor function can be potentiated through oncolytic virotherapy or the use of anti-cancer drugs **(F)**.

NK CELL-BASED CLINICAL APPLICATIONS

Altogether, these discoveries in the field of NK cell biology (**Figure 2**) (236–243) paved the way to the exploitation of these cells in different anti-tumor therapeutic approaches (**Figure 3**). Over the years important achievements have been obtained, and promising novel strategies have been designed. The most advanced clinical application exploiting the NK cell anti-tumor potential is in the field of haplo-identical HSCT (40–42, 235), in which donor-derived alloreactive NK cells (i.e., unable to recognize recipient HLA class I molecules) can exert a potent anti-leukemia effect. Moreover, the adoptive transfer of NK cells, in an autologous or allogeneic setting, can be pursued following NK cell activation and expansion with cytokines (118–120). The blockade of HLA class I-specific inhibitory receptors using human/humanized mAbs can be used to enhance killing of HLA class I^{pos} tumor cells. These mAbs can be used in combination with mAbs interfering with the PD-1/PD-L axis, as PD-1 can be expressed by human NK cells (46, 244). Another clinical approach is represented by the induction of ADCC against tumor cells by the use of antibodies specific for tumor-associated antigens (119).

More recently, the CAR technology, originally designed for T lymphocytes, has been applied also to NK cells, with promising results in the therapy of both hematological and solid tumors (118, 120). The ever-growing knowledge of activating NK receptor/ligand interactions is being applied in several strategies aimed to potentiate triggering signals through virotherapy or by the use of anti-cancer drugs capable of enhancing the expression of activating ligands on tumor cells and activating receptors on NK cells (102, 117). In conclusion, NK cell-based therapy used in combination with conventional therapeutic protocols could become more and more a powerful tool to be used in the cure of cancer.

CONCLUDING REMARKS

By revisiting the discovery of the most important NK receptors and considering the technical approaches available at that time, one might have the impression that it has been simple to obtain those results. However, experienced researchers know that, actually, relevant pieces of information leading to a new discovery must be selected from an initially confusing, and often contradictory, mass of data. Alessandro had this ability, common to many gifted scientists, but he was also endowed with the uncommon talent of catching essential information and rendering simple what actually is very complex. We think that this has been the true and most important lesson for all of us and, undoubtedly, a major legacy for Immunology and Medicine.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Recent Advances in Lung Cancer Immunotherapy: Input of T-Cell Epitopes Associated With Impaired Peptide Processing

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Recent advances in lung cancer treatment are emerging from new immunotherapies that target T-cell inhibitory receptors, such as programmed cell death-1 (PD-1). However, responses to anti-PD-1 antibodies as single agents are observed in fewer than 20% of non-small-cell lung cancer (NSCLC) patients, and immune mechanisms involved in the response to these therapeutic interventions remain poorly elucidated. Accumulating evidence indicates that effective anti-tumor immunity is associated with the presence of T cells directed toward cancer neoepitopes, a class of major histocompatibility complex (MHC)-bound peptides that arise from tumor-specific mutations. Nevertheless, tumors frequently use multiple pathways to escape T-cell recognition and destruction. In this regard, primary and acquired resistance to immune checkpoint blockade (ICB) therapy was associated with alterations in genes relevant to antigen presentation by MHC-class I/beta-2-microglobulin (MHC-I/β2m) complexes to CD8 T lymphocytes. Among additional known mechanisms involved in tumor resistance to CD8 T-cell immunity, alterations in transporter associated with antigen processing (TAP) play a major role by inducing a sharp decrease in surface expression of MHC-I/β2m-peptide complexes, enabling malignant cells to evade cytotoxic T lymphocyte (CTL)-mediated killing. Therefore, development of novel immunotherapies based on tumor neoantigens, that are selectively presented by cancer cells carrying defects in antigen processing and presentation, and that are capable of inducing destruction of such transformed cells, is a major challenge in translational research for application in treatment of lung cancer. In this context, we previously identified a non-mutant tumor neoepitope, $ppCT_{16-25}$, derived from the preprocalcitonin (ppCT) leader sequence and processed independently of proteasomes/TAP by a mechanism involving signal peptidase (SP) and signal peptide peptidase (SPP). We also provided *in vitro* and *in vivo* proof of the concept of active immunotherapy based on ppCT-derived peptides capable of controlling growth of immune-escaped tumors expressing low levels of MHC-I molecules. Thus,

non-mutant and mutant neoepitopes are promising T-cell targets for therapeutic cancer vaccines in combination with ICB. In this review, we summarize current treatments for lung cancer and discuss the promises that conserved neoantigens offer for more effective immunotherapies targeting immune-escaped tumor variants.

Keywords: lung cancer, cancer immunotherapy, cancer vaccine, tumor escape, antigen presentation and processing, T-cell epitope associated with impaired peptide processing

INTRODUCTION

Lung cancer is the third most frequent cancer worldwide and the leading cause of cancer-related death in Europe and the United States because of its frequency and resistance to currently available treatments (1). About 80–85% of human lung cancers belong to the category of non-small cell lung cancer (NSCLC), including squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large-cell carcinoma (LCC). The remaining 15–20% include small cell lung cancer (SCLC), the most aggressive subtype of lung cancer. In the NSCLC setting, surgery resection is the classical treatment in early stages; chemotherapy in combination with radiotherapy is proposed in locally advanced cancer, and chemotherapy has been the standard of care in advanced diseases over the last 30 years. However, under this management, fewer than 18% of patients reach 5 years of expected survival (2). Particularly in advanced disease, novel therapeutic strategies such as targeted therapies against oncogenic driver alterations or immunotherapy agents have changed the paradigm of NSCLC patients, and are currently being evaluated in combination with other treatments at all stages so as to establish the best standard of care for these cancer patients. However, tumors frequently develop resistance mechanisms to these promising new therapies. Among known mechanisms involved in tumor escape from T-cell-based immunotherapies, alterations in antigen presentation by major histocompatibility complex (MHC) molecules play an important role. In this context, therapeutic cancer vaccines based on T-cell epitopes generated independently of transporter associated with antigen processing (TAP), such as leader sequence-derived peptides, correspond to promising strategies to eradicate tumors with impaired antigen processing and presenting machinery (APM), and thus to overcome tumor escape from CD8 T-cell immunity.

CONVENTIONAL CHEMOTHERAPY AND TARGETED THERAPY IN LUNG CANCER

Platinum-based doublet chemotherapy is the standard first-line treatment for non-selected patients with advanced NSCLC who have a good performance status (3). Patients are treated either with cisplatin or carboplatin, where four to six cycles are administered independently of NSCLC histology (4). Although a benefit in time to progression was observed, this did not translate into a significant decrease in mortality. In preclinical models, it was shown that these chemotherapeutic agents can potentiate immune responses toward cancer cells by increasing the cancer cell mutational load (5) and enhancing the human leukocyte antigen (HLA) expression level (6), leading to better recognition of cancer cells by cytotoxic T lymphocytes (CTL). Moreover, reduced activity of myeloid-derived suppressor cells (7) and enhanced expression of PD-L1 on cancer cells (8) were described following neo-adjuvant chemotherapeutic treatments in patients with SCC. These data may reinforce strategies aimed at combining chemotherapy with immunotherapy in lung cancer.

Approximately 20% of NSCLC tumors have a druggable oncogenic driver, such as the epidermal growth factor receptor (*EGFR*) mutation and anaplastic lymphoma kinase (*ALK*) rearrangement, reported in 11 and 5% of NSCLC, respectively (9). Therapies with EGFR and ALK tyrosine kinase inhibitors (TKI) are standard-first line treatments in *EGFR*-mutant and *ALK*-rearranged NSCLC patients, respectively (10, 11). TKI are non-peptide compounds displaying homologies with the adenosine triphosphate (ATP), permitting competition for the ATP-binding domain of protein kinases, thereby preventing phosphorylation and activation of intracellular signaling pathways, resulting in inhibition of proliferation and apoptosis of cancer cells (12, 13). Results from different phase III clinical trials have reported that these personalized treatments improve the response rate, progression-free survival and quality of life compared to standard first-line platinum-based chemotherapy (10, 14). Improved outcome has also been reported with a combination of antiangiogenic agents plus chemotherapy over chemotherapy as second-line treatment in NSCLC patients, most likely by enhancing cancer cell death (15, 16). However, the efficacy of these drugs is limited, with a response rate of <10%.

LUNG CANCER IMMUNE CHECKPOINT BLOCKADE IMMUNOTHERAPIES

Recent advances in cancer treatment are emerging from new therapies that target T-cell inhibitory receptors, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) (17, 18). Indeed, lung cancer immunotherapy has greatly benefitted from the latest mechanistic understanding of inhibitory molecules expressed on the T-lymphocyte surface

Abbreviations: APM, antigen presentation and processing machinery; β2m, beta-2-microglobulin; CTL, cytotoxic T lymphocyte; CTLA, cytotoxic T-lymphocyte-associated antigen; ER, endoplasmic reticulum; HLA, human leukocyte antigen; IFN, interferon; ICB, immune checkpoint blockade; mAb, monoclonal antibody; MHC-I, major histocompatibility complex-class I; NSCLC, non-small-cell lung carcinoma; PD-1, programmed cell death-1; ppCT, preprocalcitonin; TEIPP, T-cell epitope associated with impaired peptide processing; TIL, tumor-infiltrating T lymphocyte; TCR, T-cell receptor.

and involved in modulating antigen-specific T-cell responses. This led to development of promising novel immunotherapies, especially blocking anti-CTLA-4 and anti-PD-1 monoclonal antibodies (mAb), which inhibit interaction between the inhibitory receptor on T cells and its ligands on cancer cells, thereby reestablishing T-cell reactivity and anti-tumor effector functions. These immunotherapies have reported an overall survival benefit compared to standard second-line chemotherapy (docetaxel), and recently, also, in first line platinum-based chemotherapy. During early clinical trials in NSCLC patients treated with anti-PD-1 or anti-PD-1 ligand (PD-L)1 mAb, response rates ranged from 16 to 23%, and some patients showed prolonged disease stability with unprecedented long-term survival (19–21). For instance, the anti-PD-1 nivolumab, as second-line therapy in advanced NSCLC compared with docetaxel, was reported to improve the response rate and overall survival in two randomized phase III trials (22, 23). Pembrolizumab, another anti-PD-1, prolongs survival compared with docetaxel as second-line treatment, with at least 1% of tumor cells expressing PD-L1 (24); and as a front line, pembrolizumab has demonstrated improved survival and response compared to platinum-based chemotherapy if $\geq$50% of tumor cells express PD-L1 (21). Since NSCLC is generally considered resistant to immunotherapy, these results are rather promising. Another attractive feature of these therapies is that both anti-PD-1 and anti-PD-L1 mAb are well-tolerated, with little grade 3–4 toxicity. Moreover, the success of the immune checkpoint blockade (ICB) appears to extend to other thoracic tumors such as SCLC, mesothelioma and epithelial thymic tumors (25).

A combination of different immune checkpoint inhibitors, such as anti-CTLA-4 plus anti-PD-1, has showed a manageable tolerability profile with anti-tumor activity and clinical benefits (26). Preliminary trials assessing combination therapies with anti-PD-1/PD-L1 plus anti-CTLA-4 have documented considerable advantages in survival indices over single-agent immunotherapy (27, 28). In particular, in lung cancer with a high tumor mutational burden (TMB), defined as $\geq$10 mutations/megabase (Mb) and used as a novel predictive biomarker of response to immunotherapy (29), a combination of nivolumab plus ipilimumab demonstrated better outcome than chemotherapy in first-line NSCLC patients (30, 31). As $CD8^+$ tumor-infiltrating T lymphocytes (TIL) express a large panel of immune checkpoints, including TIM-3, LAG-3, TIGIT, and BTLA (32, 33), several neutralizing antibodies toward these inhibitory receptors are under development for use as drugs in patients with cancer, including lung cancer. Moreover, since each of these immune checkpoints uses a particular mechanism to negatively regulate CD8 T-cell function, studies are attempting to combine different neutralizing antibodies to potentiate the response to immunotherapy. For instance, a combination of anti-PD-1 plus anti-LAG-3 is currently in phase II clinical trials in patients with several solid cancer types, including NSCLC (NCT03365791), as well anti-PD-1 plus anti-TIM-3 antibodies (NCT03708328) and anti-PD-1 plus anti-TIGIT (NCT03563716).

However, independently of the ICB subtype (24, 34, 35), the overall response rate in second-line treatment for unselected NSCLC patient populations is <20%. In addition, predictive response factors, such as PD-L1 expression $\geq$50% tested by immunohistochemistry, and immune mechanisms involved in the response to these therapeutic interventions, remain elusive and poorly elucidated. Accumulating evidence indicates that effective anti-tumor immunity is associated with the presence of T cells directed toward cancer neoepitopes, a class of MHC-bound peptides that arise from tumor-specific mutations (36–38). These neoantigens are highly immunogenic, since they are not expressed by normal tissues and thus bypass central thymic tolerance. Unfortunately, spontaneous T-cell responses to these mutation-derived tumor antigens are inefficient. Indeed, tumors frequently use multiple pathways to escape immune recognition and elimination. In this regard, primary and acquired resistance to ICB therapies was associated with alterations in genes relevant to antigen presentation by MHC class I/beta-2-microglobulin (MHC-I/β2m) complexes to $CD8^+$ T cells (39–41). Mutations in the Janus kinase (JAK)1/JAK2 and interferon (IFN) signaling pathway resulted in the inability to respond to IFN-γ, and thus, the impossibility of improving antigen presentation by MHC-I molecules (39, 42, 43). Moreover, clonal deletion of tumor-specific T cells by IFN-γ was recently reported to confer resistance to ICB therapies (44). Therefore, major efforts are currently being made to develop active immunotherapies to stimulate tumor-specific T cells and induce their proliferation, so as to eliminate malignant cells, including resistant tumor variants.

TUMOR-ASSOCIATED ANTIGEN-BASED AND MUTANT ANTIGEN-BASED THERAPEUTIC CANCER VACCINES

CTL are the main effectors of the immune system, capable of destroying transformed cells following recognition, by the T-cell receptor (TCR), of specific epitopes presented by MHC-I/β2m complexes. Thus, several active immunotherapies have been generated to trigger strong and persistent anti-tumor CTL responses in order to destroy primary tumors and metastases. Current cancer vaccines consist of activating tumor-specific T cells via therapeutic vaccination of cancer patients with tumor-associated antigens (TAA), such as cancer testis antigens and differentiation antigens (45, 46), mutant antigens (47, 48), and viral antigens (49, 50). TAA are relatively restricted to cancer cells, and, to a limited degree, to normal tissues; whereas mutant antigens, also classified as tumor-specific antigens (TSA) or neoantigens, are expressed only in cancer cells, arising from gene mutations that result in novel abnormal protein synthesis (51). Optimal therapeutic cancer vaccines include synthetic long peptides derived from these antigenic proteins administered with an adjuvant, as well as DNA and RNA vaccines delivered to both MHC-I and MHC-II molecules of professional antigen-presenting cells (APC), namely dendritic cells (DC), thereby promoting both CD8 and CD4 T-cell responses in patients with cancers (50, 52, 53).

Thus far, therapeutic vaccination with TAA, such as MAGE-A3, PRAME, or MUC1, had not revealed objective cancer

regression, nor an increase in disease-free survival in patients with NSCLC (54–57). However, improved survival in NSCLC was observed in some patients and correlated with development of CD8 T-cell responses against targeted TAA and non-targeted TAA, demonstrating induction of epitope spreading (58). The era of more successful therapeutic vaccines has arrived, with accessibility to next-generation sequencing (NGS) technology and *in silico* epitope prediction, contributing to identification of patient-specific tumor antigens generated by somatic mutations in individual tumors. These neoantigens have opened up new perspectives in therapeutic cancer vaccines against a wide variety of cancer types, and possibly also against virally induced epithelial cancer (59). Vaccination directed against these tumor-encoded amino acid substitutions resulted in an increase in antigenic breadth and clonal diversity of neoantigen-specific T lymphocytes in melanoma (60). Moreover, clinical benefits of neoantigen-based cancer vaccines have been observed in melanoma patients (47, 48). This neoantigen-based strategy is now in development in many cancers, including lung cancer, with tumor regression observed in a SCC patient (61). A feasibility study of personalized neoepitope peptide vaccination has also been performed on advanced SCLC patients, with possible prolongation of overall survival (62). In another randomized phase II study, personalized peptide vaccines in combination with docetaxel did not improve survival of patients with previously treated advanced wild-type EGFR NSCLC (63). This may be due to the multiple resistance pathways developed by the tumor itself to evade the induced neoantigen-specific T-cell response. In this regard, acquired resistance to a neoepitope-based cancer vaccine associated with the outgrowth of β2m-deficient cancer cells was observed in a vaccinated melanoma patient (47).

PROMISING NON-MUTANT NEOEPITOPE-BASED CANCER VACCINES

Most antigenic peptides derived from TAA and recognized by CD8 T lymphocytes originate from cleavage in proteasomes of intracellular proteins and their transport, by TAP, from the cytosol into the endoplasmic reticulum (ER) (64). The resulting 9-to-10-amino acid peptides bind to MHC-I molecules and are then presented on the surface of transformed cells for CD8 T-cell recognition. Among known mechanisms frequently used by cancer cells to evade CTL recognition and killing, alterations in TAP play a major role by inducing a sharp decrease in surface expression of MHC-I/β2m-peptide complexes, enabling malignant cells to become "invisible" to CD8 T cells (65–71). Downregulation of MHC-I molecules has been found to range from 16 to 50% among primary lesions from various types of human carcinomas (72). Moreover, between 39 and 88% of human tumors were reported to be MHC-I deficient, including 73% of lung cancers with a total loss of class I molecules in 38% and loss of A locus and A2 allele in 8.3 and 27% of the analyzed cases, respectively (73, 74); for a review see (75). Our earlier studies have stressed downregulation of TAP1 and/or TAP2 in lung cancer cells, resulting in resistance to TCR-dependent lysis (76). TAP deficiencies have been observed in a wide variety of human cancers, including cervical carcinoma (68), head and neck carcinoma (69), melanoma, gastric cancer (65, 66), and lung cancer, with up to 70% of NSCLC expressing low levels of TAP1 and/or TAP2 (77), and are associated with tumor escape from immune system control. Therefore, the discovery of alternative pathways for tumor antigen processing may improve anti-tumor T-cell responses and T-cell-based immunotherapy strategies. In this regard, the generation of tumor-specific CTL epitopes from PRAME and melanoma antigen MART-1 required cytosolic endopeptidases nardilysin and thimet oligopeptidase (78). A CD8 T-cell epitope derived from MAGE-A3 TAA processed independently of the proteasome by the insulin-degrading enzyme has been described (79). Moreover, a CTL epitope derived from the signal sequence of melanoma-associated tyrosinase is processed by a proteasome/TAP-independent pathway (80). These tumor epitopes appear to be promising targets for active immunotherapy to induce CTL responses toward APM-impaired tumors.

Remarkably, T Van Hall's group previously reported that T lymphocytes specific to a murine non-mutated self-epitope, derived from the C-terminus region of the TRH4 protein and defined as a T-cell epitope associated with impaired peptide processing (TEIPP), were selected in the thymus of TCR-transgenic mice and activated by peptide-based vaccination, leading to progression control of TAP-deficient tumors expressing low levels of MHC-I/peptide complexes (81). Using a combinatorial screening approach, this group more recently identified human non-mutated neoantigens presented by TAP-deficient cancer cells (82). TEIPP neoantigens are derived from ubiquitous non-mutant self-proteins that are not naturally loaded into MHC-I molecules of healthy cells, because they express standard levels of TAP. Their surface presentation is induced in transformed cells following downregulation or loss of TAP (82). Thus, targeting these non-mutated neoantigens is a promising potent approach to inducing specific responses to tumors displaying low MHC-I expression associated with downregulation or loss of TAP subunits.

In humans, we had previously identified a non-mutated tumor neoepitope recognized on a NSCLC tumor cell line by autologous CTL clones isolated from TIL of a lung cancer patient. This epitope ($ppCT_{16-25}$) is generated from the carboxy-terminal region of the preprocalcitonin (ppCT) signal sequence, and is processed by a mechanism independent of proteasomes and TAP, involving signal peptidase (SP) and signal peptide peptidase (SPP) (76, 83). It is released in the ER lumen and presented to CTL by MHC-I molecules on the surface of immune-escaped cancer cells expressing low levels of HLA-class I due to defects in TAP expression. We showed that most human lung tumors frequently express ppCT self-antigen and display altered expression of TAP molecules. Using epitope prediction software, we also showed that ppCT includes additional HLA-A2-restricted T-cell epitopes that are processed by TAP-dependent pathways. Processing occurs in the cytosol, either after retrotranslocation of a procalcitonin (pCT) substrate by the ER-associated degradation (ERAD) pathway ($ppCT_{50-59}$ and $ppCT_{91-100}$) or release of a signal peptide precursor by SPP ($ppCT_{9-17}$) (77). Thus, CD8 T-cell epitopes from signal

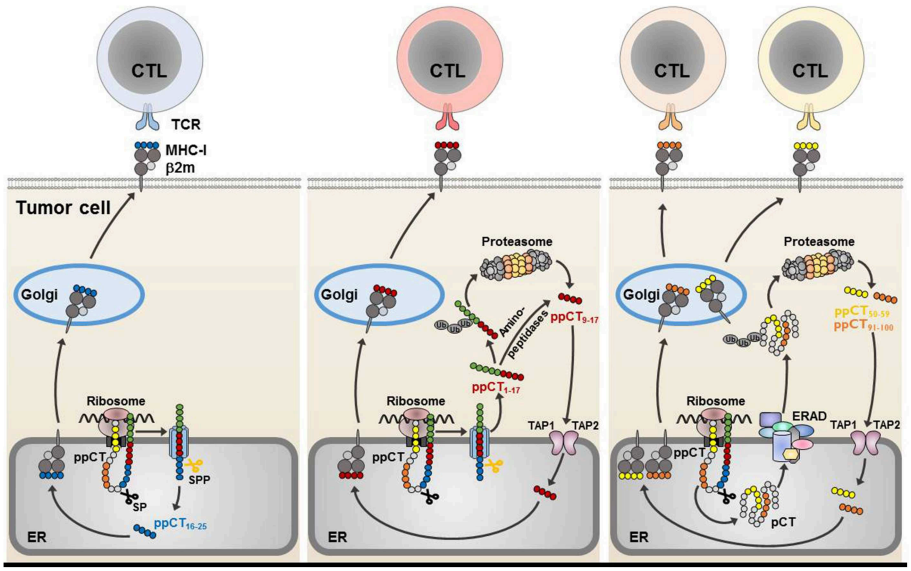

FIGURE 1 | Processing of CD8 ppCT T-cell epitopes. The ppCT signal peptide has a type II orientation (the NH2-terminal region exposed toward the cytosol and the COOH-terminal region facing the endoplasmic reticulum lumen). The $ppCT_{16-25}$ epitope is processed by SP and SPP independently of TAP, and is released directly into the endoplasmic reticulum **(left)**. After cleavage by SP and SPP, a $ppCT_{1-17}$ signal peptide fragment is released into the cytoplasm, to be processed by the proteasome/TAP pathway so as to give rise to the $ppCT_{9-17}$ epitope. The $ppCT_{9-17}$ epitope may also be generated from a $ppCT_{1-17}$ signal peptide fragment by cytosolic proteases before transport into the endoplasmic reticulum lumen by TAP **(middle)**. $ppCT_{50-59}$ and $ppCT_{91-100}$ epitopes are generated by the TAP/proteasome pathway after retrotranslocation of the pCT precursor protein from the endoplasmic reticulum lumen into the cytosol by the ERAD pathway **(right)**. All generated ppCT epitopes bind within the endoplasmic reticulum to HLA-A2 molecules before externalization to the target cell membrane for recognition and elimination by specific CD8 T lymphocytes. ER, endoplasmic reticulum; MHC-I, MHC-class I; Ub, ubiquitin.

peptides with a type II signal anchor (84), like the ppCT leader sequence (83, 85), are released into the ER lumen and presented to CTL by MHC-I in a TAP-independent manner (such as the $ppCT_{16-25}$), or into the cytoplasm where they are further processed by the proteasome or cytosolic proteases (like the $ppCT_{9-17}$). The latter epitope is then transported by TAP into the ER, where it binds to the HLA-A2 molecules and is then conveyed to the cell surface to be recognized by CD8 T cells (**Figure 1**).

To resolve the tumor heterogeneity question regarding the TAP expression level, we selected a cocktail of five ppCT immunogenic peptides, including the HLA-A2-restricted TAP-independent $ppCT_{16-25}$ neoepitope, the two ppCT epitopes processed by TAP-dependent pathways ($ppCT_{9-17}$ and $ppCT_{50-59}$) and two ppCT long peptides ($ppCT_{1-15}$ and $ppCT_{86-100}$). We provided *in vivo* proof of concept of a therapeutic cancer vaccine based on this ppCT peptide cocktail, delivered with poly(I:C) adjuvant, which was capable of inducing anti-tumor CTL responses in HLA-A*0201/HLA-DR3-transgenic (HHD-DR3) mice and in NOD-*scid-Il2rγ*^{null} (NSG) mice adoptively transferred with human HLA-A2^{+} peripheral blood mononuclear cells (PBMC), resulting in growth control of established lung tumors expressing low levels of HLA-A2/ppCT peptide complexes (77). Thus, non-mutant TEIPP, such as $ppCT_{16-25}$, represent attractive candidates for more effective cancer immunotherapies and combination therapies aimed at APM-impaired tumor variants. Signal sequence-derived peptides correspond to promising targets for therapeutic cancer vaccines against TAP-deficient tumor cells. Indeed, these TAP-independent self-peptides are not presented by normal cells displaying a standard processing status and emerge at the surface of tumor cells following alterations in APM components. This might explain their immunogenicity and ability to trigger effective anti-tumor T-cell responses.

CONCLUDING REMARKS

Personalized RNA mutanome vaccines and multipeptide neoantigen vaccines have recently demonstrated their efficacy in melanoma (47, 48). Active immunotherapies with predicted tumor neoepitopes were even more efficient when combined with anti-PD-1 mAb. However, recurrence associated with defects in APM was frequently observed. In this context, TEIPP-specific T cells are valuable effectors against immune-edited MHC-I^{low} targets that have acquired resistance to immunotherapy (86). Signal-sequence-derived peptides and their carrier proteins, such as ppCT, are attractive candidates for specific therapeutic cancer vaccines to target tumors that had downregulated HLA-class I molecules due to alterations in TAP expression. Cancer vaccines based on non-mutant neoantigens presented by TAP-deficient tumors expressing low levels of MHC-I/peptide complexes, in association with individualized tumor mutanome epitopes, represent a major challenge in lung cancer. The specificity of therapeutic vaccination with mutant and non-mutant neoepitopes combined with ICB

offers an attractive strategy for future cancer immunotherapies. A combination of such active immunotherapies with ICB would permit expansion of pre-existing antigen-specific T cells and induction of a broader repertoire of T-cell specificities, enhancing tumor progression control and leading to elimination of most cancer cells, including immune-escaped variants, and destruction of ≪ immune deserts or cold tumors ≫ that are weakly infiltrated by lymphocytes. ICB, such as anti-PD-1 and anti-PD-L1 mAb, would optimize the anti-tumor activities of cancer-vaccine-induced T cells by inhibiting the interaction of PD-1 on these activated tumor-specific T cells with PD-L1/-L2 on the target cell surface, overcoming T-cell exhaustion and resulting in malignant cell eradication.

AUTHOR CONTRIBUTIONS

ML and FM-C coordinated the writing of the manuscript and participated in drafting and editing the text and figure. All authors gave final approval to the version submitted. LM, GG, IT, IM, and SC participated in writing the manuscript.

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REVIEW
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From Natural Killer Cell Receptor Discovery to Characterization of Natural Killer Cell Defects in Primary Immunodeficiencies

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Alessandro Moretta was Professor of Histology at University of Brescia from 1994 to 1997. It was in that period that we met and started a collaboration that continued in the years to follow. He immediately involved us in the production of monoclonal antibodies (mAbs) that allowed the identification and fine characterization of novel receptor molecules that were able to activate or inhibit human Natural Killer cell function, including several antibodies specific for Natural Cytotoxicity Receptor (NCR) and Killer-cell Immunoglobulin-like Receptor (KIR) molecules. These reagents, generated in our laboratory in Brescia, contributed to complete the studies aimed to characterize innate lymphoid NK cells, that had been initiated by Alessandro and his brother Lorenzo in Genoa. Soon, we identified an anti-KIR3DL2 that was subsequently shown to be helpful for the diagnosis and treatment of various forms of cutaneous T cell lymphoma. While in Brescia, Alessandro established a partnership with those of us who were working in the Department of Pediatrics; together, in short time we tackled the goal of studying the role of NK cells in patients with primary immunodeficiencies. This collaboration led to novel discoveries that shed light on the critical role played by NK cells in the immune response against virus and tumors in humans, as best exemplified by our characterization of the molecular mechanisms of impaired control of Epstein-Barr Virus (EBV) infection in patients with X-linked lymphoproliferative (XLP) disease. After Alessandro left Brescia to return to Genoa, our collaboration continued with the same enthusiasm, and even from a distance he remained an extraordinary example of an inspirational and generous mentor. This review is a sign of our gratitude to a mentor and a friend whom we deeply miss.

Keywords: natural killer cells, primary immunodeficiencies, monoclonal antibodies, functional assays, signaling transduction

INTRODUCTION

We (Silvia and Luigi) met Alessandro Moretta for the first time in 1994, when he became full Professor of Histology at the University of Brescia. He remained there until 1997, but we continued to collaborate with him even after his return to the University of Genoa. Since then, Alessandro was my mentor, inspiring my work and transmitting to me his passion for research.

He immediately attracted my curiosity by sharing with me his passion on natural killer (NK) cells. In particular, at the time of his arrival in Brescia, Alessandro was working at the production of monoclonal antibodies (mAbs) directed against NK cells. He involved me in this project; together, we immunized mice with human NK cells that had been expanded *in vitro* with interleukin-2 (IL-2). Upon generating hybridomas from the spleen of treated mice, screening their supernatant, and isolating monoclonal antibody-producing cells through limiting dilution, we produced a large number of monoclonal antibodies directed against various NK cell receptor molecules. This work eventually resulted in the identification of novel surface molecules that modulate NK cell function. Furthermore, from the very beginning of this project Alessandro engaged another one of us (Luigi, also known as Gigi), with the aim of studying the role that NK cells may play in causing a higher risk of infections and malignancies in patients with primary immune deficiency (PID). In those years, the role of NK cells in human immune defense was not fully appreciated; in particular, it was known to what extent NK cell dysfunction contributes to the unique susceptibility to severe Epstein-Barr virus (EBV) infection that characterizes several forms of PID. If successful, these studies would help better understand human NK cells development, function and homeostasis, and could also shed some light on the development and function of other, less well-understood subsets such as adaptive and memory-like NK cells.

Over the year, the collaboration with Alessandro has been a wonderful journey that allowed us to discover the most intriguing aspects of NK cell biology.

GENERATION OF DIFFERENT ISOTYPES OF mAbs SPECIFIC FOR RECEPTOR MOLECULES THAT CONTROL AND REGULATE NK CELL FUNCTION

NK cells were originally identified on the capability of killing certain tumor cell lines in the absence of deliberate previous stimulation. More recently, it has become evident that NK cells play other important roles in immune responses, beyond cell-mediated cytotoxicity (1–3).

Upon engagement of various NK receptors and in response to certain cytokines, NK cells display regulatory functions that are especially important in the early inflammatory response that follows acute infection. After recruitment into peripheral tissues in response to chemokine gradients, NK cells must undergo a priming process in order to acquire full functional competence before migrating toward lymph nodes. NK cell priming takes place when they interact with other innate immunity cell types, that are either resident or that are recruited in peripheral tissues during inflammation, and that release a set of relevant cytokines. In addition, NK cell activity is enhanced by the recognition of virus-infected or tumor target cells (4).

A dynamic balance between inhibitory and activating NK cell receptors controls NK cell effector functions. NK cell activation can be restrained by various inhibitory receptors that include a family of strictly homologous surface molecules referred to as Killer-cell Immunoglobulin-like Receptor (KIRs) molecules, that recognize unique patterns of HLA (Human Leucocyte Antigen) class I alleles or, in the case of NKG2A/CD94 heterodimer, non-classical HLA-E alleles.

The nature and the number of ligands expressed by target cells for NK activating and inhibitory receptors is the main factor that determines susceptibility of such target cells to NK-mediated lysis (1–3). In cells undergoing viral infection or tumor transformation, alterations (and/or down-modulation) of HLA class I molecules that include either the whole HLA class I phenotype, or selected alleles, are frequently observed (5).

Since inactivation of NK cell function represents a central safety mechanism to prevent killing of self HLA class-I^+ cells, it was necessary to postulate that in order to kill self HLA class-I^+ cells under appropriate conditions (viral infection or tumor transformation), NK cells must express also activating receptors. In those years, Alessandro successfully identified three important activating NK receptors named Natural Cytotoxicity Receptors (NCRs) recognizing non-HLA ligands (6–11).

In addition to NCRs (NKp30, NKp44, and NKp46), NK cell activation can also be induced upon signaling through synergism of activating and costimulatory NK cell receptors including NKG2D, DNAX accessory molecule-1 (DNAM-1), 2B4, NTB-A, CD59, NKp80, CD2, and CD94/NKG2C. In fact, particular combination of activating receptor may trigger NK cell activation more efficiently than others. Moreover, NK cell activating receptors may differently recognize ligands expressed on target cells in qualitatively distinct events (12).

Alessandro Moretta described the NK cell subsets by his numerous publications in this way: "in human peripheral blood can be distinguished two NK cell populations characterized by different density of CD56 and CD16 expression on the cell surface: $CD56^{bright}CD16^{-/low}$ and $CD56^{dim}CD16^{bright}$ cells. These two NK cell subsets differ for the expression pattern of various other cell surface and chemokine receptors. A characteristic of immature, $CD56^{bright}$ NK cells is the expression of high levels of NKp46, CD94/NKG2A, and CCR7. By contrast, mature $CD56^{dim}$ cells express CXCR1 and KIRs at higher density. Furthermore, $CD56^{bright}$ and $CD56^{dim}$ NK cells have distinct functional properties, with $CD56^{bright}$ cells being potent producers of cytokines, and $CD56^{dim}$ cells being active mediators of natural and antibody-dependent cellular cytotoxicity, as also reflected by higher intracellular levels of perforin and granzymes. In healthy donors, $CD56^{bright}$ cells comprise a minority (5–10%) of all circulating NK cells, but because they express CCR7, they can migrate to secondary lymphoid organs where they represent the predominant NK cell subset. A subset of $CD56^{dim}$ KIR^+ NK cells, expressing CD57 represent terminally differentiated NK cells, whereas a further subset expressing the $CD56^-$ $CD16^+$ $CD57^+$ KIR^+ phenotype were thought to represent exhausted NK cells and were characterized in HIV^+ patients" (13–17).

When Alessandro arrived in Brescia, in collaboration with his brother Lorenzo, he had already produced several mAbs that had permitted to identify novel inhibitory and activating receptors and co-receptors of human NK cells. A first category of NK receptors identified with inhibitory and activating functions included p58 (KIR2DL1/L2) and p50 (KIR2DS1/S2) molecules,

identified by EB6 and GL183 mAbs, respectively. These p58 and p50 molecules are part of the family of Killer Immunoglobulin-like receptors (KIRs), and they both recognize HLA-C.

In order to continue the studies on KIR molecules, after identifying molecules characterized by three domains KIR3DL1/S1/L2 (p70 and p140), Alessandro and I (Silvia), in Brescia, produced a novel mAb, named AZ158, that was able to recognize both p70 and p140 molecules. Important features of this were: first, that it binds the same epitope shared by the KIR3DL2 in either dimeric or monomeric form, as well as the monomeric KIR3DL1 and KIR3DS1, present as monomeric (p70) or dimeric (p170) form; second, that mAb AZ158 is of IgG2a isotype, and therefore different from the other anti-KIR3D mAbs that are of IgG1 isotype. This permitted to analyze NK cell subsets characterized by clonal expression of various KIRs through multi-fluorescence flow cytometry (**Figure 1A**).

Production of a reagent that specifically binds KIR3DL2 had immediate clinical translational implications. In fact, KIR3DL2 is expressed not only by NK cells, but in patients with mycosis fungoides and Sézary syndrome it is also expressed by malignant $CD4^+$ T cells. For this reason Alessandro, in collaboration with M. Bagot and A. Bensussan and with Innate Pharma, developed clinical studies for the validation of KIR3DL2 as a tumor antigen in various forms of cutaneous T cell Lymphoma that led to a successful clinical trial (18) (2003 patent no. US 9, 828, 427 B2).

At a later time, three other non-HLA-specific receptors (NKp46, NKp30, and NKp44) were also discovered by Alessandro. These NCRs play a crucial role in the NK-mediated recognition and killing of most target tumor and/or virus-infected cell lines expressing NCR-ligands. NKp46 and NKp30 are expressed by both resting and activated NK cells, while the expression of NKp44 is restricted to activated NK cells (6, 9, 10). It is of note that NCRs display a strictly coordinated pattern of surface expression. In particular, they are collectively expressed by NK cells either at high or at low surface density; consequently, NK cells display either an NCR^{bright} or an NCR^{dull} phenotype (19). In the laboratory at the University of Brescia, we detected other mAbs specific for NCR molecules that helped perform biochemical studies to further characterize NCR function. In particular, production of mAb of IgM isotype to NKp44 and NKp46 (KS38 and KL247 mAbs, respectively) allowed us to perform NCRs masking experiments in the search of possible tumor ligands (**Figure 1C**). Specifically, the use of these IgM isotype antibodies allowed to identify the presence of the major or minor expression of NCR-ligands recognized by NK cells that showed different cytotoxic capability against various histological tumor cell lines. These masking experiments allowed to obtain new results on the functionality of NK cells and their receptors in addition to those already obtained with the redirected killing assays. In fact, in these tests, the cross-linking between NK receptor specific antibodies and FcγR expressed on P815 murine tumor cell, mimics receptor function while masking tests block receptor-ligand interaction (**Figure 1B**).

Moreover, we also generated mAbs against CD244, NTB-A, NKp80, DNAM-1, and CD59 co-receptor molecules. Use of these reagents in appropriate *in vitro* assays was critical to characterize the function of each of these molecules (20–23). The results obtained clearly demonstrated that differences in response do not reflect a functional heterogeneity of co-receptor expression but rather depend on the co-engagement of triggering receptors.

Finally, while Alessandro's brilliant imagination allowed us to produce such precious reagents and to obtain novel mechanistic insights into regulation of NK cell function, his generosity in sharing these products with the scientific community at large paved the way for a fine characterization of NK cell dysfunction in various forms of Primary Immunodeficiencies (PID).

NK CELLS AND PRIMARY IMMUNODEFICIENCIES

NK cells are innate immune cytotoxic effector cells well-known for their role in antiviral immunity and tumor immunosurveillance. To date, a growing body of evidence indicates that NK cells play an important role in anti-viral immune responses. In particular, the study of patients suffering either from rare isolated NK cell deficiencies or more profound immunodeficiency syndromes has offered novel insights into NK cell biology. In this regard, PIDs provide unique opportunities for better understanding the specific role played by individual molecules in NK cell function and anti-viral immune response in particular, as also exemplified by the results of our exciting collaboration with Alessandro at the University of Brescia.

Functional Analysis of Activating NK Cell Receptors in X-Linked Lymphoproliferative Disease (XLP1)

X-linked lymphoproliferative disease type 1 (XLP1) is a severe immunodeficiency that affects approximately 1 in 1×10^6 males and is characterized by extreme susceptibility to EBV infection, leading to fatal infectious mononucleosis, lymphoma, aplastic anemia, and/or dysgammaglobulinemia, and a high rate of death early in life (24). In 1998, several groups discovered the genetic basis of XLP1 (25–27). In particular, the gene mutated in XLP1 is named Src homology 2 domain containing protein 1 A (*SH2D1A*), and encodes for a small adaptor molecule that plays a crucial role in signaling via a number of surface molecules expressed by various cells of the immune system (28). This intracytoplasmic polypeptide of 128 amino acids, also named SAP (Signaling lymphocyte-activating molecule-Associated Protein), is expressed by T lymphocytes and NK cells, but not by normal B cells. While it was logical to assume that abnormal function of cytotoxic T lymphocytes (CTLs) and possibly NK cells could play a role in the pathophysiology of XLP1 (29), the basis of selective susceptibility to EBV infection, and the role played specifically by SAP mutations in this process, remained unclear. In the attempt to address these questions, we studied NK cells from two XLP1 patients.

The surface molecules that bind to SAP include a group of receptors belonging to the Immunoglobulin Superfamily, and in particular two NK cell co-receptors with activating function: 2B4 (CD244) and NTB-A, a novel surface molecule that had been recently identified by Alessandro's group (22, 28, 30).

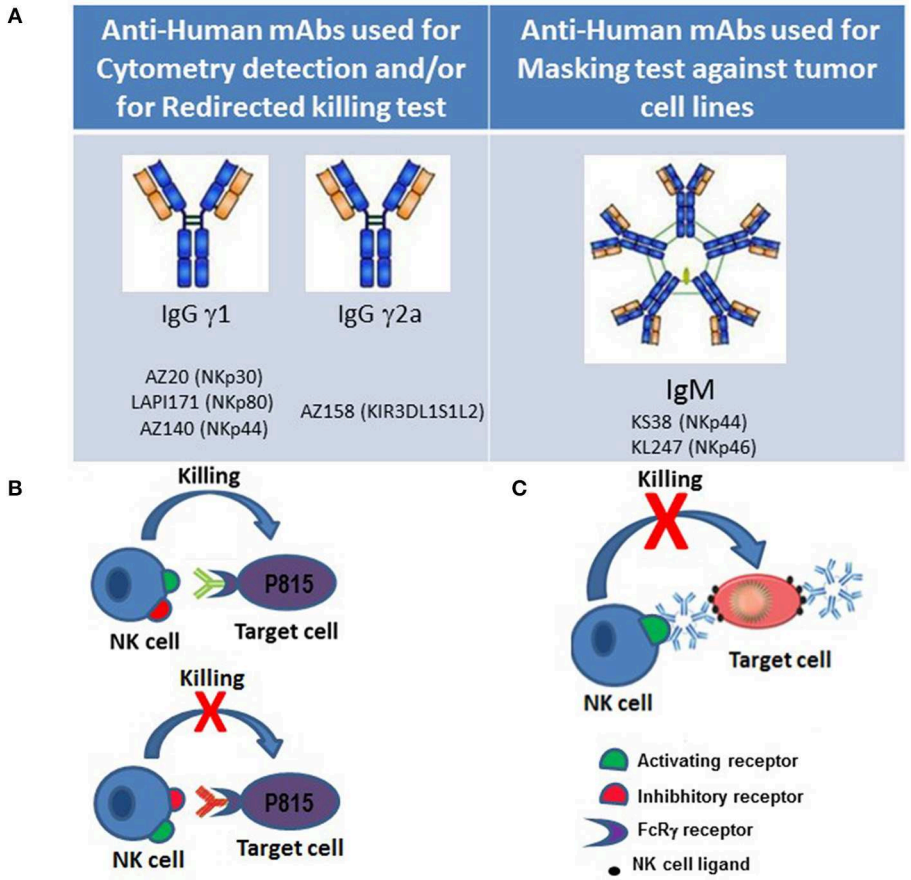

FIGURE 1 | Approaches to functional studies of NK cell receptors. **(A)** Isotypes of monoclonal antibodies against NK cell surface molecules that were generated in our laboratory and used for flow cytometry and/or cytotoxic assays against tumor cell lines. **(B)** In redirected-killing assays, the murine mastocytoma FcγR$^+$ P815 cell line is used as target. Binding of murine mAbs of γ1, γ2a, and γ2b IgG isotypes to NK receptors on one hand, and to the FcγR on P815 cells on the other, allows cross-linking of activating and inhibitory NK cell receptors, thereby permitting to study their role in NK cell-mediated cytotoxicity. **(C)** Anti-NK cell activating receptors of IgM isotype can mask receptor-ligand interactions blocking cytolysis of target cells.

Human 2B4 is a 70 kD glycoprotein expressed on NK cells and its engagement by mAb-mediated cross-linking of 2B4 or by its ligand CD48 resulted in enhancement of cytolytic activity. Because 2B4 is a co-receptor, its ability to induce NK cell activation is dependent upon the co-engagement of main triggering NK cell receptors including NKp46 (20). So, upon mAb-mediated engagement, 2B4 undergoes tyrosine phosphorylation and associates SAP. Under normal conditions, this association appears to be crucial for SHIP-1 displacement and for the transduction of the activating signals (**Figure 2C**) (28, 30).

Various studies had suggested that "SAP could act as a regulator of signaling by competitive displacing of SHP molecules from the cytoplasmic tails of the receptor. Thus, SAP was likely to prevent the generation of SHP-mediated inhibitory signals (28, 30). In our work, we reported that 2B4 function was dramatically altered in XLP1 patients carrying non-sense *SH2D1A* mutations. We demonstrated that in such patients 2B4 associates with SHIP-1, and transduces inhibitory signals. Importantly, CD48, the ligand of 2B4, is up-regulated in EBV–infected B cells (28, 30). Thus, engagement of 2B4 by CD48 determined not only an inhibition of NK-mediated lysis, but also a shutdown of activating signals engaged by other ligands in addition to CD48 recognized by NK cells, leading to the complete inability to kill EBV$^+$ targets" (30). Remarkably, the cytolytic activity of XLP1-NK cells against EBV-infected targets could be restored by mAb-mediated disruption of the 2B4/CD48 interactions. Altogether, the altered function of 2B4 may account for a general inability of different cytolytic effector cells to control EBV-infection.

Soon thereafter, Alessandro's research group discovered a novel 60-kD glycoprotein, termed NTB-A that is expressed by human NK, T, and B lymphocytes. Similar to 2B4 (CD244), NTB-A belongs to the CD2 subfamily of immunoglobulin-like molecules, and functions as an activating co-receptor. Its structure is characterized by a cytoplasmic domain containing three tyrosine-based motifs that, when NTB-A undergoes tyrosine phosphorylation, allow binding to SAP. Interestingly, in XLP1 patients, NTB-A, similar to 2B4, transduces inhibitory rather than activating signals. Furthermore, mAb-mediated masking experiments suggested that NTB-A may recognize cell

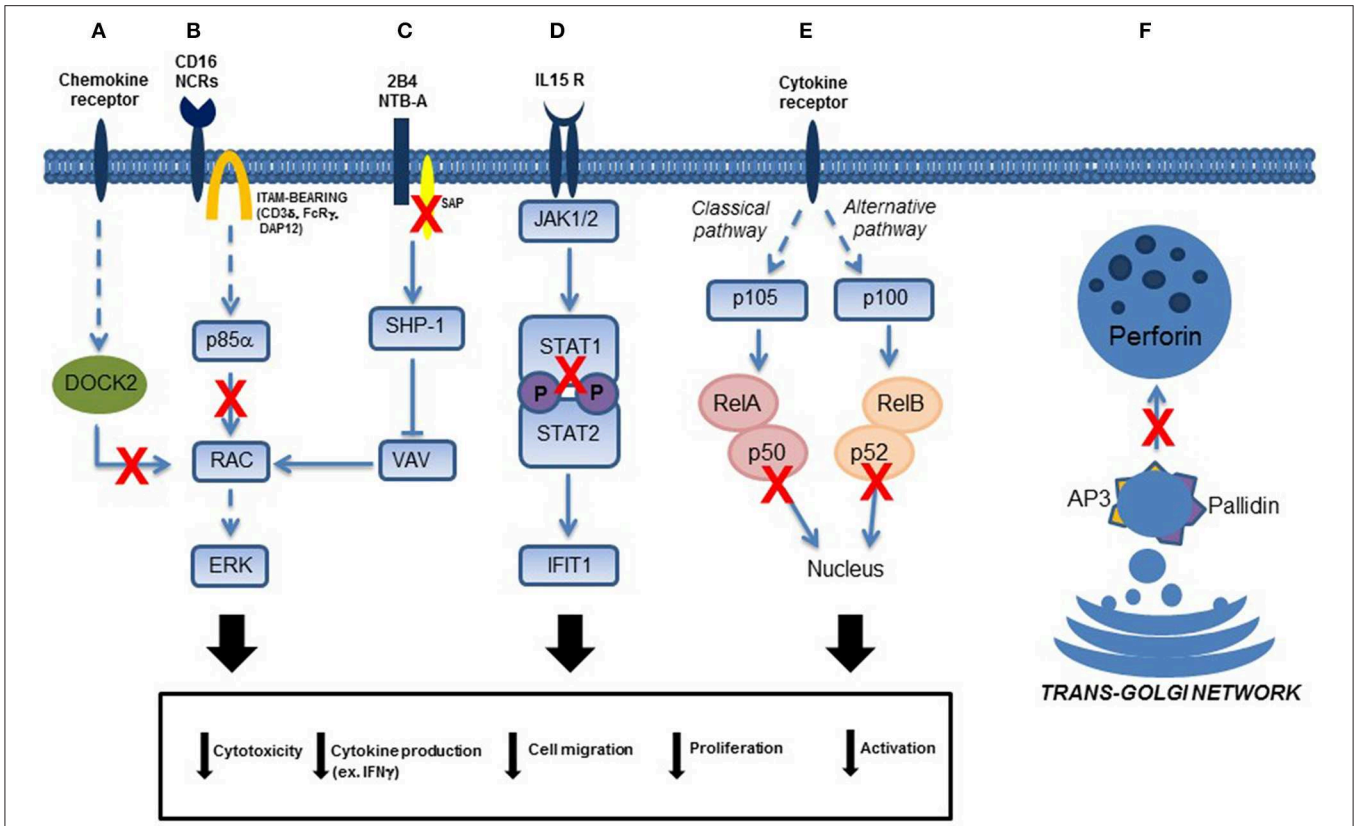

FIGURE 2 | Schematic representation of NK cell transduction pathways involved in primary immunodeficiencies. Functional NK cell defects due to: **(A)** *DOCK2* mutations, **(B)** p85α (*PIK3R1*) mutations, **(C)** SAP (*SH2D1A* gene) mutations in patients affected by XLP1, **(D)** STAT1 mutations, **(E)** p50 (in the canonical pathway of NF-κB signaling) and p52 (in the alternative pathway of NFκB signaling) mutations, and **(F)** AP-3 and Pallidin mutations in patients affected by Hermansky-Pudlak type 2 and 9, respectively. Direct and indirect effects on signaling are represented with continued or dotted lines, respectively.

surface ligand expressed on EBV-infected B lymphocytes. To further illustrate the critical role played by 2B4 and NTB-A mediated inhibitory signals in the pathophysiology of XLP1, mAb-mediated masking of these molecules resulted in virtually complete restoration of XLP1-NK cell-mediated cytotoxicity against EBV-infected target cells (22, 28, 30).

Hermansky Pudlak Syndromes Associated to AP-3 Complex and Pallidin Mutations

Pigmentary dilution disorders recognized as immunodeficiencies are characterized by partial albinism of hair, skin, and eyes, together with leukocyte defects. These disorders include Chediak-Higashi, Griscelli, Hermansky-Pudlak (HPS), and MAPBP-interacting protein deficiency syndromes (31, 32). These are recessive autosomal gene defects in which proteins with specific roles in the biogenesis and trafficking of secretory lysosomes are encoded. These intracellular granules are essential constituents of melanocytes, platelets, granulocytes, CTLs, and NK cells. Among nine genetically distinct type of HPS, only type 2 (HPS2) and type 9 (HPS9) are characterized by immunodeficiency (32).

In 2000, we analyzed two siblings who presented with oculocutaneous albinism, neutropenia, recurrent infections, and bleeding disorder. Initially, we noticed a severe defect of their NK cell cytotoxicity, but it was only some years later that we discovered that they carried a mutation in the *AP3B1* gene, encoding the β3A subunit of the heterotetrameric adapter protein (AP-)3 complex. Defects of the β3A subunit compromise stability of the entire AP-3 complex involved in cellular protein trafficking (**Figure 2F**). The absence of the AP-3 complex impairs regulated movement of protein sorting to secretory lysosomes. Consequently, regulation and function of cells related to secretory lysosomes are defective. Besides functional defects of melanocytes, platelets and CTLs, we observed impaired differentiation of neutrophils, NKT, and NK cells. In particular NK cells had reduced lysosomal pools of perforin, but normal levels of granzyme (33, 34).

After some years, the diagnosis of nodular lymphocytes predominance type Hodgkin lymphoma (NLPHL) was made in the same two young siblings affected with HPS2. By analyzing their peripheral blood immune cells, we found that CTL, NK, and iNKT cells from these patients were significantly impaired in their number and function, including tumor cell killing activity (35). The development of NLPHL suggested a possible involvement of effector function of multiple cell types including NK cells. In particular, we noted a significant reduction in the

proportion of $CD56^{bright}$ NK cells, and high expression of the NK cell maturation marker CD57 on $CD56^{dim}$ cells, in spite of reduced intracellular perforin content. We linked the high expression of $CD57^{+}CD56^{dim}$ NK cells to an expansion of a human "memory NK cells" subset, reflecting the history of recurrent viral infection in these patients, as also demonstrated previously by various authors (36). Even more important to explain the complications of their HPS2, was the demonstration of a severe NK cell cytolytic defect combined with a reduced production of IFN-γ after engagement of NK cell activating receptors. In fact, NK cells from the HPS2 patients failed to properly recognize and kill a large series of tumor cell lines *in vitro*, including Hodgkin Lymphoma cell lines (35).

Thereafter, we analyzed one HPS9 patient who manifested partial albinism, nystagmus, normal neurological development, and absence of platelet delta granules but, at variance with what observed in HPS2, the patient lacked coagulation defects (37). HPS9 is the most recently defined subtype of HPS and is caused by biallelic mutations of the *PLDN* gene, encoding for pallidin, a component of the biogenesis of lysosome-related organelles complex-1 (BLOC1). In the patient with HPS9, *PLDN* mutations led to undetectable expression of the protein product in NK cells. Comparative analysis of NK cells from the patient and healthy controls, showed that pallidin regulates the expression of lysosomal membrane proteins. In particular, freshly isolated NK cells from the patient expressed higher levels of CD107a (a marker of NK cell degranulation) and of CD63 on the cell surface; after *in vitro* activation, NK cell degranulation (measured by change in CD107a surface expression) against NK-susceptible erythroleukemia K562 and B-EBV 221 cell lines was reduced in the patient's NK cells as compared to what observed in controls, indicating impaired trafficking of cytolytic granules to the plasma membrane (37). These defects were similar, although not as severe, to those observed in HPS2.

Defective Activity of NK Cells Due to Abnormalities in Intracellular Signaling in Patients With *STAT1*, *NFKB1*, *NFKB2,* and *PIK3R1* (PI3K p85α) Gene Defects

In more recent years, Alessandro strongly encouraged us to investigate the role of NK cells in other forms of PID, and in particular in those with increased susceptibility to viral infections. His continuous support was critical for the discovery of previously unrecognized NK cell defects in a variety of PIDs.

STAT1 Mutations

Subjects affected by Signal Transducer and Activator of Transcription 1 (STAT1) deficiency suffer from life-threatening bacterial, mycobacterial, viral, and fungal infections. Complete STAT1 deficiency is inherited as an autosomal recessive disease; partial STAT1 deficiency is inherited as an autosomal recessive or autosomal dominant trait. We described a patient with homozygous STAT1 splicing mutation leading to skipping of exon 3 who developed generalized mycobacterial infections and severe viral disease sustained by cytomegalovirus (CMV) (38). We reported that the patient's cells displayed a complete defect of STAT1 DNA-binding activity after stimulation with IFN-γ and IFN-α, and failed to respond even to high doses of these cytokines. These biologic defects were associated with a partial impairment of NK cell function, which could contribute to the increased susceptibility to infections with viral and intracellular pathogens. We observed that unstimulated NK cells displayed a cytotoxicity defect against K562 target cells. It is probable that the defect of NK-mediated cytolysis was related to the immunomodulatory role of STAT1 because NK cytotoxicity is regulated by cytokines that require STAT1 for signaling. Moreover, we observed that IFN-γ production by STAT1-deficient NK cells activated with IL-15 and IL-12 in the presence of target cells, was impaired, suggesting that the IL-15 response involves STAT1 signaling (38) (**Figure 2D**).

Based on these observations, we studied NK cells in 8 patients with *STAT1* gain-of-function (GOF) mutations. This condition was initially reported to cause chronic mucocutaneous candidiasis, but was subsequently shown to cause also recurrent bacterial and viral infections. We reported that "patients with *STAT1* GOF mutations display abnormal NK cell function and proliferation (39). Upon *in vitro* activation with IL-2, NK cells from these patients show impaired cytolytic activity. Moreover, the defect of NK cytotoxic activity observed in patients with *STAT1* GOF mutations was not related to abnormal expression/function of NK receptors or to reduced perforin expression, but rather to an impaired response of these cells to IL-2 or IL-15. Indeed, NK cells from the patients produced lower than normal levels of IFN-γ after stimulation with IL-15, but normal levels upon stimulation with IL-12 and IL-18 (39). This suggests that the NK cytotoxicity defect detected also in these patients was probably related to abnormal response to the immunomodulatory cytokines IL-15 and IL-2." Interestingly, Tabellini et al. showed that "NK cells from patients with *STAT1* GOF mutations, manifested enhanced STAT1 phosphorylation in response to either IL-15 or IL-2 stimulation, whereas a weak signaling is detected in normal NK cells in response to IL-2 or IL-15, even after prolonged stimulation. This suggests that *STAT1* GOF mutations can interfere with important steps in the differentiation and functions of T and NK cells, resulting in impaired generation of TH17 cells and reduced proliferation of NK cells" (39). In addition, the observation that *in vitro* activated NK cells from patients with *STAT1* GOF mutations produce reduced amounts of IFN-γ, suggest that this defect may also lead to increased susceptibility to intracellular pathogens. Overall, our data indicate that abnormalities of NK cell function may play an important role in determining the clinical phenotype of this condition (39).

NFKB1 and NFKB2 Gene Defects

The NF-κB (NF-kappaB: nuclear factor of kappa light polypeptide gene enhancer in B cells) signaling pathways play an important role both in the innate and in the adaptive immune system (40).

The NF-κB transcription factor family consists of five members: NF-κB1, NF-κB2, RelA, RelB, and c-Rel. We had the chance to study patients with mutations of the *NFKB1* gene (that encodes the precursor p105 which is processed to the mature p50)

and one patient with *NFKB2* mutation, whose gene encodes the precursor p100 and the mature p52.

Two pathways of NF-κB signaling have been described. The canonical pathway, which includes NF-κB1, mediates numerous immunological and inflammatory cellular responses, whereas the non-canonical pathway, which involves NF-κB2, has more restricted immunological functions mainly focusing on B cell homeostasis and regulation of self-tolerance by medullary thymic epithelial cells (**Figure 2E**).

The NF-κB1 and NF-κB2 proteins were recently reported to be mutated in a limited number of common variable immunodeficiency (CVID) patients, some of which were analyzed by our group (41, 42). CVID is the most common symptomatic primary immunodeficiency characterized by low immunoglobulin serum levels, low vaccine responses, and recurrent infections. Several genetic mechanisms have been reported to account for CVID in the last few years, and involve mutations in CD19, MS4A1 (CD20), CR2 (CD21), ICOS, TNFRSF13C, TNFRSF13B, PLCG2 (phospholipase Cg2), CD81, LRBA, and PRKCD (protein kinase CD) as well as in NF-κB1 and NF-κB2.

We had the opportunity to study one patient affected with CVID due to a *de novo* heterozygous non-sense mutation (p.Arg853*) in *NFKB2* and demonstrated impaired NK cell cytotoxic activity despite normal NK cell counts and normal expression of different NK receptors (41). Moreover, we observed a normal expression of the maturation marker CD57 on $CD56^{dim}$ NK cells. This is the first description of impaired NK-cell activity associated with *NFKB2* mutations. These findings broaden the immunologic defects in NF-κB2 deficiency, confirm the heterogeneous and complex immunologic and clinical phenotype in disorders in which NF-κB components are defective, and underline an important role for NF-κB in NK-cell cytotoxic activity.

Subsequently, we had the possibility to analyze NK cells from patients with NF-κB1 deficiency (42). This was especially important, since until then, data on the role of NF-κB signaling in NK cells were largely limited to observations in mice. In particular, we provided evidence that monoallelic *NFKB1* mutations affect both maturation and effector functions of human NK cells. In fact, *NFKB1*-mutated NK cells showed reduced percentage of $CD56^{+}$ NK cells expressing KIRs, NKp46, CXCR1, CCR7, and CD16, when compared to healthy controls. Expression of CD57, a classical maturation marker, was also downregulated. By studying $CD56^{bright}$ and $CD56^{dim}$ NK cells, we noticed a similar proportion of $CD56^{dim}$ $CD57^{low}$ cells in patients and controls. In contrast, the population of more mature $CD56^{dim}$ $CD57^{bright}$ NK cells was significantly reduced in individuals with *NFKB1* mutations (42). These results, described in our work, indicate an impaired peripheral maturation of NK cells in patients with *NFKB1* haploinsufficiency and they were supported by a significant reduction of CD62L (an additional marker of maturation) on the $CD56^{dim}$ $CD57^{+}$ NK cell subsets, as well as accumulation of $CD56^{bright}$ $CD62L^{+}$ NK cells, a finding not observed in healthy controls. Together, these data suggest that the canonical NF-κB pathway orchestrates unique aspects of human NK cell maturation stages (42).

Finally, *NFKB1*-mutated NK cells showed impaired cytotoxicity, IFN-γ production, and proliferation. These observations indicate that defective maturation and function of NK cells may play an important role in the increased susceptibility to viral infections that has been reported in NFκB1 haploinsufficient patients (42).

PIK3R1 (p85α) Defects

Recently, novel insights indicating the role that PI3K signaling plays in human NK cell maturation and lytic function are suggested by the identification of patients with phosphoinositide-3-kinase (PI3K)-signaling pathway mutations that can cause primary immunodeficiency (43). Class I PI3Ks are divided into class IA (p110α, p110β, p110δ) and class IB (p110γ) kinases, which interact with the regulatory subunits p85α, p50α, p55α, p85β, and p55γ (for class IA kinases), and with p101 and p84 (for class IB kinases) (44). Many authors showed an involvement of PI3K in multiple functions of NK cell biology, including development/maturation, homing, priming, and function. In human NK cells, the PI3K-signaling pathway plays a direct role in signaling downstream from activating receptors, including 2B4 and KIR receptors (45–47). The recruitment of p85α, in combination with Grb2, is also necessary and sufficient for the propagation of signaling, promoting cytotoxicity upon engagement of NKG2D associated with the DAP10 adaptor (47). In addition, "PI3K activates a Rac1–MEK–ERK pathway that is a key signaling pathway for actin reorganization and cellular polarization (48) (**Figure 2B**). The central role of PI3K in mediating cell polarization is consistent with PI3K-induced activation of CDC42 at the NK cell immune synapse; in particular, p85α acts as a scaffold to target and position PI3K, and subsequent recruitment of guanine nucleotide exchange factors to the membrane (49). As such, the role of PI3K signaling in cytotoxicity and NK cell migration can be through the control of actin remodeling, polarization, and even granule exocytosis, which requires intracellular calcium store mobilization" (50).

Following the discovery of *PIK3CD* GOF mutations, that result in constitutive p110δ activation in patients with Activated PI3K Delta Syndrome type 1 (APDS1) (51), monoallelic GOF *PIK3R1* mutations were identified in 12 patients affected with a hyper–IgM-like primary immunodeficiency/immune dysregulation condition associated with T- and B-cell maturational and functional defects (52). These mutations deprive p85α from its regulatory function, unleashing p110δ, and thereby causing APDS type 2 (ADS2) (51). However, when the first cases of APDS2 were described, the impact of this p85α mutant protein on the maturation and function of human NK cells had not yet been studied. To address this issue, we investigated NK cell phenotype and function in two patients with APDS2 (53).

We showed that NK-cell maturation from *PIK3R1*-mutated patients is normal (53). This is in contrast with maturational defects described in the animal model. We then tested NK cell function, including degranulation, IFN-γ secretion, and cytolytic activity against of EBV-infected target cells (53).

Upon IL-2 stimulation, patients' NK-cell degranulation against the human erythroleukemia cell line K562 was

significantly reduced as compared to what observed with healthy control NK cells. This impairment was also confirmed by a classical chromium release killing assay, underscoring an important role for p85α in this process in humans, similar to what was observed in p85α knockout mice (53). Moreover, IFN-γ production was significantly reduced in patients' as to healthy control NK cells. After demonstrating that NK cell activating receptors function normally in APDS2 patients (as shown by redirected killing assays), we observed that IL-2 activated NK cells from these patients display reduced degranulation against the 721.221 EBV^+ lymphoblastoid cell line, and failed to upregulate CD107a upon engagement of autologous EBV-infected B cells (53). These results indicate an essential role for p85α in human NK-cell lysis of EBV-infected cells, thereby providing mechanistic insights into the increased susceptibility to EBV infection and EBV-associated lymphoproliferative disorders that patients with APDS1 and APDS2 manifest (53).

Impaired NK Cell Function in Patients With DOCK2 Deficiency

A few years ago, we performed genetic and immunological investigations in five unrelated children of different ethnic origin who manifested severe susceptibility to infections sustained by a broad spectrum of pathogens. The immunological phenotype of the patients included severe lymphopenia and functional defects affecting T, B, and NK cells. Through whole exome sequencing, we established that these patients carried bi-allelic mutations in the *DOCK2* gene (54).

DOCK2 is a large protein involved in intracellular signaling network, that specifically activates isoforms of the small G protein RAC in response to engagement of various cell surface receptors, including T and B cell receptors, chemokine receptors, and various NK cell receptors (**Figure 2A**). We demonstrated that T, B, and NK cells from the patients manifest defective chemokine-induced migration and actin polymerization, and that RAC1 activation was impaired in T cells (54).

We also showed that DOCK2 deficiency impairs NK-cell degranulation against K562 target cells. The ability of NK cells to fight viral infections and the onset of tumors correlates with the functionality of a variety of activating NK cell receptors interacting with the distinct adaptor (DAP10, DAP12) and signaling (CD3ζ and FcεRγ) molecules (55). Furthermore, triggering of activating NK cell receptors induces actin polymerization, phosphatidylinositol-3-OH kinase activation and phosphorylation of MEK and ERK, ultimately promoting NK-cell cytotoxicity. Based on this knowledge, we analyzed NK cells from DOCK2 deficient patients. In particular, we studied NK cell degranulation upon engagement of CD16, NKp30, NKp46 (all of which utilize CD3ζ and FcεRIγ for signaling), or NKG2D (which recruits the DAP10 adaptor), and observed severely impaired degranulation in a patient, and moderately impaired in another patient with residual amounts of DOCK2 protein. Degranulation was also impaired in patient-derived IL-2 activated polyclonal NK cells upon engagement of NKp44 (which utilizes DAP12) (54). We observed reduced levels of F-actin in patient NK cells upon CD16 and NKp46 stimulation, reminiscent of similar observations in *Dock2*$^{-/-}$ mice reflecting impaired tonal signaling through antigen and chemokine receptors. Moreover, we noticed reduced phosphorylation of ERK1/2 and MEK, and impaired actin polymerization in polyclonal NK cells derived from patients upon cross-linking of activating receptors. Finally, we showed that upon stimulation with IL-12 and IL-18, the proportion of NK cells expressing IFN-γ was markedly reduced in patients when compared with IFN-γ production in normal NK cells (54). These data demonstrated that DOCK2 deficiency is a combined immunodeficiency affecting the function of multiple white blood cell types, including NK cells, further supporting the notion that hematopoietic stem cell transplantation (HSCT) should be considered soon after diagnosis to prevent life-threatening infections and early death.

Altered Phenotype and Function of NK Cells Derived From Patients With RAG and NHEJ Defects

In the last years of collaboration with us, Alessandro had shown much interest in a research proposed by Gigi concerning the phenotypic analysis of NK cell subsets in patients carrying mutations in the recombinase-activating genes *RAG1* and *RAG2*. The RAG1 and RAG2 proteins play a critical role in V(D)J recombination and therefore in T and B cell development, but are dispensable for the development of NK cells (56). Consistent with this, null mutations in the RAG genes are associated with T^- B^- NK^+ severe combined immune deficiency (SCID). However, we and others have shown that hypomorphic mutations in humans are associated with a broad range of clinical and immunological phenotypes, whose severity correlates with the residual recombination activity of the mutant RAG proteins (57). Nonetheless, irrespective of the phenotype, human RAG deficiency is associated with a dismal prognosis, with death early in life in infants with SCID, and severe infections, autoimmunity and inflammation later in life in patients with leaky forms of the disease. Therefore, HSCT represents the mainstay of treatment for patients with RAG deficiency; however, as compared to other forms of SCID and related diseases, HSCT for RAG deficiency is associated with a high rate of graft rejection (58, 59). Based on the observation that NK cells play an important role in graft-vs.-leukemia and graft rejection after HSCT (40), it had been hypothesized that they may also contribute to the increased risk of graft rejection after unconditioned HSCT for RAG deficiency. However, why this would be true for RAG deficiency and not for other forms of NK^+ SCID, remained unclear.

Although RAG genes are not required for NK cell development, data in mice indicated that RAG deficiency affects NK cell phenotype and function. It had been shown that expression of the *Rag* genes begins in common lymphoid progenitor cells that give rise to T, B, and NK cells (60). A seminal work by Joe Sun at Memorial Sloan Kettering had shown that NK cells from Rag-deficient mice have an abnormally activated phenotype and display enhanced cytotoxicity, associated with reduced cellular fitness (61). The hypothesis was put forward that *Rag* gene expression in common lymphoid progenitor cells

provides preferential advantage to cells with superior DNA repair capacity, as suggested by mixed bone marrow chimera transplantation experiments, in which Rag-sufficient NK cells outcompeted Rag-deficient cells (56).

These observations paved the way to explore the hypothesis that NK cells from RAG-deficient patients could be dysfunctional, and that their possibly hyperactivated status might play a role in promoting rejection after HSCT. In collaboration with Alessandro and taking advantage of a large repository of specimens from patients with RAG deficiency and other forms of PID, we decided to test this hypothesis.

With his help and generosity, we utilized a large panel of mAbs (many of which he had generated) that define various steps in the differentiation process from $CD56^{bright}$ to $CD56^{dim}$ cells to study NK cells in 66 patients with defects in the *RAG* genes or in other genes involved in non-homologous end joining (NHEJ) genes (*DCLRE1C, LIG4, NHJ1*), or with other forms of PID with selective T or B cell deficiency unrelated to defects in VDJ recombination and DNA repair, and in healthy donors of comparable age (62). In our research, we observed that "NK cells from patients with mutations in *RAG* and NHEJ genes have an immature phenotype, with significant expansion of $CD56^{bright}$ $CD16^{-/int}$ $NKG2A^{+++}$ $CD57^{-}$ cells, and a reduced percentage of $CD56^{dim}$ $CD16^{hi}$ cells expressing CD57, KIRs, and CXCR1 than observed in age-matched healthy controls (62). These observations suggest that NK cells from patients with RAG/NHEJ defects have a more immature phenotype when compared to age-matched healthy controls and to SCID not due to RAG/NHEJ defects. These data contrast with findings in $Rag^{-/-}$ mice whose peripheral NK cells display a more mature phenotype (62). However, we found that in spite of their immature phenotype, NK cells from patients with RAG/NHEJ defects have enhanced degranulation capacity and express higher amounts of perforin as compared to control NK cells." This hyperactivation status of NK cells resembles what observed in $Rag^{-/-}$ mice (56) and may indeed contribute to enhanced graft rejection activity after HSCT. Inclusion of serotherapy targeting NK cells in the HSCT conditioning regimen for RAG deficiency may therefore be beneficial to reduce the risk of graft rejection.

At the same time, because many of the patients included in our study suffered from CMV infection, this provided an opportunity to assess whether these patients have an increased proportion of "memory" NK cells (63), a topic that Alessandro was particularly interested in. It is well-known that CMV can drive expansion of $NKG2C^{+}$ NK cells that produce high amounts of IFN-γ as compared to $NKG2C^{+}$ NK cells from CMV-seronegative subjects (64). These $NKG2C^{+}$ NK cells may represent "memory" NK cells capable of prompt responses upon secondary exposure to CMV. We failed to observe an expansion of $NKG2C^{+}$ NK cells in CMV-infected patients with RAG/NHEJ defects. It is possible that T cells (that are defective in number in these patients) be required help in this process and for controlling CMV infection.

CONCLUSIONS

The study of NK cell phenotype and function in patients with PID has provided unanticipated mechanistic insights into the pathophysiology of these diseases, and offered important novel information on the role of individual molecules in human NK cell biology. This long journey, which we have embraced with enthusiasm and dedication, would have not been possible without Alessandro's thoughtful guidance, scientific curiosity, and generosity. We remain inspired by his work, and will also miss his irony, the best remedy at times when our work seemed lost on a dead track.

AUTHOR CONTRIBUTIONS

SP and LN contributed conception and design of the study and wrote the first draft of the manuscript. GT, OP, KD, VL, MB, DC, AP, and RB wrote sections of the manuscript.

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REVIEW
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On the Way to Become a Natural Killer Cell

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Natural Killer (NK) cells are innate lymphocytes playing pivotal roles in host defense and immune-surveillance. The homeostatic modulation of germ-line encoded/non-rearranged activating and inhibitory NK cell receptors (NKRs) determines the capability of these innate lymphocytes to either spare "self" cells or to kill viral-infected, tumor-transformed and heterologous cell targets. However, despite being discovered more than 40 years ago, several aspects of NK cell biology remain unknown or are still being debated. In particular, our knowledge of human NK cell ontogenesis and differentiation is still in its infancy as the majority of our experimental evidence on this topic mainly comes from findings obtained *in vitro* or with animal models *in vivo*. Although both the generation and the maintenance of human NK cells are sustained by hematopoietic stem cells (HSCs), the precise site(s) of NK cell development are still poorly defined. Indeed, HSCs and hematopoietic precursors are localized in different anatomical compartments that also change their ontogenic commitments before and after birth as well as in aging. Currently, the main site of NK cell generation and maturation in adulthood is considered the bone marrow, where their interactions with stromal cells, cytokines, growth factors, and other soluble molecules support and drive maturation. Different sequential stages of NK cell development have been identified on the basis of the differential expression of specific markers and NKRs as well as on the acquisition of specific effector-functions. All these phenotypic and functional features are key in inducing and regulating homing, activation and tissue-residency of NK cells in different human anatomic sites, where different homeostatic mechanisms ensure a perfect balance between immune tolerance and immune-surveillance. The present review summarizes our current knowledge on human NK cell ontogenesis and on the related pathways orchestrating a proper maturation, functions, and distributions.

Keywords: natural killer cell, ontogenesis, hematopoietic stem cell, natural killer cell receptors, cytokines, self-tolerance, education

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INTRODUCTION

Natural Killer (NK) cells were first described as large granular lymphocytes with a natural ability to kill tumor cells without a previous activation (1). Currently, it is well-known that NK cells mediate immune-surveillance not only via cytotoxic effector-functions, but also by serving as regulatory lymphocytes able to secrete cytokines and to interact with both innate and adaptive immune cells, such as monocyte/macrophages, dendritic cells (DCs), and T lymphocytes (2–5). These activities are governed by a balance between activating and inhibitory NK cell receptors (aNKRs and iNKRs)

expressed on cell surface (6–9). Under homeostatic conditions, NK cells remain in a resting state due to the engagement of iNKRs [i.e., inhibitory Killer Immunoglobulin-like receptors (iKIRs), the C-type lectin receptor NKG2A, Ig-like transcripts (ILTs), and the leukocyte Ig-like receptors (LIRs)], that recognize a broad spectrum of classical and non-classical Human Leukocyte Antigen (HLA)-I molecules constitutively expressed of autologous "self" cells (10, 11). Viral infected, tumor-transformed or allogeneic "non-self" cells down-regulate, lack or express different HLA-I alleles, thus boosting the NK cell-mediated killing of these dangerous targets via the engagement of aNKRs, that includes Natural Cytotoxicity Receptors (NCRs) (NKp30, NKp46, and NKp44), C-type lectin receptors (NKG2C, NKG2D), DNAM-1 and activating KIRs (aKIRs) ("missing self hypothesis") (6, 12–14).

NK cells comprise two main subsets defined on the basis of CD56 and CD16 surface expression: the cytotoxic $CD56^{dim}/CD16^{pos}$ ($CD56^{dim}$) population accounting for up to 90% of circulating NK cells and the regulatory $CD56^{bright}/CD16^{neg}$ ($CD56^{br}$) NK cell subset producing high amount of pro-inflammatory cytokines, such as interferon (IFN)-γ. These two NK cell populations also differ for the expression of several NKRs that determine their ability to respond to different stimuli (15). Both genetic and environmental (i.e., infections and microbes) factors also contribute to generate NK cell diversity in terms of NKR repertoire and functions (16). Indeed, extensive flow-cytometry and mass-spectrometry data clearly showed that a large diversity in the phenotype of NK cell subsets can coexist especially at tissue levels (17–20). This heterogeneity is also associated with the different microenvironments in which NK cells develop and reside (21). However, although these cells are widely distributed in several tissues and organs of human body, most of the current knowledge on these innate lymphocytes is limited to peripheral blood (PB-) NK cells (22). In this context, how and to what extent NK cells are exchanged between blood and human tissues and which anatomic compartments host tissue-resident NK cells represent important matters of scientific debate.

In this review, we discuss our current knowledge of the several steps of human NK cell ontogenesis with a special focus on those related mechanisms regulating their development, tissue homing and residency.

TISSUE SITES OF NATURAL KILLER CELL DEVELOPMENT

The production and the maintenance of NK cells in the blood are sustained by $CD34^{pos}$ hematopoietic stem cells (HSCs). However, the exact sites of NK cell development are poorly defined, as hematopoietic cell precursors have been found in different anatomic compartments of the human body both in intra-uterine and adult lifespan (23). In the embryo and fetus the hematopoiesis takes place in the yolk sac, aorta-gonad-mesonephros region, and liver, while in adults bone marrow (BM), thymus, spleen, omentum, and liver are considered the main sites of blood cell development (24).

BM has been considered for long time the major site of NK cell generation and differentiation after birth. Indeed, this immunological niche is highly enriched of $CD34^{pos}$ HSCs and hematopoietic progenitors, including NK cell ones (25). Herein, NK cell development is supported through interactions with stromal cells, cytokines, growth factors, and other soluble molecules. However, whether NK cell ontogenesis occurs exclusively or primarily in the BM niche is still being debated.

In this regard, tissue-specific NK cell development had been reported and even the so-called NK cell "education" ensuring self-tolerance can occur in certain tissues (25). Indeed, several lines of evidence demonstrated that, while the early phases of NK cell development occur in the BM, later stages of NK cell differentiations can take place in secondary lymphoid tissues (SLTs), PB, liver, mucosa-associated lymphoid tissues (MALTs), and uterus (22, 26–29). In particular, tonsils, spleen, and lymph nodes (LNs) are considered those SLTs hosting the main extra-medullary sites of NK cell development and maturation. The para-follicular T cell regions of LNs are one of the main anatomical districts enriched with NK cells. Here, more than the 90% of tissue-resident NK cells have a $CD56^{br}$ phenotype and they are able to differentiate in mature $CD56^{dim}$ NK cells following stimulation with interleukin (IL)-2, as circulating $CD56^{br}$ NK cells (15, 30). These findings suggest that LNs might be one of the major peripheral tissue sites of NK cell development. This working hypothesis is further corroborated by other evidences showing that human LNs contain $CD34^{dim}/CD45RA^{br}$ hematopoietic precursors that likely origin from HSCs in the BM and then traffic in this SLT via the PB. Once in LNs, they can differentiate in $CD56^{br}$ NK cells upon activation mediated by LN-resident T cells (26).

The existence of $CD34^{pos}$ lymphoid precursors endowed with the ability of differentiating in NK cells *in vitro* have been also reported in human thymus (31). However, patients either affected by the Di George syndrome (32) or undergone thymectomy (33, 34) and splenectomy (35) have normal frequencies of circulating NK cells, that are also phenotypically and functionally similar to those of healthy donors (30). For that reason, thymus as well as spleen are not considered major sites of NK cell ontogenesis. Although it is possible that these unaltered frequency, phenotype and functions could be due to the redundancy of NK cell developmental pathways.

Fetal liver certainly represents one of the major tissue of NK cell development and this solid organ also retain a residual ability to generate NK cells even after birth (36). Indeed, human liver is highly enriched in tissue-resident NK cells that are phenotypically and functionally distinct from their circulating counterparts (29, 37–39). Moreover, it has been reported that human liver perfusates and biopsies contain all NK cell developmental stages from multipotent $CD34^{pos}$ hematopoietic progenitors to terminally differentiated cells. In addition, liver-resident NK cell precursors retain the ability to generate *in vitro* fully mature and functional NK cells (29). Taken together, these data further support the hypothesis that adult liver represents an important tissue site for NK cell development *in vivo* even in the adult life.

Another peripheral organ highly enriched of tissue-resident NK cells is the uterus. Here, the so-called uterine NK (uNK) cells hold a unique phenotypic/functional profile and they are present at high frequencies in the decidua to ensure mother tolerance vs. the implanted fetus. uNK cells play also a primary role in angiogenesis, tissue remodeling, and immune modulation mainly during the first trimester of pregnancy (40–42). In this regard, a population of $CD34^{pos}$ cells able to differentiate in NK cells either following *in vitro* stimulation with several cytokines or upon co-culture with decidual stromal cells had been described in human decidua (43, 44).

Although ∼10–20% of total lymphocytes in human lungs are NK cells, they share a very similar phenotype with circulating $CD56^{dim}$ NK cell subset and express very low levels of tissue-residency markers. This observation thus suggests that lung NK cells, different from liver and uterus, likely migrate in this tissue from the PB (21).

NATURAL KILLER CELL PRECURSORS AND ONTOGENESIS

Our current knowledge on immune cell hematopoiesis postulates that the earliest step of HSCs to undergo the NK cell differentiation relies on their commitment toward the lymphoid/myeloid lineage rather than the erythroid/megakaryocyte one. Then, $CD34^{pos}$/$CD133^{pos}$/$CD244^{pos}$ cells acquire the expression of CD45RA to become Common Lymphoid Progenitors (CLPs), which have the potential to generate B, T and innate lymphoid cells (ILCs) (45). This process requires cell-to-cell interactions with stromal cells in the context of a peculiar microenvironment characterized by the presence of the stem cell factor (SCF), the ligand for the fms-like tyrosine kinase 3 (FLT3L), and IL-7 (46).

CLPs can then further differentiate in NK cell progenitors (NKPs) that are classified in three sequential stages of maturation named NK cell progenitors (stage 1), pre-NK cells (stage 2), and immature NK (iNK) cells (stage 3) (**Figure 1**) (47, 48). The commitment of CLPs toward NKPs had been first postulated for analogy with B and T cells progenitors and it is characterized by the down-regulation of CD34 and by the acquisition of CD122, the common IL-2 receptor subunit β shared by IL-2 and IL-15 signaling pathways. The induced expression of CD122 marks the irreversible fate of CLPs toward the NK cell differentiation (22, 49, 50). Indeed, both NKPs and pre-NK cells still express CD34 and retain the ability to differentiate in T cells, DCs and other ILCs. On the opposite, $CD34^{neg}$/$CD122^{pos}$ iNK cells loose this development potential, thus representing the real NKPs (**Table 1**) (47, 51).

More recently, two distinct and additional stages of pre-NK cells have been described on the basis of their negative (stage 2a) or positive (stage 2b) expression of both IL-1β and IL-2β receptors (**Figure 1** and **Table 1**). Stage 2a is mainly enriched in certain tissues (i.e., SLTs and PB) and retains the ability to give rise to T cells and DCs, while stage 2b represents the so-called common ILC progenitors, since its commitment is restricted to the generation of ILCs, including NK cells (48, 52). The transition from stage 2b to stage 3 is then marked by the acquisition of aNKR expression (i.e., NKG2D, NKp30, and NKp46) (**Table 1**).

All the developmental stages of NKPs have been mainly characterized in the context of the BM niche. However, it is still an important matter of debate whether distinct organ-specific NKPs also exist and could undergo a "peripheral ontogeny" able to generate tissue-resident NK cells (25). In this regard, a subset of putative NKP has been recently identified in BM, PB and SLTs, where it can give rise to all members of ILC lineage. Differently from the above-mentioned stage 2b pre-NK cells, these latter NKPs are characterized by a $CD34^{pos}$/$CD45RA^{pos}$/$CD38^{pos}$/$CD10^{pos}$/$CD7^{pos}$/$CD123^{neg}$/$CD127^{neg}$ phenotype sharing several surface markers with both stage 1 and stage 2 NKPs (53). Finally, an additional $CD56^{pos}$ subset of $CD34^{neg}$/$CD117^{pos}$ precursors able to generate NK cells and ILC3s, but not ILC2s, has been described in tonsils (54).

In our currently accepted linear model of maturation (**Figure 1**), the sequential expression of specific markers on the surface of iNK cells (stage 3) parallels the acquisition of NK cell self-tolerance and effector-functions. In particular, the shift from NKPs to mature NK cells is associated with the sequential acquired expression of CD56, CD94, and of the Killer C-type lectin receptor CD161 (55). While the functional roles of the expression of both CD161 and CD56 have not yet been fully clarified, the acquisition of CD94 surface expression is essential to allow the formation of the heterodimeric C-type Lectin receptors. Hence, the $CD34^{neg}$/$CD117^{pos/neg}$/$CD94^{pos}$/$HLADR^{neg}$/$CD10^{neg}$/$CD122^{pos}$/$CD94^{pos}$/$NKp44^{low}$/$NKG2D^{pos}$/$CD161^{pos}$ phenotype defines mature NK cells that can be then further distinguished into in the 2 final developmental stages according to the expression of CD56 and CD16 (**Table 1**) (25, 56, 57).

$CD56^{br}$ NK cell represents the immune-regulatory and cytokine producer stage 4, characterized by a $CD34^{neg}$/$CD117^{low}$/$CD94^{pos}$/$CD16^{neg}$ phenotype (**Table 1**). More recently, 2 distinct stages 4 of NK cells have been described in SLTs: 4a and 4b stages that differ for the induced expression of NKp80 on the latter subset (58, 59). The stage 4a $NKp80^{neg}$/$CD56^{br}$ NK cell subset is characterized by constitutive high expression of NKG2D, NKp30, and NKp46, CD94/NKG2A, CD161, and is not endowed with potent effector-functions (**Table 1**). On the opposite, its stage 4b counterpart can produce IFN-γ and mediate perforin-dependent cytotoxicity *in vitro* (48). Both 4a and 4b NK cell stages are then considered precursors of the terminally-differentiated and cytotoxic $CD56^{dim}$ NK cells (stage 5) (25) (**Figure 1**). Indeed, the $CD56^{br}$ NK cell subset does not express KIRs and CD57 and it is more immature as also confirmed by the longer length of its telomeres (60). Additional experimental evidence demonstrated that the transition from $CD56^{br}$ to $CD56^{dim}$ NK cells is progressive as the latter terminally-differentiated subset gradually acquires the expression of CD16, KIRs, and cytotoxic granules by generating a transitory population of $CD56^{bright}$/$CD16^{pos}$ NK cells (61). During this transition, stage 4 $CD56^{br}$ NK cells lose the expression of CD117, CD127, and CD94/NKG2A receptor, while acquiring CD94/NKG2C and down-regulating CD56 (60, 62) (**Table 1**). Finally, it has been also recently proposed that stage 5 $CD56^{dim}$/KIR^{pos} NK cells can be further distinguished from

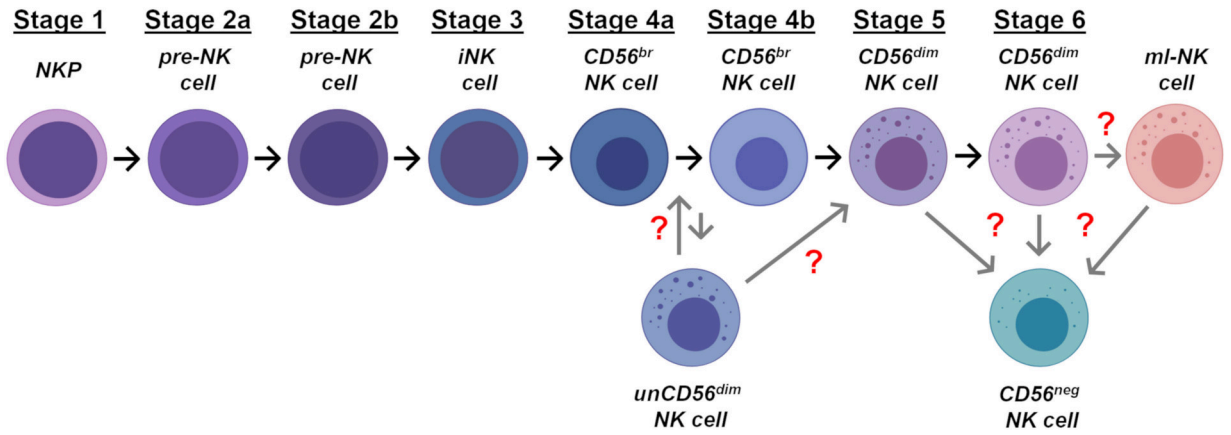

FIGURE 1 | Stages of NK cell ontogenesis. Schematic representation of the different stages of NK cell differentiation in human bone marrow and secondary lymphoid tissues. Gray arrows and red question marks indicate the possible location in NK cell development of unCD56^{dim}, CD56^{neg}, and ml-NK cells.

TABLE 1 | Principal surface markers differentially expressed on NK cell developmental intermediates.

Surface marker	Stage 1	Stage 2a	Stage 2b	Stage 3	Stage 4a	Stage 4b	Un CD56^{dim}	Stage 5	Stage 6	ml-NK	CD56^{neg}
CD34	+	+	+	–	–	–	–	–	–	–	–
CD10	+	+/–	+/–	–	–	–	n.d.	–	–	–	–
HLA-DR	+	+	+	–	–	–	n.d.	–	–	+	+
CD117	–	+	+	+	+/low	low/–	–	–	–	–	–
CD127	+	+	+	+	–	–	–	–	–	–	+
CD45RA	+	+	+	+	+/–	+/–	n.d.	–	–	–	–
IL-1 βR	–	–	+	+	+/low	low/–	n.d.	low/–	low/–	low/–	–
CD122	–	–	+	+	+	+	n.d.	+	+	+	+
CD161	–	–/low	–/low	–/+	+	+	n.d.	+	+	low/–	+
CD56	–	–	–/low	–/low	++	++	+	+	+	+	–
CD94	–	–	–	–	+	+	+	+/–	+/–	+	+
NKG2A	–	–	–	–	+	+	+	low/–	low/–	low/–	low/–
NKG2D	–	–	–	–/low	+	+	+	+	+	+	+
NKp30	–	–	–	–/low	+	+	+	+	+	low/–	low
NKp46	–	–	–	–/low	++	++	–/low	+	+	low	low
NKp80	–	–	–	–	–	+	n.d.	+	+	+	+
NKG2C	–	–	–	–	low/–	low/–	low/–	+	+	++	+/low
CD16	–	–	–	–	–	–	–	+	+	+	+
KIRs	–	–	–	–	–	–	low	+	+	+	+
CD57	–	–	–	–	–	–	–	–	+	+	+

n.d., not determined.

stage 6 based on the expression of CD57, a surface marker of replicative senescence (**Figure 1** and **Table 1**). Although this is still a matter of scientific discussion, a recent study confirmed at transcriptome, epigenome, and proteomic levels that this linear developmental trajectory starts from CD56^{br} NK cells and ends with the final acquisition of CD57 (63).

ADDITIONAL STAGES OF NK CELL MATURATION

Memory-like NK Cells

While stage 6 CD56^{dim} NK cells show a poor responsiveness to cytokine stimulation, they retain high degree of cytotoxicity and can expand in response to several viral infections (64, 65). In this regard, it has been shown that some viruses can change the NKR repertoire and can also induce the clonal expansion of peculiar NK cell subsets endowed with adaptive features. These latter populations display higher effector-functions when re-encountering the same virus and they are defined "memory-like" NK (ml-NK) cells (**Figure 1**) (16, 19, 66, 67). ml-NK cells are characterized by a peculiar KIRs^{pos}/CD57^{pos}/NKG2C^{pos} phenotype and lack the expression of CD161, NKp30, and CD7 (68–70). Among the main viruses inducing the expansion of these NK cells endowed with adaptive traits there is the Human Cytomegalovirus (HCMV) that drives a profound epigenetic reprogramming in ml-NK cells. This HCMV-driven mechanism increases the IFN-γ production when ml-NK cells are re-exposed to the same viral pathogen (71–75).

ml-NK cells have been described not only in PB but also in tissues and associated with different antigens. Indeed, a subset of a hepatic CXCR6^{pos} NK cells with adaptive properties against haptens or viral antigens had been also reported. This latter subset of ml-NK cells is liver-resident and express a CD49a^{pos}/DX5^{neg} phenotype (76, 77).

CD56^{neg} NK Cells

Besides the induced expansion of ml-NK cells, viral infections can also drive the emergence of another dysfunctional CD56^{neg}/CD16^{pos} (CD56^{neg}) NK cell subset. These cells had been firstly described during the course of HIV-1 infections and then in other viral diseases, in autoimmune-disorders and in elderly. CD56^{neg} NK cells are present at very low frequency in the PB of healthy subjects, but they represent the majority of total NK cells in AIDS patients showing high levels of HIV-1 viremia (74, 78–81). Despite being identified and characterized more than 2 decades ago, the ontogenesis of this subset is still unknown. The repertoire of NKRs expressed on CD56^{neg} NK cells shared several similarities with that of stage 3 iNK cells. However, the high constitutive expression of CD94/NKG2A, NKG2D, and CD16 together with the retention of a certain degree of cytotoxicity represent phenotypic and functional differences that do not allow a completely overlap between CD56^{neg} NK cells and stage 3 iNK cells (**Table 1**) (79, 82). Indeed, the high surface levels of KIRs, CD57 and CD107a degranulation marker on CD56^{neg} NK cells suggest that they rather represent exhausted lymphocytes that already engaged target cells (**Figure 1**) (83).

Unconventional CD56^{dim} NK Cells

The latest NK cell subset to be identified and characterized is represented by the so called unconventional NK cells holding a CD56^{dim}/CD16^{neg} phenotype (unCD56^{dim}) (84). This neglected population is extremely rare under homeostatic conditions, although it displays a significantly higher cytotoxicity compared to that of CD56^{br} and CD56^{dim} NK cell subsets. However, a very few studies characterized the homeostasis, the phenotype and the functional relevance of unCD56^{dim} NK cells subset although there is not yet a consensus on its name and classification (85–89). Unexpectedly, other and we recently reported that, in the context of the lymphopenic environment of patients affected by hematologic malignancies and undergone haploidentical stem cell transplantation (haplo-HSCT), unCD56^{dim} NK cells are by far the largest subset of NK cells immune-reconstituting in the first 2–4 weeks after the transplant (88, 90). Indeed, in this short window after haplo-HSCT the very low frequency of the conventional cytotoxic CD56^{dim} NK cells are compensated by the high expansion of unCD56^{dim} NK cells that lack the expression of CD34, CD117, and CD127 (**Table 1**). These data demonstrate that unCD56^{dim} NK cells cannot be classified as NKPs, but are rather differentiated cells expressing several NKRs as well as lytic granzyme and perforin. Moreover, the transcriptional profile of unCD56^{dim} NK cells revealed that they are placed within an intermediate stage of differentiation between CD56^{br} and CD56^{dim} NK cells as also functionally assessed with time-course *in vitro* experiments of NK cell differentiation (88). Furthermore, those unCD56^{dim} NK cells highly expanded early after haplo-HSCT also have a transient high expression of CD94/NKG2A, an iNKR also involved in NK cell differentiation. This phenomenon makes this subset anergic only in this particular human setting *in vivo*. Hence, the use of an immunotherapeutic strategy to block this inhibitory checkpoint, unleashing NK cells thus improving the clinical outcome of haplo-HSCT early after the infusion of HSCs is currently under clinical investigation (84). Taken together, these data highlight the key role played by unCD56^{dim} NK cells in the mechanisms of immune-reconstitution and also show that this unconventional NK cell subset could represent and additional or alternative stage of NK cell differentiation (**Figure 1**).

SIGNALS AND MECHANISMS REGULATING THE DIFFERENTATION OF NK CELLS

Cytokines

NK cell differentiation is finely tuned by different cytokine signals (48, 91). As previously mentioned, HSC survival and proliferation are preserved by FLT3L and SCF. Indeed, mice lacking their receptors FLT3 and c-Kit (CD117) show a consistent reduction in the frequency of CLPs (46, 92–94). In addition, the engagements of FLT3/FLT3L and c-Kit/SCF axes induce the expression of CD122 and/or IL-15Rα (CD215), thus increasing the sensitivity of NKPs to IL-15 (51, 95). Although both IL-15 and IL-2 stimulation promote the maturation of CD56^{br} toward CD56^{dim} NK cells *in vitro* (96), only IL-15 is involved in NK cell differentiation both in humans and mice.

This is confirmed by the experimental evidence showing that mature NK cells are nearly absent in mice lacking any of the 3 different subunits that compose the IL-15 heterotrimeric receptor (IL-15R) complex: CD215, CD122, and CD132 (γ_c chain) (97–101). Accordingly, patients showing an X-linked gene mutation in the γ_c gene (*il2rg*) are affected by a severe combined immune-deficiency characterized by a high susceptibility to infections due to developmental defects of lymphocytes (including NK cells) (100). Despite the γ_c chain of IL-15R is shared by other relevant cytokines (i.e., IL-2, IL-4, IL-7, IL-9, and IL-21) for their downstream signaling (102), dysfunctions of this subunit affects only the IL-15 pathway. Indeed, knockout mice lacking IL-2, IL-2Rα, IL-7, IL-7Rα, and IL-21R have normal frequencies of mature circulating NK cells (103–106).

The production of IL-15 at NK cell developmental site is mainly exerted by BM stromal and myeloid cells (107). The binding of soluble IL-15 to CD215 on the surface of surrounding cells mediates the trans-presentation of this complex to NK cells expressing CD122 and CD132 heterodimer (108–110). This engagement of IL-15R on NK cells induces the activation of JAK1/3 downstream cascade that, in turn, activates STAT3/5 and the mitogen-activated protein kinase (MAPK). These signaling pathways mediate NK cell survival via both the up-regulation of anti-apoptotic B cell lymphoma 2 (BCL-2) family members and the down-regulation of pro-apoptotic proteins (111–114). Indeed, both *Stat5*-deficient and NK cell-specific *Stat5*-deficient

mice show a marked reduction of circulating mature NK cells (115, 116). In humans, a similar severe reduction in NK cells is observed in patients with a mutation of *STAT5b*, one of the two highly conserved *Stat5* human genes (117, 118).

NK cell responsiveness to IL-15 during NK cell development is also influenced by the expression of phosphoinositide-dependent kinase-1 (PDK1) that connects IL-15 signaling to the activation of both E4BP4 (also known as Nfil3) and Inhibitor of DNA-binding 2 (ID2) transcription factors (119–121).

An additional cytokine playing a critical role in the development of NK cells is IL-21. Indeed, IL-21 stimulation *in vitro*, together with FLT3 and IL-15, promotes the differentiation and the expansion of cytotoxic $CD56^{dim}$ NK cells from BM progenitors (104, 122). In addition, IL-21 induces rapid maturation of human NK cells and the acquisition of a KIR^{pos} mature phenotype from $CD34^{pos}$ cell precursors (123). On the other side, IL-7 is key in promoting the survival and early differentiation of NKPs (106). As a matter of fact, although mice lacking IL-7 or its receptor (CD127) keep a relatively normal NK cell development (105), the correct engagement of CD127 expression is key in the early stages of NK cell differentiation and in the retention of NKPs in SLTs (52). Moreover, those NK cells enriched in thymus are characterized by high constitutive expression of CD127 and require IL-7 for their homeostasis (100).

IL-4 has been recently described of being able to induce the development of tissue-resident NK lymphocytes in mice by converting $CD49a^{pos}/Eomes^{neg}$ NK cells into their functional $CD49a^{pos}/Eomes^{pos}$ counterparts (124). Since $CD49a^{pos}/Eomes^{neg}$ NK cell subset is considered a liver-resident NK cell subset in mice, these findings could be relevant for a better understanding of the specific tissue-resident generation of NK cells (125). However, other than expressing high levels of Eomesodermin (Eomes) transcripts, human liver-resident NK cells appear to be much more phenotypically heterogeneous compared to their murine counterparts (126).

IL-12 can also promote differentiation of NK cells and can enhance their cytotoxicity *in vitro* (127). Interestingly, an alternative pathway of NK cell development that bypasses the above-mentioned γ_c-signaling relies on the engagement of IL-12 in response to viral infections. Indeed, the stimulation of NKPs with this pro-inflammatory cytokine in the BM generates an unconventional but yet functional NK cell subset. However, this pathway is still not exploited in humans and might be highly relevant in patients with SCID (128). IL-12, together with IL-18, has been also described for its ability to induce the differentiation of ml-NK cells. In this regard, IL-12 and IL-18 have been studied as co-stimulatory factors for the generation of CMV-specific murine $Ly49H^{pos}$ ml-NK cells (129). In particular, the IL-12/STAT4 signaling pathway is required for the formation and the expansion of these NK cells with adaptive traits (130). Similarly, the expansion of $NKG2C^{pos}$ ml-NK cells in humans upon HCMV infection has been shown to be IL-12- plus IL-18-dependent (131, 132). In this regard, *in vitro* activation of both murine and human NK cells with IL-12, IL-18, and IL-15 supports the generation of cytokine-induced ml-NK cells (133, 134). This mechanism has been recently employed in a clinical trial to boost the expansion of adaptive NK cells showing enhanced anti-tumor responses against myeloid leukemia (135).

Transcription Factors

The commitment and differentiation of hematopoietic stem cells/precursors toward NK cell lineage require the expression of specific transcription factors (TFs). In this context, the current knowledge on NK cell development derives from experimental findings mainly generated either *in vitro* or in animal models and very little is known in human setting *in vivo*. However, it is widely accepted that several TFs are required by CLPs for their transition to both NKPs and iNK cells. These mechanisms are not specific for NK cell development as the same TFs are used to commit CLPs toward different cell lineages (136, 137). Ets-1 and PU.1, members of the Ets TF family, are involved in the transition of CLPs to NKPs and they are broadly expressed in multiple hematopoietic-derived lineages (138–141). Interestingly, Ets-1-deficient mice have a severe decrease of circulating NK cells, while knocking out PU.1 in murine models does not affect the frequency of NK cells in PB. These latter animals are also characterized by an up-regulation of Ets-1, thus suggesting the existence of compensatory mechanisms to ensure a correct ontogenesis and maturation of NK cells (142, 143).

As previously mentioned, the transition to NKPs also requires the expression of CD122 that induces STAT5 phosphorylation, dimerization and nuclear translocation (115–117). Although the specific gene targets of STAT5 in NK cells have not yet been clarified, more than 15,000 STAT5 DNA binding sites have been identified in T cells, including genes required for lymphocyte proliferation and survival (144). In addition, the expression of CD122 in NK cells is regulated by the Runx family of TFs that represent key regulators of lymphocyte lineage-specific gene expression (145, 146). In particular, Runx3 has been reported to play an important role both in NK and $CD8^{pos}$ T cell development, thus indicating its specific involvement in transcriptional programs of cytolytic lymphocytes (146). Similarly, Thymocyte selection-associated high mobility group box protein (Tox) and the interferon-regulatory factor (IRF) families regulate the transition toward NKPs as well as toward B and T cells, ILCs and myeloid lineages (147–149). On the contrary, several other TFs regulating the early steps of NK cell differentiation are much more restricted to the development of innate lymphocytes. Indeed, E4BP4-deficient mice lack only NK cells and ILCs, as the expression of this TF is required to tune the expression of Eomes and ID2 in early progenitor cells (150–154). Other reports also claimed the existence of alternative and E4BP4-independent development pathways for immature and tissue-resident NK cells (125, 155, 156).

Another TF required for the differentiation of ILCs is ID2, whose expression is controlled by Ets-1 (138). ID2-deficient mice show a block of NK cell development between NKPs and mature NK cells with the subsequent lacking of circulating NK cells (120, 121). Recently, it has been also reported that ID2 regulates NK cell responsiveness to IL-15 through the modulation of DNA-binding helix-loop-helix E proteins (E2A) (157).

In later stages of NK cell maturation, T-box protein 21 (T-bet) and Eomes play a major role in promoting the expression of cytolytic and IFN-γ production machineries (158, 159). Indeed, mice deficient for both these latter TFs have a systemic lack of circulating mature NK cells (160, 161). However, whether or not Eomes and T-bet act in the same pathway is not yet clear as these two members of the T-box family are believed to function in a sequential manner during NK cell maturation. T-bet is required for the production of iNK and it is detectable just prior this development stage (154). Indeed, T-bet deficiency results in an accumulation of iNK cells in BM (161, 162). On the contrary, Eomes tunes the differentiation of mature NK cells from iNK cells and it is also critical to discriminate between NK and ILC1 subsets (158, 159). In addition, T-bet and Eomes have been reported to regulate NK cell development at different anatomical site as T-bet is primarily required for the production of NK cell at extramedullary sites (125, 163, 164).

SURFACE MOLECULES REGULATING NK CELL TRAFFICKING AND MATURATION

Very little is known about the mechanisms orchestrating the trafficking of human NKPs and mature NK cells from PB to tissues/organs and *vice versa* (165). Several lines of evidence indicate that this trafficking is governed by several adhesion molecules, such as integrins, selectins, and chemokine/cytokine receptors. Among them, CXCR4, the alpha-chemokine receptor specific for the stromal derived factor-1, has a role in maintaining HSCs in the BM niche (166–168). Indeed, it has been shown that the treatment with a CXCR4 antagonist promotes the progenitor mobilization from the BM (169, 170). CXCR4 appears to also play a key role in the first steps of NK cell ontogenesis since it is highly expressed by NKPs and iNK cells, while its surface levels gradually decrease during NK cell maturation (171).

Differently from CXCR4, the down-modulation of CX3C chemokine receptor 1 (CX3CR1) in response to stimulation with transforming growth factor (TGF)-β prevents the NK cell egress from the BM (172, 173). Furthermore, CX3CR1, together with CC chemokine receptors (CCRs)-7 and−5, tunes NK cell maturation as the acquisition of a $CD56^{dim}$ phenotype is associated to its induced expression (174, 175). Similar to CX3CR1, the sphingosine-1-phosphate receptor 5 (S1P5) is involved in the NK cell release in the bloodstream and in NK cell differentiation. Indeed, terminally differentiated stage 6 NK cells up-regulate S1P5 and migrate in response to sphingosine-1-phosphate (S1P) (176). The active role of this bioactive sphingolipid in determining the NK cell trafficking is also suggested by the observation that S1P creates a gradient with highest concentrations in the blood and lymph, while its levels are maintained low in tissue parenchyma (177).

Several other adhesion molecules and chemokine receptors regulate the preferential localization of $CD56^{br}$ and $CD56^{dim}$ NK cells in SLTs, PB and inflamed tissues (178). Indeed, while S1P5 seems to be involved in retaining $CD56^{dim}$ NK cells in the bloodstream, CD62L, CCR7, and CXCR3 are involved in the selective homing of $CD56^{br}$ NK cell to LNs. Indeed, these latter surface molecules are either absent or expressed at very low levels on $CD56^{dim}$ NK cells (30, 62, 179, 180). Furthermore, CD69 is now considered not only as a marker of cell activation, but also as a tissue-residency one. Indeed, $CD56^{br}$ NK cells in tissues (i.e., liver, uterus, LNs) express high levels of CD69, while their counterparts in PB are $CD69^{neg}$ (38, 181). Moreover, highly cytotoxic $CD56^{dim}$ cells infiltrating metastatic LNs express CD69 and CCR7 and can upregulate CXCR1 (182–184). CD103 and CD49a are other tissue-residency markers that are up-regulated by NK cells in response to TGF-β (185, 186). CD103 heterodimerized with β7 and binds to E-cadherin on epithelial cells, thus retaining NK cells in tissues (187). Moreover, the heterodimer β1-CD49a is involved in the tissue retention of NK cells via the binding to collagen (188). Those $CD56^{dim}$ NK cells preferentially migrating toward inflamed tissues, express a different patterns of cytokine/chemokine receptors that include CXCR1, CXCR2, and ChemR23 (59, 175, 189).

NK CELL EDUCATION AND ACQUISITION OF TOLERANCE TO SELF

Although NK cell ontogenesis and education are two separated processes, there are quite a few interconnections between these two key mechanisms of NK cell homeostasis. The acquired expression of iNKRs together with their binding to self-HLA molecules in BM during NK cell development represent the mechanism generating functional NK cells that are also tolerant against autologous targets (190). Indeed, the direct cell-to-cell interactions with "self"-MHC-I educate NK cells to sense the down-regulation or lack of matched HLA alleles on target cells in order to mount an efficient effector-responses only against threatening viral-infected or tumor-transformed or allogeneic targets (190–192). Hence, the so-called process of "NK cell education" relies on the avidity of binding between NKRs and self-HLA molecules and on the level of response of an NK cell to activating signals (i.e., stress ligands, inflammatory cytokines, and Fc receptor engagement) (193, 194).

In addition to the recognition of self-HLA/MHC antigens in *trans* on neighboring cells, the expression of MHC-I molecules on the NK cell itself has been shown to play an important role in regulating NK cell activity and licensing in mice, by Ly49 interaction in *cis* (195, 196). In agreement, evidences in literature indicate that KIR:HLA interactions could occur both in *trans* and in *cis* in humans too. While the HLA-I trans-presentation seems to be mainly involved in NK cell education, the *cis* interaction in humans could play a major role in the maintenance of NK cell effector potential (190). However, since, unlike Ly49, KIRs do not have a flexible stalk, it has been proposed that this *cis* interaction between HLA-C and KIR2DL could occur in endosomes rather than on the cell surface (197).

Each individual shows a highly stochastic but tolerant HLA-I specific repertoire of iNKRs, which can be shaped by the subject-specific immunological history. These phenomena are regulated by several "licensing" iKIRs, that recognize HLA-A/B/C, and by CD94/NKG2A, that binds HLA-E (9, 198, 199). During NK cell maturation, the NKR repertoire is selected to the expression of at

least one iNKRs specific for self-HLA-I haplotype on each mature NK cell. This makes NK cells able to recognize target cells, thus avoiding autoreactivity (200). CD94/NKG2A is the first HLA-I-specific iNKR expressed on differentiating NK cells prior to the appearance of KIRs. Indeed, it is present on $CD56^{br}$ NK cells and to a less extent on $CD56^{dim}$ NK cells (201). Moreover, *in vitro* differentiating NK cells from immature post-natal thymocytes express high levels of CD94/NKG2A that prevents the lysis of autologous cells expressing self-HLA-I alleles (202). However, it is widely accepted that $iKIR^{pos}$ cells represent the main subset of "educated" NK lymphocytes. Indeed, during NK cell differentiation, the surface levels of CD94/NKG2A decrease while the expression of KIRs increases only on terminally differentiated $CD56^{dim}$ NK cells (201). In this context, the human KIR gene family shows a certain degree of diversity due to both the high variability of KIR gene contents and allelic polymorphisms (203, 204). KIR and HLA genes are located on different chromosomes, and are inherited independently. This phenomenon might affect the selective evolutionary pressure as well as the NK cell-mediated susceptibility toward infections and diseases, as it is possible that KIR genes can be inherited in the absence of the cognate HLA ligand. Moreover, as previously mentioned, only NK cells expressing at least one KIR can be considered fully "licensed" (200, 205).

Although the mechanism tuning the process of NK cell education has not yet been fully disclosed, NK cell responsiveness is acquired in a finely regulated manner through KIR–KIR ligand interactions during development and several working hypotheses are currently being discussed on this matter. The first one relies on the concept of "arming" in which a given iKIR recognizes its cognate self-HLA-I allele, thus allowing the fully maturation of NK cells. In this regard, an NK cell expressing more than one iKIR should receive a stronger inhibitory signal, but this cell should also mediate a more potent alloreactivity when encountering a non-self-target (206). An opposite theory is instead based on the idea that NK cells expressing iKIRs mismatched with self HLA-I alleles are not clonally deleted, but are rather kept "unlicensed" both in PB and tissues in a state of hypo-responsiveness to ensure self-tolerance (194, 207, 208). This so called "disarming" working hypothesis states that, in the absence of self-iKIRs, the chronic stimulation of a still undetermined aNKR is associated with NK cell anergy (207).

An additional iNKR involved in NK cell education is ILT2/LIR1, which recognizes HLA-G and other shared epitopes present in all human HLA-I molecules. It has been shown that the expression of LIR1 by NK cells is able to prevent the secretion of IFN-γ (199). This iNKR appears to be expressed by mature NK cells and its surface level increases upon cytokine stimulation or HCMV infection, thus representing a possible escape mechanism from NK cell immune-surveillance (209–211). Moreover, the LIR1-mediated inhibition of NK cell effector-functions has been proposed to be also important in regulating the maternal-fetal immune tolerance during pregnancy (212).

Besides HLA-I specific iNKRs, several additional surface molecules have been reported to be involved in NK cell licensing to prevent their cell activation against self-cells. These additional mechanisms likely ensure a multi-layered and complementary system of immune tolerance and education of NK cells. In this context, the appearance of NKp46 and NKp30 before HLA-I specific iNKRs during development could ensure an HLA-I independent self-tolerance at early stages of NK cell differentiation (198, 213, 214). This hypothesis is also supported by clinical evidence in human HLA-I-deficient individuals, in which NK cells do not kill autologous cells although the engagement of HLA-specific iNKRs is either impaired or lacking (215, 216). In line with this last theory, it has been demonstrated that 2B4 could be involved in NK cell education by being expressed early on the $CD34^{pos}$ NKPs. As a matter of fact, although 2B4 is an aNKR in mature NK cells, it can exert an inhibitory function when expressed on immature NK cells (198, 213).

Finally, another mechanism possibly preventing NK cell autoreactivity relies on the differential/asynchronous expression of NK cell receptors and ligands. A classic example is the expression of NKG2D, an aNKR able of modulating NK cell receptor activation with different thresholds (217). In adults, it has an important role in eliminate potentially dangerous cells expressing NKG2D ligands including tumor-transformed and viral-infected target cells. On the contrary, NKG2D is not expressed in embryonic life, when the soluble and exosome-bound ligands MIC-A and MIC-B are produced by human placenta. This mechanism thus contributes to prevent the activation of mother NK cells against the fetus (218, 219).

CONCLUDING REMARKS

Although our current knowledge on the mechanisms tuning human NK cell ontogenesis greatly advanced over the past 2 decades, several questions still remain unanswered. In particular, the signals and pathways involved in NK cell development in SLTs and in other anatomic compartments remain to be clarified. Furthermore, the intracellular processes by which an NK cell is able to discern between self and non-self are still elusive. Emerging evidence from high-throughput technologies highlighted that NK cell diversity is more complex than expected and it is determined by genetic and environmental determinants. Thus, it is possible that this phenotypic NK cell diversity and apparent redundancy, within the same tissue and between the different tissues, could be the result of NK cell plasticity and could mirror the different NK cell functional properties rather than mere developmental intermediates.

Future efforts in understanding NK cell differentiation, effector-functions and heterogeneity in both physiological and pathological conditions will provide insights for the prevention and the treatment of human diseases. In particular, a better understanding of NK cell development in malignancies and other diseases will facilitate the design and implementation of NK cell-mediated immunotherapies.

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Alessandro Moretta and Transporter Associated With Antigen Processing (TAP) Deficiency: On Giant's Shoulders

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The laboratory hosting me for my Ph.D. described in 1994 the first human cases of TAP deficiency in two siblings with recurrent bacterial airway infections and a negative Human Leukocyte Antigen class I (HLA) serotyping. At this time, it became clear that natural killer (NK) cells interact with HLA class I molecules which inhibit them. Inhibitory receptors were postulated, and Alessandro Moretta was the first to generate monoclonal anti-human NK cell antibodies that bound to such molecules, which he characterized in detail (Killer Immunoglobulin-like receptors—KIR). Natural killer cells from healthy donors preferentially kill targets with absent HLA class I molecules ("missing self" concept), whereas we observed that the NK cells from the TAP-deficient patients were hypo-responsive and did not lyse the HLA class I-negative leukemia cell line K562. Moreover, they were not very active in antibody-dependent cellular cytotoxicity assays. To address the question if such NK cells would express KIR or not, my thesis supervisor requested the anti-KIR antibodies from Alessandro Moretta, who was kind enough to provide us generously with aliquots. It turned out that the NK cells from the TAP-deficient individuals expressed most of these inhibitory receptors normally. We then had the privilege to receive almost every new antibody generated in the Moretta lab and to complete the phenotypic studies of the NK cells from our patients. I had the great chance to meet Alessandro Moretta at several occasions. He deeply impressed me each time and strongly influenced my way of thinking.

Keywords: TAP deficiency, natural killer cells, HLA class I, cytotoxicity, antibodies

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INTRODUCTION

It is likely that most scientists meet, especially during the early phase of their career, more advanced colleagues that guide them and leave a deep impression on them for the rest of their life. My case was no exception, as I was lucky enough to find two of such mentors, namely my Ph.D. thesis supervisor, Dr. Henri de la Salle, and Professor Alessandro Moretta.

In 1995, after my medical studies in Strasbourg, France, and 2 years working at an Emergency Department, I realized that I would not want to continue spending my lifetime under such conditions, and decided to give a new orientation to my career by acquiring a Master Degree in Immunology, a field in which I always had been interested. I found an

internship position at the Blood Transfusion Center in Strasbourg, in the lab of Daniel Hanau and Henri de la Salle. They had described in 1994 two siblings with a very low cell surface expression of HLA class I molecules (negative serotyping), chronic bacterial infections of the respiratory tract and bronchiectasis, found to have a homozygous mutation in the TAP2 gene (1, 2). The transporter associated with antigen processing (TAP) is a heterodimer (TAP1 and TAP2 subunits) inserted into the membrane of the endoplasmic reticulum (ER) that transports endogenous peptides from the cytosol into the ER lumen, where they are loaded onto newly synthesized HLA class I molecules. Peptide acquisition is necessary for their stabilization, export from the ER and migration to the cell surface (3). Consequently, in the absence of a functional TAP, these processes do not efficiently occur.

In 1994, it was already established, in the context of the "missing self-hypothesis" formulated by Ljunggren and Kärre, that natural killer (NK) cells, the third type of lymphocytes besides B and T cells, preferentially killed targets not expressing HLA class I molecules (4). However, peripheral blood mononuclear cells from the TAP-deficient patients (containing the NK cell fraction), which contained a normal percentage of NK cells compared to healthy donors, were not cytotoxic to HLA class I negative K562 leukemia cells (1). Mouse models of TAP1- and β2-microglobulin (β2m) deficiency confirmed the observation, as NK cells from these animals were likewise hyporesponsive (5, 6).

NK CELLS FROM TAP-DEFICIENT PATIENTS EXPRESS INHIBITORY NK CELL RECEPTORS

The project for my master thesis aimed at studying NK cells from the two TAP-deficient patients in more detail, as Alessandro Moretta previously had described several monoclonal anti-human antibodies of murine origin that identified NK cell subpopulations and whose target molecules were clonally distributed (indeed, the Moretta lab was successful in cloning NK cells) (7–9). This work later revealed that the structures recognized by the antibodies were inhibitory NK cell receptors (IR), able to negatively regulate NK cell functions.

Henri de la Salle asked Alessandro Moretta for these antibodies (GL183, EB6, Z27, and XA185), which were not yet commercially available, and received generous amounts of hybridoma cell culture supernatants in the context of a scientific collaboration. My role was first to check if the patient's NK cells, drawn from peripheral blood, did express the IR recognized by the antibodies. We did not purify the NK cells but identified them by three-color flow cytometry after staining with anti-CD3 (specific T cell marker), anti-CD19 (specific B cell marker), and anti-IR antibodies. Natural killer cells were considered as the CD3-CD19- events in the lymphocyte gate which allowed to evaluate the percentages of cells positive for the different IR. As controls, we used PBMC from the unaffected father of the patients (heterozygous for the TAP mutation) in addition to those from 10 unrelated healthy donors. The results clearly showed that the percentages of IR+ NK cells were in the same range for the patients, their father and the healthy donors (10), of course by taking into consideration the substantial inter-individual variability in IR expression. Patient's NK cells were no outliers, although the percentages of cells positive for the antibody XA185 were rather high (10).

A second readout for receptor expression in flow cytometry is the mean fluorescence intensity (MFI), which reflects the density of expression of a given molecule. Regarding this parameter, it turned out that the MFI for the Killer Immunoglobulin-like Receptors (KIR) recognized by the antibodies EB6 and Z27 was again in the normal range, whereas for GL183, the MFI was slightly higher for the NK cells from the patients and their father, suggesting rather a genetic cause than a relationship to the TAP deficiency. However, and interestingly, the MFI of XA185 was much more pronounced in the case of the patients' NK cells than in their father and the ten normal donors (10).

In the meantime, Prof. Moretta had developed additional anti-NK cell antibodies called Q66, FES172, and Z270. Whereas, the former two were directed against a KIR and an activating isoform, respectively, the latter was specific for CD94/NKG2A only and not for all CD94-bound receptors like XA185. CD94 is a chaperone protein necessary for the surface expression of different NKG2 molecules, and among them, the inhibitory receptor NKG2A (8). Again, we received aliquots of these antibodies, and they confirmed prior results in that the anti-KIR Q66 antibody and the anti-NKG2A Z270 antibody stained percentages of NK cells in the patients comparable to those of the father and the healthy volunteers, whereas the frequency of FES172+ cells was extremely low except in three healthy donors (10). Such a pattern was compatible with the previously observed fact that only one third of the analyzed donors had a subset of FES172+ NK cells. It is now clear that this receptor is an activating KIR isoform which binds to a bacteria-derived epitope on HLA-C (11). Furthermore, even if the MFI of Q66 was in the lower normal range, the one of Z270 was strongly increased on patients' NK cells (even slightly more than for XA185) (10).

Table 1 summarizes the different IR and, if applicable, their activating counterparts. The presence or absence of activating KIR isoforms depend on the KIR haplotype that an individual has inherited (12).

FIRST MEETING WITH ALESSANDRO MORETTA

The first time I had the honor and pleasure to meet Alessandro Moretta was in November 1996, at the Annual Congress of the French Society for Immunology held at the Pasteur Institute in Paris, France. Henri de la Salle was present too, and we listened to the brilliant presentation of Alessandro Moretta about the antibodies allowing to distinguish different NK cell subpopulations and clones. During the coffee break, he was literally assaulted by colleagues who wanted his antibodies. Fortunately, Henri de la Salle had fixed an appointment during the lunch break, and there I was introduced to Alessandro. I had read almost all his papers and, as lots of young researchers,

TABLE 1 | Different anti-human NK cell antibodies with their antigens and the HLA class I ligand(s) of the antigens.

Antibody	Antigen(s) current name(s)	Antigen(s) former name(s)	Ligand(s)
EB6	KIR2DL1/S1	p58.1/p50.1	HLA-C group C2
GL183	KIR2DL2/L3/S2	p58.2/p50.2	HLA-C group C1
Z27	KIR3DL1/S1	p70/NKB1	HLA-B group Bw4
Q66	KIR3DL2	p140	HLA-A*03/HLA-A*11/HLA-F
FES172	KIR2DS4	p50.3	HLA-C alleles/HLA-F
XA185	CD94		HLA-E
Z270	CD94/NKG2A		HLA-E

List of monoclonal antibodies generated in the Moretta lab and used for the study of NK cells in TAP-deficient patients. The molecules recognized by these antibodies are members of the killer immunoglobulin-like receptor (or KIR) family (first five cells in column 2) or of the C-type lectin superfamily (last two cells in column 2). All the receptors mentioned are IR, except p50.1, p50.2, and p50.3, which are AR.

thought that I was already very good in my field. Nevertheless, I was nervous when I was introduced to him, but his kindness and nonchalance together with an impressive scientific rigor, had an anxiolytic effect on me. He gave us interesting ideas to try out. Then, I had to present my poster describing my data. Alessandro followed with interest and asked a lot of challenging questions.

INVESTIGATION OF THE CYTOTOXICITY OF PATIENTS' NK CELLS

Back in Strasbourg, we continued the investigation of the cytotoxic properties of patients' non-activated NK cells by using ^{51}Chromium release assays. Natural killer cells were purified after depletion of monocytes and T cells with anti-CD14 and anti-CD3 magnetic beads, respectively. They confirmed the absence of K562 killing, but in addition, antibody-dependent cellular cytotoxicity (ADCC) against rabbit antiserum-coated Raji Burkitt's lymphoma cells was also quite low compared to the NK cells from a healthy donor. Raji without antiserum was used as a negative control due to its resistance to unstimulated NK cells, and indeed neither the NK cells from the healthy donors nor those from the patients were cytolytic to this target. The EBV-transformed B lymphoblastoid cell lines (B-EBV) from the patients were killed to a substantial amount by healthy donor NK cells, fully in accordance with the "missing self" concept [(10) and unpublished data].

It had been described that the stimulation of NK cells with the cytokine interleukin (IL)-2 leads not only to their proliferation, but also to a strong increase in their cytotoxic properties toward targets already killed by their non-activated counterparts, and in addition to an extension of the spectrum of susceptible cell lines. Thus, Raji and Daudi, which both resist to *ex vivo* NK cells, are killed by activated ones (10).

The most efficient method for NK cell expansion and activation at that time was the one described by Perussia et al.: total PBMC are co-cultured with irradiated Burkitt's lymphoma Daudi feeder cells (at a 5:1 ratio) and IL-2 for 6 days, identically re-stimulated and then grown for a few additional days (13). We usually harvested the cells at day 12, purified the NK cells with anti-T cell immunomagnetic beads (as some T cells likewise expand under these conditions) and then used them in cytotoxicity assays. In preliminary experiments, we noticed that the method worked even better with B lymphoblastoid cell lines like ST-EMO derived from one of the patients. Later, we found that the best stimulator cell line was TND-3 (unpublished data), derived from a Japanese patient with a TAP1 deficiency (14). We have to date no clear explanation for the phenomenon that TAP-deficient B-EBV cell lines induce higher expansion rates than cell lines derived from healthy volunteers.

When we cultured normal and TAP-deficient PBMC under these conditions, we observed that the latter expanded much less well (10). Normal activated NK cells very significantly killed K562, Daudi and Raji and performed a strong ADCC against Raji. Interestingly, the TAP-deficient activated NK cells were likewise efficient in terms of natural cytotoxicity and ADCC against the same target cell lines, which was a bit surprising for us. We then tested the killing of autologous B-EBV cell lines from five healthy donors and from the patients. These experiments revealed that the percentage of lysis of the normal B-EBV cells never exceeded 15 %, whereas it reached 35% in the case of patients' cell lines (10). This observation suggests some degree of auto-aggressivity exerted by the activated NK cells of these individuals.

NK CELL-MEDIATED KILLING OF TAP-DEFICIENT FIBROBLASTS

Having demonstrated this autologous killing performed by TAP-deficient NK cells (in the meantime I had become a PhD student), we went on to check if it was also the case against primary cells, namely skin fibroblasts. We cultured them with HLA class I-inducing cytokines (IFN-α, IFN-γ, IFN-γ + TNF-α) and used them as targets for purified activated autologous NK cells from three healthy donors and one of the patients (15). As expected, the patient's fibroblasts only marginally increased their surface HLA class I expression (W6/32 antibody), in contrast to the normal fibroblasts. As a consequence, the former were strongly lysed by their autologous NK cells, whereas healthy fibroblasts were protected either completely or at least to a significant extent. We then thought to demonstrate that the masking of HLA class I by the A6-136 antibody (IgM isotype) from the Moretta lab restored (normal cells) or increased (patient's cells) the killing of the fibroblasts This was clearly the case for the healthy fibroblasts but not for the TAP-deficient ones, for whom no effect could be observed (15).

In the next step, T lymphoblasts from phytohemagglutinin (PHA)-stimulated cultures were used as targets. When originating from healthy donors exposed to autologous activated NK cells, almost no lysis was observed, whereas such NK cells efficiently killed TAP-deficient PHA-blasts. The latter were surprisingly only killed by healthy volunteers' NK cells but not by autologous patients' NK cells, in contrast to our previous observations with B-EBV lymphoblastoid cell lines and skin fibroblasts. When the A6-136 antibody was added to the

cytotoxicity assays, autologous T-PHA blasts from the healthy donors were significantly lysed by their autologous activated NK cells. Inversely, the patients' activated NK cells did not attack autologous T-PHA blasts.

During all this work, Henri and me regularly discussed the data by phone (Skype didn't yet exist) with Alessandro who was very interested in the topic and gave useful advice. To facilitate the exchanges, I traveled twice to his lab where I received a very warm welcome from him and his collaborators. We shipped PBMC and fibroblasts to Genova to be able to do further experiments there. I still precisely remember the scientific discussions we had at these occasions, because they represented high quality lessons in immunology during which I learned a lot. On the last evening of my stays, Alessandro took us to nice restaurants where I became aware even more what a fascinating personality Alessandro had and where I was delighted to enjoy local pasta specialties in a lot of different variations.

EXPRESSION OF ACTIVATING RECEPTORS

It had become clear that NK cells need not only IR, but also activating receptors (AR) to efficiently kill targets. Here again, Alessandro was the first to identify a family of them, namely the natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46 (16). We received the corresponding antibodies before their appearance on the market and could show that these molecules were expressed totally normally by the NK cells of the patients. Regarding NKG2D, Alessandro was scooped by another group (from whom we could not obtain an antibody aliquot), although his lab later developed antibodies against this AR, too. It is likewise expressed by TAP-deficient NK cells.

The work in Alessandro's lab further allowed to demonstrate that TAP-deficient activated NK cells are able to produce IFN-γ but difficult to clone (17), and we completed together the phenotypic studies of our patients in 2007, focusing this time on the expanded CD56bright population and the overexpression of the broad-spectrum IR, NKG2A, and ILT2 (18).

FURTHER CASES OF TAP DEFICIENCY

Other groups identified TAP-deficient patients as well and overall confirmed our data (19, 20). We then described a patient with cytotoxic NK cells at the baseline (21). This individual was heavily infected in the airways at the time of blood drawing, and thus her NK cells might have been activated *in vivo*. Over the years, several additional patient observations appeared in the literature, focusing more on the clinical aspects (22–29). Based on the number of published cases, TAP deficiency is extremely rare, but there are probably much more patients, as the frequent diagnosis of idiopathic bronchiectasis only seldomly leads to a check for normal HLA class I expression (30), and as the diagnosis is probably often not made in regions with a difficult access to medical structures and a high consanguinity rate.

The last time I met Alessandro was in 2012, when I organized a scientific meeting in Luxembourg, entitled "HLA class I molecules in Health and Disease," bringing together an impressive international speaker panel composed of first-class immunologists. There was a session exclusively dedicated to TAP deficiency, whereas Alessandro gave a talk about his current work. For the gala dinner, I arranged to sit at his table, and once more, we had very interesting and stimulating discussions.

SUMMARY AND OUTLOOK

In the light of the vertiginous evolution Immunology has taken in recent years, can we give a new interpretation to our findings about TAP deficiency? It is now obvious that NK cells from TAP-deficient patients are not educated (not licensed), but Henri de la Salle and myself were not creative enough to go deeper into the hyporesponsiveness mechanisms of these cells, and we also did not resolve the molecular basis of the cytotoxic activity after cytokine stimulation.

Most of the patients described to date have serious and chronic bacterial infections of the respiratory tract (leading to bronchiectasis), whereas viral infections of course occur but are not exceptionally severe (3). In addition, most of them also suffer from deep skin ulcers on the legs that are healing very slowly and are difficult to treat (3, 27, 29). They might even develop granulomatous lesions of the nose, leading to the complete destruction of the nasal cartilage. It is known that NK cells are involved in antibacterial defense but that their effects can be beneficial as well as deleterious, depending on the pathogen and the precise context (31–33). Natural killer cells in tissues are different, at least in part, from those circulating in peripheral blood (34). Therefore, we cannot say for the moment if lung NK cells from the patients are dysfunctional in terms of antibacterial defense and/or if they destroy bronchial tissues due to their hypothetical (over-)activation in the infectious situation. Likewise, skin NK cells might be at the origin of the skin ulcers, where they have indeed been found (19). The nasal lesions resemble lethal midline granuloma which is now called NK/T cell lymphoma, nasal type, and this suggests that, once activated, NK cells destroy autologous tissues which in the case of TAP deficiency do not express sufficient amounts of HLA class I molecules to inhibit these cytolytic effectors. Recently, we found that TAP-deficient patients have a higher percentage of the newly described CD56dimCD16dim NK cell subset than healthy controls (35), and this might be the result of a slightly delayed maturation of patients' NK cells. The Malmberg group, in collaboration with our lab, also showed that a polyclonal expansion of adaptive NKG2C+ NK cells occurs in the patients, which might partially explain their resistance to severe viral infections (36). Furthermore, we analyzed in detail the repertoire of the HLA class I-binding receptors KIR, NKG2A and CD8 and found a correlation between the presence/absence of HLA class I molecules and the coexpression of their receptors (37).

In summary, Alessandro Moretta has made substantial contributions to the in-depth description of TAP-deficient NK cells. Without his antibodies, I might not have been able to publish in high quality journals during my PhD thesis and to find a postdoc position in the lab of Prof. Werner Held

at the Ludwig Institute in Lausanne, Switzerland. Interestingly, Alessandro had started his career at this same Institute, in the lab of his brother Lorenzo.

His work has not only theoretical but also practical clinical implications, as illustrated by the well-known phenomenon of KIR-ligand mismatch in semi-allogeneic bone marrow grafts (38). Very recently, a NK cell engager targeting NKp46, CD16 and a tumor antigen was described by the Vivier group (39), and this tri-specific engager holds quite some promise for cancer immunotherapy.

Alessandro Moretta was an outstanding scientist and a very nice person. I am sad that we lost him, but I am proud and honored to have known him.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

JZ conceived and wrote the article.

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