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MOLECULAR MECHANISMS REGULATING CYTOTOXIC LYMPHOCYTE DEVELOPMENT AND FUNCTION, AND THEIR ASSOCIATIONS TO HUMAN DISEASES

Topic Editors
Konrad Krzewski and Yenan Bryceson

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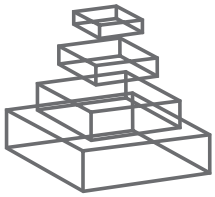

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MOLECULAR MECHANISMS REGULATING CYTOTOXIC LYMPHOCYTE DEVELOPMENT AND FUNCTION, AND THEIR ASSOCIATIONS TO HUMAN DISEASES

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Cytotoxic lymphocytes, comprised of NK cells and cytotoxic T cells, play a pivotal role in immune defense. By directed release of perforin-containing lytic granules, NK and cytotoxic T cells can eradicate pathogen-infected, tumorigenic, and otherwise stressed cells. By the virtue of cytokine and chemokine secretion, they can influence other cells of the immune system. Through these processes, cytotoxic lymphocytes also contribute to the maintenance of immune homeostasis. In recent years, much progress has been made with respect to the mechanisms by which cytotoxic lymphocytes develop, differentiate, and exert their effector functions.

In a clinical perspective, a wide variety of mutations impairing cytotoxic lymphocyte development and/or function have been associated with immunodeficiency and severe diseases in humans. Aberrant activity of cytotoxic T cells and/or NK cells has been linked to an increased susceptibility to viral infections, persistent inflammation, cancer and autoimmunity. In addition, lymphocyte cytotoxic activity may be harnessed therapeutically to target tumor cells in different adoptive cellular therapy regimes, or through the use of recombinant antibodies. Still, a number of questions remain in regards to how cytotoxic lymphocytes develop, their relationships and plasticity, as well as the mechanisms dictating target cell discrimination, lytic granule release and induction of target cell death.

In this Research Topic we encouraged submission of research articles, reviews, perspectives, or methods on cytotoxic lymphocyte development and function, their relation to the pathogenesis or treatment of different diseases, as well as comparison between similarities and/or differences in their effector functions. Considering the clinical significance of NK cells and cytotoxic T cells, we aimed to provide a range of articles summarizing the current knowledge on the identification and elucidation of the mechanisms governing cytotoxic lymphocyte activity.

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EDITORIAL
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Molecular mechanisms regulating cytotoxic lymphocyte development and function, and their associations to human diseases

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Keywords: cytotoxicity, lytic granules, secretory lysosomes, perforin, granzyme, immunological synapse, hemophagocytic histiocytosis, immune therapy

Cytotoxic lymphocytes, encompassing cytotoxic T cells and natural killer (NK) cells, play a pivotal role in immune defense. By directed release of perforin-containing lytic granules, cytotoxic lymphocytes can eradicate pathogen-infected, tumorigenic, and otherwise stressed cells. By the virtue of cytokine and chemokine secretion, they can also influence other cells of the immune system. In addition, cytotoxic lymphocytes can kill activated immune cells to limit excessive immune reactions and maintain homeostasis. In recent years, much progress has been made with respect to the mechanisms by which cytotoxic lymphocytes develop, differentiate, and exert their effector functions. In this Research Topic, we collected several exciting articles that highlight the mechanisms controlling the development and effector function of cytotoxic lymphocytes, as well as the role these cells play in several disease conditions.

We open the Research Topic with a review providing an up-to-date view on the development and differentiation of NK cells, and their relationships to other innate lymphoid cells. The authors describe in detail NK cell developmental progression through distinct stages before reaching the mature phenotype, and discuss possible reasons behind functional and phenotypic plasticity among NK cells from different tissues (1). With respect to differentiation, NK cells receive cues from several other cell types. Of particular interest is the ability of NK cells and dendritic cells to influence each other's function during early immune responses. Such reciprocal interactions are the focus of a fascinating review by Chijioke and Munz (2).

Next, we take a closer look at series of molecular events shaping the response of cytotoxic lymphocytes. A research article by Olofsson et al. (3) demonstrates how stimulation by IL-2 influences the migration of individual NK cells and their recognition and killing of target cells. Then, a review by Galandrini et al. (4) discusses the mechanisms underlying activation of cytolytic machinery following target cell recognition. Two complementary reviews focus on the maturation and release of lytic granules, and the high-resolution techniques that allow for visualization of lytic granules and quantification of their subsequent fusion with the plasma membrane at the contact site with target cells (5, 6). The following research article shows how inhibitory signals interrupt the formation of activating synapses formed by primary NK cells, thus disrupting NK cell cytotoxicity (7). Finally, Baginska et al. (8) present a fascinating view from the opposite, target cell side, providing us with a comprehensive review of how tumor cells can affect the function of NK cells and evade cell death; these include secretion of multiple tumor-derived immunosuppressive factors and metabolites, or autophagy-mediated resistance to NK-mediated killing.

In a clinical perspective, a wide variety of mutations impairing cytotoxic lymphocyte development and/or function have been associated with severe diseases in humans. Impaired activity of cytotoxic lymphocytes has been linked to an increased susceptibility to viral infections, persistent inflammation, cancer, and autoimmunity. In the middle part of our Research Topic, we gathered articles that describe defects in cytotoxic lymphocyte biology and their relation to different human disease conditions. Focusing on NK cells, Ham and Billadeau (9) summarize a variety of human immunodeficiency syndromes that affect lymphocyte cytotoxicity, bridging the molecular, and clinical side of this Research Topic. A Hypothesis and Theory article introduces the interesting concept of "perforinopathy" (10). The authors discuss how immune dysregulation and immunopathology are caused by, or related to decreased perforin activity and/or delivery to the target cells, and present compelling evidence that this may be more common cause of human disease than previously assumed. Sieni et al. (11) provide an extensive view of hemophagocytic lymphohistiocytosis, an immunodeficiency caused by defective lymphocyte cytotoxicity, and describe how the knowledge gained by characterization of patients with hemophagocytic syndromes contributes to our understanding of cytotoxic lymphocyte function. This in-depth review is followed by two outstanding papers that illustrate the effect of mutations affecting cytotoxic lymphocyte activity in hemophagocytic lymphohistiocytosis. Jessen et al. (12) compare different molecular defects in lytic granule-associated lytic pathway with the clinical phenotype of hemophagocytic lymphohistiocytosis; based on the degree of impairment of cytotoxic lymphocyte activity a gradient of disease manifestations is distinguished, both in human hemophagocytic lymphohistiocytosis and the mouse models of the disorders. Muller et al. (13) characterize a novel missense mutation in syntaxin-11, and demonstrate that this mutation abrogates binding between syntaxin-11 and Munc18-2,

an interaction critical for release of lytic granules from cytotoxic lymphocytes. Thus, the authors identify an important mechanism that could underlay the development of the familial hemophagocytic lymphohistiocytosis. The last article in this part of the Research Topic focuses on CD57 molecule in NK cell function, its expression pattern during NK cell differentiation and, importantly, in several pathologies, such as cancer, autoimmunity, and infections. The review compiles our current knowledge about the role of CD57 as a marker for NK cell function and disease association (14).

Lymphocyte cytotoxic activity may be harnessed therapeutically to target tumor cells in different adoptive cellular therapy regimes or through the use of recombinant antibodies. In this regard, Mentlik James et al. (15) describe and discuss different therapies available to enhance NK cell anti-tumor responses, with a particular focus on the use of a variety of therapeutic antibodies and immuno-modulating drugs to fight hematologic malignancies. Della Chiesa et al. (16) review the response of NK cells to human cytomegalovirus. The authors summarize evidence for how cytomegalovirus infection can promote NK cell development and maturation, which could actually be beneficial for the hematopoietic stem cell transplant therapies, due to accelerated anti-leukemic or even anti-viral responses. In addition, the aforementioned review by Chijioke and Munz (2) highlights the potential therapeutic benefits of dendritic cell–NK cell interactions, from enhancing anti-tumor NK cell responses, to dendritic cell-mediated expansion of tumor-specific T cells, to decreasing graft-versus-host-disease in different transplantation settings.

We hope that the reader will find this Research Topic very interesting and informative. We invite you to read the following articles and immerse yourself in the fascinating world of cytotoxic lymphocytes and their function.

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Signatures of human NK cell development and terminal differentiation

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Natural killer (NK) cells are part of the innate lymphoid cell (ILC) family and represent the main cytotoxic population. NK cells develop from bone marrow common lymphoid progenitors and undergo terminal differentiation in the periphery, where they finally gain their cytotoxic competence as well as the ability to produce IFN-γ in response to engagement of activating receptors. This process has been at least partially elucidated and several markers have been identified to discriminate different NK cell stages and other ILC populations. NK cell terminal differentiation is not only associated with progressive phenotypic changes but also with defined effector signatures. In this essay, we will describe the phenotypic and functional characteristics of the main stages of NK cell development and terminal differentiation and discuss them in light of recent discoveries of novel ILC populations.

Keywords: NK cells, ILC, differentiation, IFN-γ, CD62L, CD57, NKG2A, KIR

NATURAL KILLER CELLS AND THE INNATE LYMPHOID CELL FAMILY

Natural Killer (NK) cells are innate lymphocytes with the capability to rapidly kill virus-infected or transformed target cells and to produce type 1 cytokines, such as IFN-γ. Over the past years, other innate lymphoid effectors have been identified, which share defined developmental requirements with NK cells, such as the dependence on the transcriptional repressor inhibitor of DNA binding 2 (Id2), but clearly differ for phenotype and effector properties. The heterogeneity displayed by the different innate lymphocyte subsets closely resembles the one observed among $CD4^+$ T helper cells. Thus, a new nomenclature was adapted to collectively define the family, which has been named innate lymphoid cells (ILCs). According to this classification, ILCs have been divided in three main groups: Group 1 ILCs, including NK cells, are defined by the expression of the T-box transcription factors Eomesodermin (*EOMES*) and/or T-bet (*TBX21*) and produce mainly IFN-γ in response to infected or transformed cells; Group 2 ILCs, expressing GATA3, produce IL-13 and IL-5 for the defense against helminthic infections; Group 3 ILCs are characterized by the expression of the orphan nuclear receptor transcription factor RORγt (*RORC*) and include fetal lymphoid tissue-inducer (LTi) cells and adult LTi-like cells, also named ILC3 (1). NK cells likely do not represent the only member of Group 1 ILCs. Indeed, NK cells residing in different organs, such as liver, lung, uterus, or intestine are quite dissimilar among each other. It is still unclear whether this peripheral heterogeneity originates from tissue-specific signals influencing either NK cell *in situ* development or terminal differentiation. Alternatively, tissue resident NK cells might also represent other Group 1 ILC members emerging independently of NK cell precursors. In line with this hypothesis, other potential Group 1 ILC members have been identified recently in the intestine (2, 3). Hence, in the present review we will give an overview on classical human NK cell development and terminal differentiation, and discuss the current knowledge in the frame of this emerging diversity of ILCs.

HUMAN NK CELL DEVELOPMENT

Natural killer cells develop during fetal life as well as after birth from hematopoietic stem cells (HSCs) through a common lymphoid progenitor (CLP). Although most markers used in the mouse and human systems are different, we will revise the major findings of human NK cell development also in light of murine data. Fetal as well as bone marrow (BM) CLP still have the potential to give rise to B, T, NK, and dendritic cells (DCs). As the development of all mouse ILC group members relies on Id2, it was proposed that a common $Id2^+$ ILC progenitor might exist, but still needs to be identified (4). Interestingly, fetal as well as BM HSC can give rise to both, Group 3 ILCs and NK cells in mouse. In particular, expression of the integrin $\alpha_4\beta_7$ defines a mouse fetal CLP subset with a developmental potential restricted to T, NK, and LTi (Group 3 ILC) cells (5, 6). In line with these data, mouse fetal liver $\alpha_4\beta_7{}^+$ CLP can up-regulate RORγt and differentiate toward LTi cells (7–9). Similarly, after birth, mouse BM precursors expressing integrin $\alpha_4\beta_7$ and CXCR6 can give rise to Group 3 ILCs as well as to NK cells, although with low efficiency, but have lost B cell as well as T cell differentiation ability, thus potentially represent a common or a mixture of progenitors committed toward Group 3 ILC and NK cell lineage (8). However, it would be relevant to test the differentiation potential of these cells toward Group 2 ILC, which can also be generated from BM CLP (10–12). In humans, a pioneer study from the group of Verneris has clearly shown that total umbilical cord blood-$CD34^+$ cells can give rise to both NK cells and Group 3 ILCs (13); however, these populations might originate from fetal CLP, and the identification of a common human ILC progenitor is not yet identified, similar to mouse. In addition to the CLP,

mouse NK cell lineage committed progenitors have been initially identified among Lin$^-$ CD122$^+$ NK1.1$^-$ DX5$^-$ NK cells (14) and more recently redefined according to the expression of CD27 and CD244 or Id2 (15, 16). Nevertheless, as to date it has not been tested whether these cell populations are also able to differentiate toward Groups 2 or 3 ILCs, it still remains to be formally proven whether they represent or not the earliest NK cell committed precursor. Although the exact phenotype is not yet completely established in humans, CLP are enriched among CD34$^+$ CD45RA$^+$ CD38^{lo} CD10$^\pm$ CD7$^\pm$ cells. In particular CD34$^+$ CD7$^+$ CD45RA$^+$ and CD34$^+$ CD10$^+$ CD45RA$^+$ cells are preferentially biased to develop into T/NK and B cells, respectively (17–20). CLP-like cells with NK cell commitment potential and expressing β_7 integrin, similar to mice, have also been described in human peripheral blood (PB) and shown to be enriched in extramedullary sites, such as secondary lymphoid organs (SLOs) (21–23), which were therefore proposed as putative sites of human NK cell development. According to this concept, human CLP-like CD34$^+$ β_7 integrin$^+$ CD45RA$^+$ (c-Kit$^-$ CD94$^-$) cells identified in SLO still display T cell and DC potential and have been termed "stage I" NK cells (23). Human NK cell differentiation in SLO would then proceed through "stage II" (CD34$^+$ CD45RA$^+$ c-Kit$^+$ CD94$^-$) and "stage III" (CD34$^-$ c-Kit$^+$ CD94$^-$) NK cells, which would finally give rise to "stage IV" NK cells, also defined as CD56^{bright} CD16$^{neg/lo}$ NK cells (23). More recently, it has been shown that most putative "stage III" human NK cells (CD34$^-$ c-Kit$^+$ CD94$^-$) isolated *ex vivo* in SLO are actually mature ILC3 (24, 25). As mature human ILC3 mostly coexpress c-Kit and CD127, it was proposed that among CD34$^-$ c-Kit$^+$ cells only a minor fraction, characterized by the lack of CD127 might represent "stage III" human NK cells (25). Thus, due to the complex overlapping of markers between human NK cells and ILC3, a more detailed analysis of *in vitro* differentiation of "stage III" and "stage II" NK cell precursors toward different ILC subsets would be of great help. Nevertheless, these studies have contributed crucially to support the idea that extramedullary compartments might indeed represent important sites of NK cell development (22, 23). The unique milieu available in different tissues might influence *in situ* differentiation of NK cells and could actually explain the large phenotypic and functional heterogeneity found among NK cells derived from different tissues, such as liver, lung, or uterus. NK cell precursors might migrate very early to the tissues and develop *in situ* influenced by tissue-specific signals, such as cytokines, stromal or epithelial cells, and environmental cues. However, we cannot exclude that this large heterogeneity might actually rather reflect potentially novel Group 1 ILC subsets, displaying NK-like phenotype. Along this line, extramedullary compartments might represent preferential developmental sites not only for most Group 1 but also for Group 3 ILCs. It has been shown that precursors of murine Group 3 ILCs do not up-regulate RORγt expression in the BM and might migrate very early to the periphery, especially to the intestinal lamina propria (LP), for their development/terminal differentiation (8, 26). However, it still remains an open question at which developmental stage (CLP? common ILC, Group 1 ILC, or NK cell precursor?) NK cells migrate to peripheral tissues to undergo further differentiation. **Figure 1** illustrates markers associated with early NK cell developmental stages derived from human CLP.

HUMAN NK CELL TERMINAL DIFFERENTIATION AND RELATED EFFECTOR FUNCTIONS

Despite the presence of NK cells or NK cell-like populations in different organs, most of our knowledge on NK cell biology originates from mouse splenic and human PB-derived NK cells. Even within the same compartment, NK cells are heterogeneous, and one possible interpretation of this observation is that this diversity might reflect a process of terminal differentiation occurring in the periphery. Along this line, different markers have been used in humans and mouse to describe the stages of final NK cell maturation. In mouse, several authors have contributed to delineate a model in which splenic NK1.1$^+$ NK cells can be mainly dissected according to CD27 and CD11b, which enable to foresee the following maturation progression: CD27$^-$ CD11b$^-$ → CD27$^+$ CD11b$^-$ → CD27$^+$ CD11b$^+$ → CD27$^-$ CD11b$^+$ NK cells (27–29). Conversely, most of the data concerning this process in humans relies on the analysis of circulating PB-derived NK cells. In this compartment, two main NK subsets were originally described on the basis of the differential expression of CD56, which has no homolog in mice and were named CD56^{bright} and CD56^{dim} NK cells (30, 31). CD56^{bright} and CD56^{dim} NK cells differ in phenotype, functional capabilities, and preferential locations. CD56^{bright} NK cells are only a minority of PB-NK cells, whereas they represent the majority of NK cells in SLO. CD56^{bright} NK cells have been described as preferentially CD62L$^{-/lo}$ CD94/NKG2A$^+$ CD62L^{hi} CD57$^-$ KIR$^-$ and commonly termed as the immunomodulatory subset, because of their potential to produce high levels of cytokines like IFN-γ and TNF. In contrast, they express only very low amounts of cytolytic granules and display poor cytotoxic capability. This feature is a characteristic of CD56^{dim} NK cells, which express high amounts of perforin and granzymes and are able to kill target cells like virus-infected and transformed cells. However, the idea that CD56^{bright} are the cytokine producers whereas CD56^{dim} NK cells represent the cytotoxic subset, might be actually misleading as both NK cell subsets can produce large amounts of IFN-γ. CD56^{bright} NK cells produce high amounts of IFN-γ and extensively proliferate in response to DC-derived cytokines like IL-2, IL-15, IL-12, and IL-18 (31), but are unable to produce IFN-γ in response to target cell recognition (32, 33). Conversely, CD56^{dim} NK cells are less efficient in proliferating and producing IFN-γ in response to cytokines (31), but become the main IFN-γ producers after target cell encounter (32, 33). A detailed analysis of CD56^{dim} functional properties and phenotype has actually revealed a consistent heterogeneity also among CD56^{dim} NK cells. When CD56^{dim} NK cells were dissected according to the expression of either CD62L, CD94/NKG2A, or lack of CD57, a subset of NK cells with intermediate phenotype and properties between CD56^{bright} (CD94/NKG2A^{hi} CD62L^{hi} CD57$^-$) and CD56^{dim} CD94/NKG2A$^-$, CD62L$^-$, or CD57$^+$ cells could be identified (33–39). These data were also confirmed at global transcriptome level (33). CD56^{dim} NK cells displaying this intermediate signature (CD94/NKG2A$^+$, CD62L$^+$, or CD57$^-$) combine the ability to produce IFN-γ and proliferate in response to cytokines, characteristic for CD56^{bright} NK cells, with the capacity to kill and produce IFN-γ upon engagement of activating receptors (actRs), typical of CD56^{dim} NK cells. The developmental relationship between CD56^{bright} and the different CD56^{dim} NK cell subsets

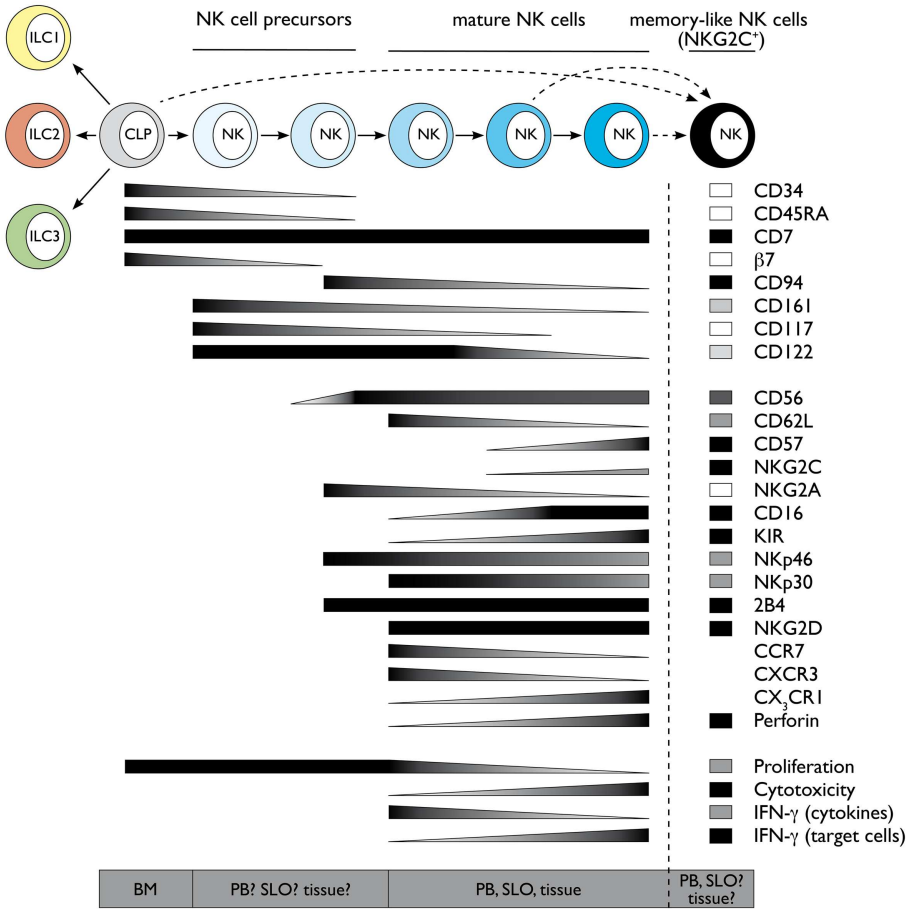

FIGURE 1 | Schematic representation of human NK cell development and terminal differentiation. The development of human NK cells from a common lymphoid progenitor over NK cell precursors to terminally differentiated NK cells is depicted from left to right. The acquisition and loss of indicated surface markers and functional properties propose a linear model of human NK cell development and terminal differentiation. Protein expression levels are depicted as black for high expression and white for no expression, gray indicates intermediate levels. Functional properties are indicated correspondingly. Abbreviations: common lymphoid progenitor (CLP), bone marrow (BM), peripheral blood (PB), secondary lymphoid organs (SLO). See also Ref. 59–61.

has been controversially discussed, but several studies support the concept that CD56^{bright} cells might represent the progenitors of CD56^{dim} NK cells. CD56^{bright} NK cells display longer telomeres compared to CD56^{dim} NK cells and can acquire the expression of CD16 and killer Ig-like receptors (KIR), as well as of other markers, *in vitro* as well as *in vivo* after transfer into immunodeficient mice (33, 36). Further observations support a model of linear maturation from more immature CD56^{bright} toward terminally differentiated CD56^{dim} (CD94/NKG2A^{-}, CD62L^{-}, or CD57^{+}) NK cells passing through CD56^{dim} NK cell subsets displaying intermediate signatures (CD94/NKG2A^{+}, CD62L^{+}, or CD57^{-}). As previously mentioned, this phenotypic sequence is also associated with progressive loss of responsiveness to cytokines and gradual acquisition of responsiveness to actR engagement (33–35) (Luetke-Eversloh and Romagnani, unpublished data). The capability of the more immature CD56^{bright} and CD56^{dim} NK cell subsets to respond to cytokine stimulation strictly correlates to the expression of CD94, CD62L, or CD57 and is reflected by higher expression of cytokine receptors and STAT4 activation (33, 37, 39, 40). Conversely, the ability of the CD56^{dim} subsets to kill and produce IFN-γ in response to actR mainly correlates to the expression of self MHC class I binding inhibitory receptors, in humans belonging to the KIR family or CD94/NKG2A (41–44). This phenomenon, termed education or licensing, was originally described in mice for NK cells expressing self MHC class I specific inhibitory Ly49 receptors or CD94/NKG2A (41–43). According to this concept, during their differentiation or the course of immune responses, NK cells are licensed by self MHC class I molecules through engagement of their inhibitory receptors and this interaction results in NK cell functional competence in response to actR. Thus, licensed NK cells are not only functionally competent but also tolerant because they display at least one inhibitory receptor for self MHC. Conversely, NK cells which do not have a self MHC-specific inhibitory receptor are less functional and therefore still tolerant toward self (41, 45–47). The molecular mechanisms underlying this process have been only partially clarified. In mice, NK cell education seems

to require functional immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of self MHC class I binding Ly49 receptors, but to be independent of SHP-1 signaling (41–43). In addition, educated mouse NK cells have an altered membrane distribution of actR, which is instrumental to functional competence in response to actR engagement (41, 45–47). Although further research is needed to clarify the site, the stimuli, and the time of NK cell education, it is now clear that this process is not an on-off switch occurring during BM development, but rather a fine tuning determined by the number and the affinity of MHC-inhibitory receptor interactions (41, 45–48). However, the stage of NK cell terminal differentiation might also contribute to finely tune functional competence in response to actR engagement. Terminally differentiated NK cell subsets are enriched in cells expressing more than one inhibitory receptor (39), and thus in educated cells, suggesting that differentiation and education might not be entirely uncoupled processes. In **Figure 1**, we summarized several markers defining the phenotype and functions of the different NK cell subsets.

In contrast to individual markers, the combination of expression of CD16, KIR, CD57, CD62L, CD94/NKG2A does not identify three main populations but results in a complex number of intermediate, terminally differentiated, educated, and non-educated NK cell subsets. Although this implies even a larger heterogeneity of PB-NK cells, it does not exclude that NK cell terminal differentiation still proceeds following a linear progression. In support of this model, we have observed that these markers are not acquired or lost synchronously in time during reconstitution from HSC during transplantation and this might contribute to explain NK cell peripheral variety (M.K. and C.R., unpublished data). Moreover, diverse signals, such as homeostatic and pro-inflammatory cytokines or engagement of defined activating or inhibitory receptors at steady state or during infections, might influence NK cell terminal differentiation and/or education and determine the quality and the intensity of this process. One clear example of how environmental stimuli can dramatically poise human NK cell final maturation is cytomegalovirus (CMV) infection, which can globally accelerate NK cell terminal differentiation (49). CMV infection induces the expansion and persistence of a unique NK cell subset, expressing the actR NKG2C and being preferentially positive for CD57 and self MHC class I binding KIR (50–52). Although $NKG2C^+$ NK cells undergoing expansion during CMV infection tend to resemble phenotypically and functionally terminally differentiated NK cells, it has been also suggested that they might represent the human correspondent of the previously described $Ly49H^+$ memory-like murine NK cells expanding and persisting after CMV infection (53, 54) (**Figure 1**). Interestingly, expansion of $NKG2C^+$ NK cells occurs also in CMV-seropositive patients with chronic hepatitis B or C virus or during hantavirus infection (55, 56). Further experiments are required to understand their origin and developmental relationship with other PB-NK cell subsets.

Interestingly, different stages defined according to CD16, NKG2A, and KIR can also be identified among fetal NK cells, suggesting that similar forces likely govern the process of differentiation before and after birth. However, even in fetal lung where NK cells are strongly enriched in more mature KIR^+ cells, CD57 expression is very low and only NKG2A, but not self MHC specific KIR, mediate NK cell education, possibly suggesting that fetal tissue milieu influence NK cell differentiation and functional properties (57).

Despite of all these suggestive evidences, it is however difficult to completely exclude that human NK cell peripheral heterogeneity might not rather represent developmentally unrelated members of the Group 1 ILC family. $CD56^{bright}$ NK cells uniquely express CD127, CD117, and GATA3, which are hallmarks of other ILC subsets and have been also proposed to correspond to mouse thymus-derived NK cells (58). Further investigation employing different experimental approaches might help us to revisit this issue and elicit some surprises.

"It's a rare occasion when your plans and expectations come down just exactly how you planned them. Who can say, anyway? Time will tell" ("Time will tell," Tower of Power).

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Dendritic cell derived cytokines in human natural killer cell differentiation and activation

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Dendritic cells (DCs) and natural killer (NK) cells shape each other's functions early during immune responses. DCs activate NK cells and NK cells can mature or kill DCs. In this review we will discuss which DC and NK cell subsets are mainly affected by this interaction, where these encounters might take place and which signals are exchanged. Finally, we will point out what the clinical benefit of understanding this interaction might be and how it changed our view on NK cells as innate lymphocytes.

Keywords: IL-18, IL-12, IL-15, IFN-alpha, immunological synapse, NKp30, DNAM-1

INTRODUCTION

Natural killer (NK) cells have originally been described by their function to spontaneously lyse tumor and infected cells (1–3). However, it has recently become apparent that they require mostly cytokine mediated activation to differentiate into effector cells and execute different effector functions – at least in humans – dependent on their differentiation stage (4–6). Three prominent cell populations that trigger this NK cell activation and differentiation have been identified. These are dendritic cells (DCs), neutrophils, and $CD4^+$ T cells (7–12). In this review we will focus on the interaction of NK cells with DCs, with an emphasis on its role in augmenting NK cell function during the innate phase of immune responses.

Human DCs are composed of different subpopulations (13) of which the two main subpopulations are conventional and plasmacytoid DCs (cDCs and pDCs). While pDCs are primarily found in primary and secondary lymphoid tissues, including bone marrow, thymus, lymph nodes, and spleen as well as blood in steady-state conditions, cDCs can be found both in lymphoid tissues and peripheral organs. Human cDCs can be subdivided in two additional subsets, $CD1c^+$ ($BDCA1^+$) and $CD141^+$ ($BDCA3^+$) DCs, which have now been found in the skin, liver, and lung, in addition to primary and secondary lymphoid tissues (14). In addition to these constitutive DC populations, which are at least to a substantial part dependent on Flt3L in their development (15, 16), inflammatory DCs can develop from monocytes. This DC lineage is dependent on GM-CSF for its development and therefore, GM-CSF constitutes an integral component of human monocyte-derived DC differentiation *in vitro* (16). Finally, Langerhans cells constitute a human DC population in the epidermis and at least in mice their steady-state maintenance is dependent on stromal IL-34 (17, 18). To fulfill their function DCs are equipped with molecules that sense the environment and in contrast to mice, the human DC populations have quite restricted expression patterns of pathogen associated molecular pattern (PAMP) receptors (19). For example, the toll-like receptor (TLR) nine for unmethylated DNA, which can be stimulated by CpG oligonucleotides, is only expressed by pDCs in humans, as is TLR7 for single-stranded RNA. In contrast, the double-stranded RNA receptor TLR3 is highest expressed on $CD141^+$ cDCs and elicits high IL-12 and IFN-α/β/λ production from this subset. Interestingly, the IFN-α production by $CD141^+$ cDCs reaches similar levels as IFN-α production from pDCs after TLR7 stimulation (20, 21). Therefore, all of these human DC populations need to be considered for NK cell activation and differentiation and will be discussed below.

HUMAN NATURAL KILLER CELL SUBSET DISTRIBUTION

Natural killer cell reactivity is guided by the balance of activating and inhibitory receptors (22). Both are acquired sequentially during development where inhibitory receptors are also instructive in NK cell education (23, 24). NK cell differentiation can in part be driven by both IL-15 and IL-2 in humans (25, 26). It is now assumed that the first functionally competent NK cell subset are $CD56^{bright}CD16^-$ NK cells, which have lost c-kit (CD117) and IL-7Rα (CD127) expression (26, 27). These seem to acquire the intermediate affinity activating FcγRIII/CD16, successively down-regulate the inhibitory HLA-E receptor NKG2A/CD94 and acquire more and more inhibitory killer immunoglobulin-like receptors (KIRs) upon differentiation (28). Interestingly, at any stage CD57 expression seems to terminally differentiate the respective NK cell subset and diminish its capacity to further expand. While $CD56^{bright}CD16^-$ NK cells respond to cytokine stimulation primarily with cytokine production, further differentiated $CD56^{dim}CD16^+$ NK cells display increased cytotoxicity and can produce a rapid, but transient cytokine burst upon tumor or infected cell encounter (25, 29). Interestingly, the successive

up-regulation of KIRs seems to influence the reactivity of the later NK cell differentiation stages, depending on the expression of the cognate HLA class I ligands (24). Namely, NK cells with KIRs specific for self-HLA class I molecules have a higher reactivity against HLA class I negative tumor cell targets (30). These so-called licensed NK cells accumulate preferentially during some viral infection, primarily during persistent human cytomegalovirus (HCMV) infection (31). Some of these NK cell subset expansions allow for a more rapid response to secondary challenge with the same pathogen, which could be interpreted as an immunological memory function of the NK cell compartment (32, 33). These infection-experienced NK cells have been suggested to be enriched in the CXCR6 expressing hepatic NK cell subset, at least in mice (34).

The different NK cell differentiation stages have been found to be enriched at distinct anatomical sites (35). While $CD56^{dim}CD16^{+}$ NK cells predominate in the blood, most other tissues harbor high frequencies of $CD56^{bright}CD16^{-}$ NK cells. This IFN-γ producing NK cell subset has been originally found to mainly populate lymph nodes, tonsils, and splenic white pulp (25, 36–38). However, this NK cell differentiation stage has recently also been found to be enriched in liver, skin, uterus, joints, and tumor tissue (39–43). The CXCR6 positive NK cell subset with memory-like features might preferentially home to liver (34). Therefore, different NK cell differentiation stages can be preferentially found in distinct organs and their location might determine with which human DC populations they can preferentially interact.

SITES OF INTERACTION BETWEEN HUMAN DCs AND NK CELLS

While DCs can be found in all tissues, after activation, also called maturation, by for example TLR ligands, they migrate to or remain in secondary lymphoid tissues (13). Therefore, the interaction between mature DCs and resting NK cells would probably preferentially take place in secondary lymphoid tissues. Consistent with this notion, human NK cells and cDCs have been found to be enriched in the T cell zones of lymph nodes (36, 44). Moreover in mice, activation of NK cells in different infectious settings required DCs and homing of NK cells to secondary lymphoid tissues (45). Furthermore, injection of mature DCs resulted in the attraction of NK cells to secondary lymphoid tissues in mice (46) and brief contacts of NK cells with DCs have been observed in lymph nodes after adoptive transfer of mature DCs or *in vivo* activation with TLR3 and 4 ligands (47). Human secondary tissues might be especially predestined for these interactions, because $CD56^{bright}CD16^{-}$ NK cells preferentially home to these sites via CCR7 and CD62L expression and are enriched at these sites (36, 44). Moreover, mature monocyte-derived DCs preferentially stimulate $CD56^{bright}CD16^{-}$ NK cells to proliferate and produce cytokines (37, 44, 48). Thus, NK cell activation by mature DCs probably happens primarily in the T cell zones of secondary lymphoid tissues including lymph nodes.

Once activated, these NK cells might then leave secondary lymphoid tissues and home to sites of inflammation. Indeed, it has been observed that NK cells and DCs co-localize in inflamed skin (49). These activated NK cells might kill immature DCs at this site in order to prevent them from transmitting tolerogenic signals to secondary lymphoid tissues (50). Indeed, NK cell killing of preferentially immature DCs has been observed, especially when activated NK cells outnumber DCs (51, 52). In light of the fact that in most peripheral human tissues, inflamed organs, or tumor microenvironment, it has been shown that $CD56^{bright}CD16^{-}KIR^{-}$ NK cells are enriched (35), it is interesting that this NK cell subset again might be preferentially killing DCs in the autologous setting without compromised MHC class I expression (53). However, NK cell reactivity might be curbed by regulatory T cells at these sites, who have been suggested to impair IL-2 and IL-15 mediated expansion and activation in mice (54–56). Furthermore, myeloid derived suppressor cells (MDSCs) might inhibit anti-tumor NK cell responses (57, 58) and their depletion by chemotherapeutika could augment their reactivity in tumors (57, 59). Only in conditions of infection induced down-regulation of MHC class I, as for example during HCMV infection, terminally differentiated $KIR^{+}NKG2C^{+}CD57^{+}$ NK cells might accumulate and then be enriched in peripheral tissues (33). Thus, NK cells might be stimulated in secondary lymphoid tissues by mature DCs and afterward might kill immature DCs at peripheral sites. Since both immature and mature DCs express significant levels of MHC class I molecules as ligands for inhibitory NK cell receptors, like KIRs, preferentially $CD56^{bright}CD16^{-}KIR^{-}$ NK cells might be involved in both interactions.

SIGNALS IN HUMAN DC INTERACTION WITH NK CELLS

According to these two different sites of interactions for DCs and NK cells, the stimulatory and killing signals that are exchanged require different molecules (**Figure 1**). In secondary lymphoid tissues, probably primarily cytokines are exchanged. Interleukin-12 and -18 have mainly been identified to activate cytokine production by NK cells (37, 44, 60). In response to IL-12, NK cells primarily produce IFN-γ, TNF-α, and GM-CSF. In contrast, IL-15 is involved in DC-stimulated NK cell proliferation, survival, and pre-activation (44, 45, 61). Interestingly, IL-15 only reaches the cell surface of the producing cell in complex with IL-15Rα (62), and trans-presentation might facilitate cell contact dependent IL-15 signaling. Finally, type I IFN augments NK cell cytotoxicity (37, 63). Depending on their cytokine secreting potential, different DC subsets are therefore capable of triggering one or the other NK cell function. PDCs stimulate primarily NK cell cytotoxicity via their type I IFN producing function (63). $CD1c^{+}$ conventional and monocyte-derived human DCs are capable of producing IL-12, particularly after maturation with a TLR3 agonist (37, 63). Finally, Langerhans cells can support NK cell survival via their ability to present IL-15 on their surface (64). Thus, different human DC subsets stimulate distinct NK cell effector functions primarily via secretion of cytokines.

The respectively activated NK cells can then however signal back to DCs and presumably spread immune activation to neighboring secondary lymphoid tissue resident DCs (**Figure 1**). It has been shown that NK cell produced TNF-α can mature DCs (52, 65). This maturation can initiate adaptive T cell mediated immune responses against for example tumors (66, 67). Moreover, NK cell produced IFN-γ can assist in the polarization of Th1 responses by DCs (46, 68–70). Particularly, IL-18 activated NK cells up-regulate secondary lymphoid tissue homing markers like CCR7, and can

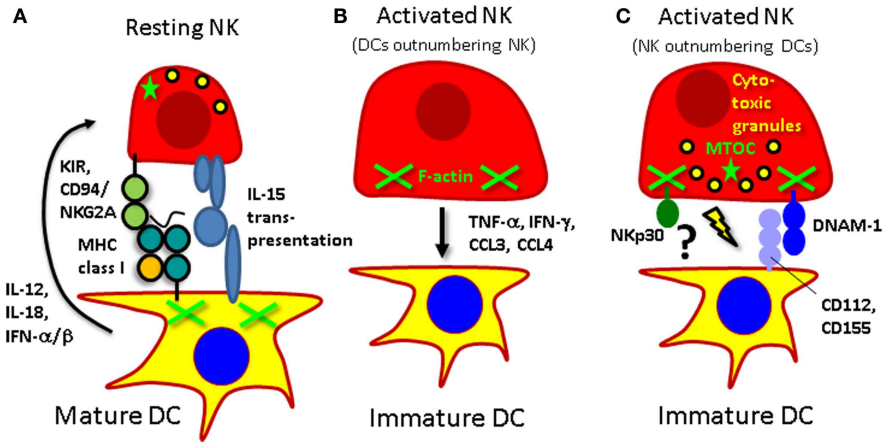

FIGURE 1 | Interactions of human NK cells with DCs. **(A)** Mature DCs activate resting NK cells via IL-12, IL-15, and type I IFN. At the same time NK cells receive an inhibitory signal via killer immunoglobulin-like receptors (KIRs) or CD94/NKG2A to prevent them from killing mature DCs. **(B)** Activated NK cells can mature DCs via secretion of TNF-α, polarize them to produce IL-12 for Th1 induction with IFN-γ, and attract them via the CCR5 ligands CCL3 and CCL4. **(C)** If they, however, outnumber immature DCs they can kill these targets by perforin mediated lysis after engagement of the activating receptors NKp30 and DNAM-1.

stimulate IL-12 production by DCs (71). These NK cell stimulated DCs also up-regulate CCR7 and migrate in response to its ligand CCL21 (72). Furthermore, these so-called "helper" NK cells can also stimulate DCs to produce chemokines, primarily CXCL9, CXCL10, and CCL5, which allows attraction of effector $CD8^+$ T cells (73). Therefore, NK cells can mature DCs to preferentially home to secondary lymphoid tissues and prime Th1 responses.

Finally, the third outcome of interactions between DCs and NK cells is killing of DCs (**Figure 1**). It has been shown that this occurs between activated NK cells and immature DCs (51–53). At least monocyte-derived DCs are recognized by activated NK cells via their NKp30 and DNAM-1 activating receptors (51, 74–76). Mature DCs are protected from this NK cell lysis by up-regulation of MHC class I molecules, including the non-classical HLA-E molecule (51, 53). Thus, DCs express ligands for activating receptors on human NK cells, but are after maturation protected from NK cell lysis by increased expression of MHC class I molecules.

IMMUNOLOGICAL SYNAPSES THAT MEDIATE NK CELL INTERACTION WITH DCs

Natural killer cells interact with target cells usually via the establishment of one of two types of immunological synapses. If activating signals dominate the interaction, an activating immunological synapse is observed with actin polymerization in the NK cell, polarization of the microtubule organizing center (MTOC) to the synapse and cytotoxic granule release through the center of the synapse, which leads to the killing of the target cell (77). On the contrary, if inhibitory signals prevail, inhibitory immunological synapses do not mature with cytoskeleton rearrangement, are short lived and the NK cell dissociates from the target cell without mobilizing any effector functions (78). NK cells also interact with DCs through immunological synapses (61, 79–81). However, the outcome of the interaction between mature DCs and NK cells is NK cell activation without killing of the conjugated DC. Therefore, we termed this immunological synapse regulatory. It seems to be designed to efficiently exchange paracrine IL-12, IL-18, and IL-15 from DCs to NK cells, in order to stimulate cytokine production and survival of NK cells (61, 79, 80). This becomes especially important when maturation stimuli allow DCs only to produce limited amounts of these cytokines and other leukocyte populations in the lymph node environment can consume these cytokines in addition to NK cells (37, 79). At the same time, inhibitory interactions are exchanged at the regulatory immunological synapse between mature DCs and NK cells. Inhibitory receptors like KIRs accumulate in other membrane domains than NK cell stimulatory IL-15/IL-15Rα complexes, although both are located in the center of immunological synapses of mature DCs with resting NK cells (61). This compartmentalization of inhibitory and activating domains occurs rapidly within 5 min after interaction between these two leukocyte populations. Upon longer interaction, the immunological synapse between mature DCs and NK cells is then stabilized by cytoskeletal rearrangements, including actin polymerization at the synapse in the conjugated DC (81). Interestingly, these cytoskeletal rearrangements seem to primarily support the inhibitory signals that are exchanged at the synapse between DCs and NK cells, because inhibition of actin polymerization in DCs by for example decreasing the expression of Wiskott Aldrich Syndrome Protein (WASP), which organizes the actin cytoskeleton at immunological synapses, leads to conversion of the immunological synapse into an activating NK cell synapse with actin polymerization in the conjugated NK cells and killing of DCs. Therefore, human DCs seem to coordinate their interaction with NK cells via a regulatory immunological synapse, which allows exchanging at the same time stimulatory signals for NK cells and signals that inhibit them from killing DCs.

While these long-lasting synapses have been observed with human cells *in vitro*, DCs, and NK cells establish only short interactions, usually below 3 min, in mouse lymph nodes (47). It is so far unknown, which species differences might cause these divergent interaction kinetics. One possibility, however, could be that

the possible NK cell subpopulation counterpart in mice (82) of the $CD56^{bright}CD16^{-}$ NK cell population, which preferentially forms conjugates with human mature DCs (61), engages in these long-lasting synapses, and the respective murine NK cell subset is too rare to be readily observed in mouse lymph nodes *in vivo*. Alternatively, however, the short interactions *in vivo* could also result from additional stimuli like chemokine gradients that could sustain NK cell mobility and shorten NK cell interactions with DCs. Further *in vivo* imaging studies could clarify such heterogeneity of immunological synapse formation between DC and NK cell subpopulations.

THERAPEUTIC POTENTIAL OF NK CELL INTERACTIONS WITH DCs

Both NK cell activating as well as DC restricting functions in the interaction of NK cells with DCs might be harnessed for therapeutic benefit. NK cell activation by DCs during vaccination might generate a stimulatory environment for the priming of Th1 responses. Along these lines, TLR3 agonists mature DCs for optimal NK cell stimulation *in vitro* (37). Moreover, synthetic double-stranded RNA induced a profile beneficial for NK cell stimulation in healthy volunteers (83) and was able to augment NK cell responses against tumor cells in mice with reconstituted human immune system components (84). Therefore, the right choice of adjuvant could harness NK cells during vaccination. Cytokine production by activated NK cells can improve maturation of DCs to expand tumor specific T cells more efficiently and acquire homing markers for secondary lymphoid tissues (72). Thus, NK cell activation by DCs during vaccination could feed-back to antigen presenting cells to increase their Th1 polarizing potential.

However, a completely different clinical benefit of DC interaction with NK cells was revealed when it was noticed that alloreactive NK cell therapy by haploidentical bone marrow transplantation against acute myeloid leukemia (AML) relapse also diminished graft-versus-host-disease (GvHD) (85, 86). It was noted that NK cells were not only able to target HLA mismatched leukemia cells, but also allogeneic DCs, which then no longer can prime donor T cells, specific for the host MHC allotype, to attack the host. This NK cell reactivity against MHC mismatched DCs might also be beneficial in other transplantation settings. At least in experimental animal models, it is well documented that alloreactive NK cells eliminate DCs from allogeneic grafts (87–90). In MHC mismatched skin, pancreatic β-islet and lung transplantation, it was shown that host NK cells eliminate donor DCs from the transplant, which subsequently led to decreased priming of host derived alloreactive T cell responses. The resulting diminished rejection allowed the respective transplants to survive longer and to perform better. These data suggest that allogeneic DC targeting by NK cells that lack KIRs against the MHC haplotype of the graft can ameliorate GvHD by donor NK cell cytotoxicity or transplant rejection by host NK cell cytotoxicity. These clinical benefits might be augmented by adoptively transferring alloreactive NK cell lines, which could be either stimulated with TLR3 agonist matured DC populations or their cytokines *in vitro*. IL-12, IL-15, IL-18, and IFN-α should be considered as stimulatory monokines that could be used to expand functionally competent NK cell lines *in vitro*. Adoptively transferred NK cell lines that have been activated and expanded in this fashion might confer protection against leukemia relapse and GvHD in a haploidentical transplantation setting until NK cell populations have reconstituted from transplanted hematopoietic progenitor cells.

CONCLUSION

In recent years it has become apparent that DCs can in addition to being superior antigen presenting cells for T cell priming, activate innate lymphocytes (69). In fact, the parallels between $CD8^{+}$ T cell priming and NK cell activation by DCs are quite striking. For both lymphocyte populations, activation happens in secondary lymphoid tissues, is dependent on IL-12 and requires IL-15 for survival (91). DCs form immunological synapses with $CD8^{+}$ T cells and NK cells, which are stabilized by the DC cytoskeleton. Furthermore, both of them acquire cytotoxicity through this activation and loose initial cytokine production during further differentiation. Alongside, and presumably as a protective mechanism against immunopathology mediated by these cytotoxic lymphocytes, both $CD8^{+}$ T cells and NK cells up-regulate inhibitory receptors, which safe-guard their activation upon target cell encounter. Finally, they both can kill DCs either after viral antigen presentation or virus induced MHC class I down-regulation. Therefore, it is tempting to speculate that NK cells are the evolutionarily older cousins of $CD8^{+}$ T cells. However, it still needs to be clarified if they can also develop some sort of memory to infections through for example NK cell subset expansion in response to pathogens (33).

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Distinct migration and contact dynamics of resting and IL-2-activated human natural killer cells

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Natural killer (NK) cells serve as one of the first lines of defense against viral infections and transformed cells. NK cell cytotoxicity is not dependent on antigen presentation by target cells, but is dependent on integration of activating and inhibitory signals triggered by receptor–ligand interactions formed at a tight intercellular contact between the NK and target cell, i.e., the immune synapse. We have studied the single-cell migration behavior and target-cell contact dynamics of resting and interleukin (IL)-2-activated human peripheral blood NK cells. Small populations of NK cells and target cells were confined in microwells and imaged by fluorescence microscopy for >8 h. Only the IL-2-activated population of NK cells showed efficient cytotoxicity against the human embryonic kidney 293T target cells. We found that although the average migration speeds were comparable, activated NK cells showed significantly more dynamic migration behavior, with more frequent transitions between periods of low and high motility. Resting NK cells formed fewer and weaker contacts with target cells, which manifested as shorter conjugation times and in many cases a complete lack of post-conjugation attachment to target cells. Activated NK cells were approximately twice as big as the resting cells, displayed a more migratory phenotype, and were more likely to employ "motile scanning" of the target-cell surface during conjugation. Taken together, our experiments quantify, at the single-cell level, how activation by IL-2 leads to altered NK cell cytotoxicity, migration behavior, and contact dynamics.

Keywords: natural killer cells, cell migration, single-cell, fluorescence imaging, microchip

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes capable of clearing both virus-infected and transformed cells. They have conventionally been classified as part of the innate immune system, but this picture is currently changing as studies have shown that NK cells display features of immunological memory normally ascribed to adaptive immunity (1). NK cell-mediated cytotoxicity is controlled by the integration of activating and inhibitory receptor signaling at the NK cell immune synapse (IS) formed between NK and target cell (2, 3). NK cells can also respond by producing cytokines, e.g., interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α), and are known to be activated by cytokines like interleukin (IL)-2, IL-12, and IL-15.

Interleukin-2, initially called T-cell growth factor for its capacity to maintain *in vitro* cultures of primary T cells (4–6), has been widely used to augment the cytotoxic activity of NK cells *in vitro* (7). The immunostimulatory properties of IL-2 have been used in cancer treatment (8) where it has also been shown to selectively lead to NK cell expansion when given in relatively low doses over extended periods of time (9). It is poorly understood under what conditions NK cells can be stimulated by endogenous IL-2, even though cross-talk between NK cells and IL-2-producing T cells has been reported, linking the innate and adaptive immune systems (10–12).

Interleukin-2 shifts the gene and cell surface receptor expression of NK cells. Activating receptors, such as DNAM-1, NKp44, and KLRB1, are upregulated while inhibitory receptors, like KIR2DL2 and KIR3DL3, are downregulated after exposure to IL-2 (13, 14). The expression of adhesion molecules is also higher on IL-2-activated cells, consistent with the observation that they form stronger conjugates than resting NK cells (12, 15). Increased cell–cell adhesion has been directly coupled to cytotoxicity, partly explaining why IL-2-activated NK cells show higher cytotoxic potential than resting NK cells. IL-2 stimulation has also been observed to restore the formation of filamentous (F)-actin and cytotoxicity in NK cells from patients suffering from Wiskott–Aldrich syndrome (WAS) (16).

Although IL-2 activation generally enhances NK cells' ability to lyse target cells, resting NK cells can also efficiently lyse some target-cell types, e.g., the leukemia cell line K562 (13). Bryceson et al. used resting NK cells in a redirected lysis assay to systematically decipher the role of individual activating receptors in combination with LFA-1 (that was triggered by expression of ICAM-1 on the P815 target cells). Engagement of CD16 led to cytotoxicity, whereas none of the receptors NKp46, NKG2D, 2B4, CD2, or DNAM-1 triggered a cytotoxic response. In IL-2-activated NK cells, individual engagement of these receptors was sufficient to trigger cytotoxicity. Interestingly, when resting NK cells were

stimulated through combinations of these receptors, e.g., NKG2D and 2B4, or 2B4 and DNAM-1, cytotoxic responses could be triggered (13). Thus, resting NK cells are able to lyse target cells but require the right combination of activating signals, and, therefore, seem more tightly regulated than IL-2-activated NK cells.

An emerging theme at the border between technology and biology is the development of methods probing the dynamics of many individual cells in parallel. This can be achieved, for example, by using microchip-based tools trapping cells over extended periods of time (17–20). Such approaches have provided insights into NK cell heterogeneity in terms of cytokine production, killing behavior, and migration (21–23). We also recently reported significant heterogeneity among individual IL-2-activated NK cells in terms of migration and cytotoxicity and, here, compare this data with resting NK cells (21, 24). We report dramatic differences in morphology, contact dynamics, and target-cell killing, but less obvious differences in migration dynamics between resting and IL-2-activated cells.

MATERIALS AND METHODS

CELLS

Peripheral blood mononuclear cells were obtained from buffy coats of anonymous healthy donors and all experiments were performed in accordance with local ethics regulations. NK cells were isolated by negative selection according to manufacturer's instructions (StemSep, StemCell Technologies, Grenoble, France; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in IMDM or RPMI supplemented with 10% human serum, 50 U/ml penicillin-streptomycin, 1 × non-essential amino acids, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (in some cultures only). Resting NK cells were used within 24–48 h of isolation. Activated NK cells were cultured in the same medium as above supplemented with 100 U/ml recombinant IL-2. Activated cells were used after 7–16 days. The purity of $CD3^-CD56^+$ cells was assessed by flow cytometry and was >95% for all experiments except one, for which $CD3^-CD56^+$ was >85%. For all isolations, the fraction of contaminating $CD3^+CD56^-$ T cells was <1%.

Human embryonic kidney (HEK) 293T (ATCC, Manassas, VA, USA) cells were used as target cells and were maintained in high-glucose RPMI-1640 supplemented with 10% FBS and 50 U/ml penicillin-streptomycin and for some experiments with additions of 1 × non-essential amino acids, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol.

CELL LABELING

Natural killer cells and HEK293T target cells were labeled for 10 min at 37°C in serum-free medium with 0.32–1 μM calcein red–orange and 1 μM calcein green (both Invitrogen), respectively. Cells were washed three times in serum-free medium prior to seeding in microwells.

MICROWELL MIGRATION AND CELL–CELL INTERACTION IMAGING ASSAY

The microchip-based imaging assay has been described in detail for IL-2-activated NK cells (21, 24). For resting NK cells, washed and sterilized microchips (vertical walls of 300 μm and base area 450 × 450 μm^2) were rinsed with filtered PBS and coated with a 25 μg/ml fibronectin solution for 1 h at room temperature. Thereafter, the chip was mounted into a holder, rinsed with PBS, and covered with complete cell culture medium. Approximately 40,000 HEK 293T target cells were seeded onto the microwell chip and left to sediment and adhere to the glass bottom for 1.5–3 h at 37°C, 5% CO_2. Afterward, approximately 20,000 NK cells were seeded onto the chip and left to sediment for 5 min. The upper layer of the medium was aspirated and replaced with new medium 10 times to remove unseeded NK cells, reducing the number of NK cells falling down into the wells during imaging. The average number of target and NK cells in each well was approximately 120 and 35, respectively. Fluorescence imaging was performed using a confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) with an open pinhole to maximize detected light. Up to four individual microwells in three separate experiments were imaged every 2 min for up to 12 h.

EFFECTS OF DIFFERENCES IN EXPERIMENTAL PROTOCOLS

Of note, the experimental setup differed slightly between resting and three out of four experiments of IL-2-activated NK cells. For all experiments in the resting condition and one in the IL-2-activated ($n = 48$), wells were coated with fibronectin and had slightly smaller base area (450 × 450 vs. 650 × 650 μm^2). Fibronectin coating has been shown to facilitate migration of human NK cells through transwell systems (25) and maintain the survival of murine NK cells *in vitro* (26). Thus, any effects caused by fibronectin coating could be expected to lead to more migration of the resting NK cells and therefore decrease the overall differences in migration observed between the two conditions. Furthermore, NK cells close to the microwell walls could experience some restriction in their migration. Based on NK cell size, it is reasonable to assume that only NK cells within 15 μm from the walls would be affected by interactions with the walls. This area corresponds to 7 and 5% of the total area for the smaller and larger wells, respectively. Thus, any edge effects caused by the different well sizes can be expected to be small. β-mercaptoethanol was used in three out of four cultures of activated cells and although any effects are assumed to be negligible they cannot be ruled out.

CELL TRACKING AND ANALYSIS

Natural killer cells were tracked manually in the image analysis software packages Volocity (PerkinElmer, Waltham, MA, USA) and ImageJ. In total, 265 resting NK cells from four different donors ($n = 113, 55, 69, 28$) and 221 IL-2-activated NK cells from four different donors ($n = 48, 50, 75, 48$) were tracked in 8 h-long time-lapse movies. Each NK–target cell interaction was scored for duration (conjugation and attachment time) and outcome (killing/non-killing). Target-cell death was determined by examining both intracellular calcein fluorescence decrease as well as visible signs of death, like plasma membrane blebbing or cell swelling as previously described (24). Unless otherwise stated in figure legends, the data presented derive from all resting and activated NK cells.

MIGRATION ANALYSIS

Natural killer cell migration behavior was analyzed from individual cell trajectories similar to what has been described previously

(20, 21). In short, cellular speed was calculated by comparing consecutive coordinates in the trajectories. Properties of each NK cell trajectory was quantified by calculating a local mean-square displacement (MSD) and migration coefficient using a sliding window of 25 time points centered around the time point to be calculated. Based on transient values of the migration coefficient and curvature of the MSD function, each NK cell trajectory was divided into different modes of migration [random movement, directed migration, and transient migration arrest periods (TMAPs)]. TMAPs are characterized by low motility that is typically confined to a small area. Directed migration is generally marked by higher and directionally persistent migration. Random movement is defined as neither TMAP nor directed migration, i.e., motion that appears stochastic and consistent with random-walk.

STATISTICAL ANALYSIS

The non-parametric two-sided Mann–Whitney U-test was used to evaluate statistical significance for all data except for that presented in Figure S6 in Supplementary Material, where the Wilcoxon signed-rank test was used. p-values < 0.05 were considered statistically significant. Data shown are mean $\pm$ standard deviation unless otherwise stated.

RESULTS

ACTIVATED NK CELLS EXHIBIT MORE DYNAMIC MIGRATION, WHICH IS RELATED TO MODES OF MIGRATION

The average mean migration speed of the resting NK cells over the 8-h assay was $\pm 0.6\,\mu$m/min while it was $\pm 0.7\,\mu$m/min for activated NK cells ($p < 0.005$). A histogram of mean migration speeds revealed substantial differences in the mean speeds of individual cells within subsets and that fast-migrating NK cells were more common in the activated subset (**Figure 1A**).

We then set out to investigate if there were any detectable differences in the migration behavior of activated and resting NK cells. To this end, we applied a previously developed method to subdivide cell trajectories into three distinct modes of migration, i.e., TMAPs, directed migration or random movement (21). Overall, a majority of NK cells spent considerable time in TMAPs with the average fractions of time 89% for resting and 71% for activated NK cells (**Figure 1B**). Strikingly, approximately 72% of resting NK cells spent between 90 and 100% of the assay in TMAPs, compared to 38% for the activated NK cells (**Figure 1C**). Thus, a large fraction of the resting cells displayed low motility while activated NK cells were significantly more motile ($p < 0.005$).

Examining directionally persistent migration showed that all resting NK cells (with the exception of one cell) spent little time in directed migration (<40% of the time), while a few activated NK cells spent >40% of the assay in directed migration (**Figure 1D**). The average times spent in directed migration were 2% for resting NK cells and 12% for activated cells (**Figure 1B**). Thus, in this assay resting NK cells almost completely lacked directionally persistent migration. The rest of the time, on average 9% for resting NK cells and 17% for activated NK cells, was spent in random movement (**Figures 1B,E**). Analysis showed that differences between resting and activated cells in the fractions of time spent in different modes of migration were statistically significant ($p < 0.005$).

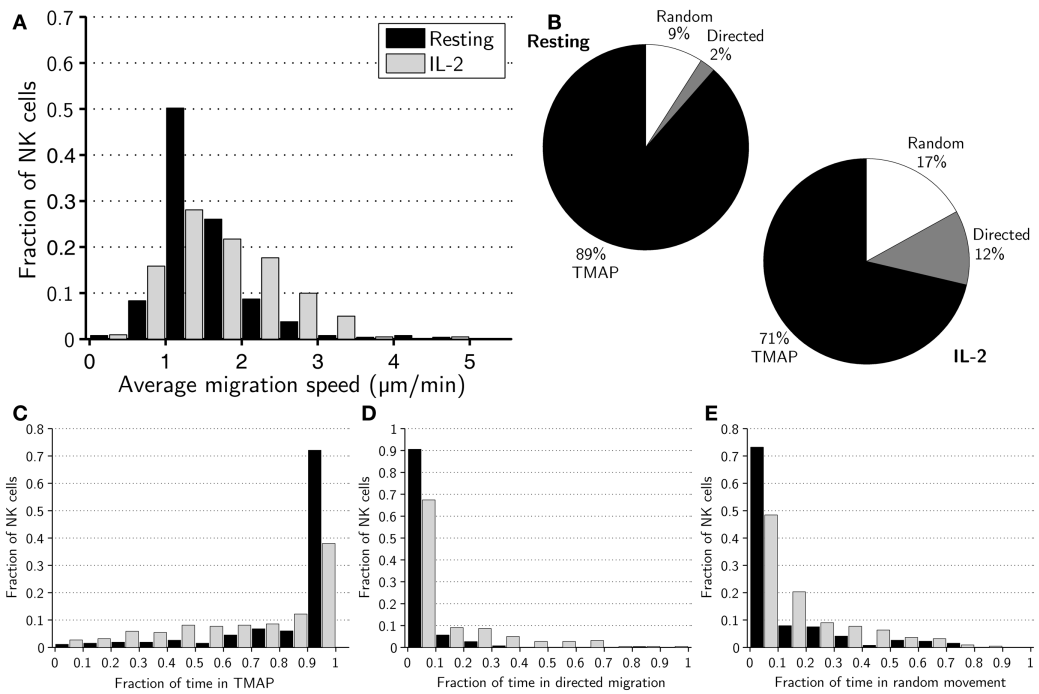

FIGURE 1 | Migration dynamics of resting and activated NK cells. **(A)** Distributions of average migration speeds for resting (black bars) and activated (gray bars) NK cells. **(B)** Pie charts showing mean fraction of time spent in TMAPs (black), directed migration (gray), and random movement (white) for resting (left chart) and activated (right chart) NK cells. **(C)** Distributions of fraction of time spent in TMAPs for resting (black bars) and activated (gray bars) NK cells. **(D)** Distributions of fraction of time spent in directed migration for resting (black bars) and activated (gray bars) NK cells. **(E)** Distributions of fraction of time spent in random movement for resting (black bars) and activated (gray bars) NK cells.

Next we compared the mean migration speeds of cells in different modes of migration and, as expected, the average mean speeds in TMAPs were considerably lower, $\pm 0.8\,\mu$m/min for resting and $\pm 0.8\,\mu$m/min for IL-2-activated NK cells ($p < 0.05$) compared to other modes of migration. In directed migration, the average mean speeds were ± 1.1 and $\pm 0.7\,\mu$m/min for resting and activated NK cells, respectively ($p < 0.005$). The random movement periods had average mean speeds of $\pm 1.1\,\mu$m/min for resting and $\pm 1.0\,\mu$m/min for activated NK cells (n.s; $p = 0.09$, Figure S1 in Supplementary Material). Thus, resting NK cells had higher average mean speeds in both directed migration and random movement and, yet, had a lower overall average mean speed.

Taken together, the observed shift in the distribution of migration modes (**Figure 1B**) for resting and activated NK cells shows that IL-2 gives the NK cells a more migratory phenotype. This difference was reflected in a slight skewing of the distribution toward higher mean speeds for activated cells but even more pronounced when looking at transient migration behavior.

ACTIVATED NK CELLS MORE FREQUENTLY ALTERNATE BETWEEN DIFFERENT MODES OF MIGRATION

Next, we investigated whether other characteristic differences in migration modes existed between resting and activated NK cells. Overall, resting NK cells switched between different modes of migration on average 1.1 times during the assay, compared to 2.8 times for activated NK cells. As an example, we studied the number and duration of TMAPs for individual cells in the experiments (**Figure 2**). All NK cells studied had at least one TMAP, which was also the most frequent number of TMAPs in both subsets with decreasing frequency up to five TMAPs, which was the maximum observed. Looking at average values, resting NK cells made fewer (1.3 vs. 1.8, $p < 0.005$), but longer (272 vs. 169 min, $p < 0.005$) TMAPs compared to activated NK cells (**Figures 2A,B**). Additionally, approximately 30% of the TMAPs from the resting NK cell subset, threefold more than activated NK cells, lasted almost the entire assay indicating low to no motility. In contrast, activated NK cells showed approximately 30% more TMAPs that lasted less than 2 h compared to resting NK cells. This shows that while both cell populations exhibited stop-and-go behavior, it was more characteristic of activated NK cells.

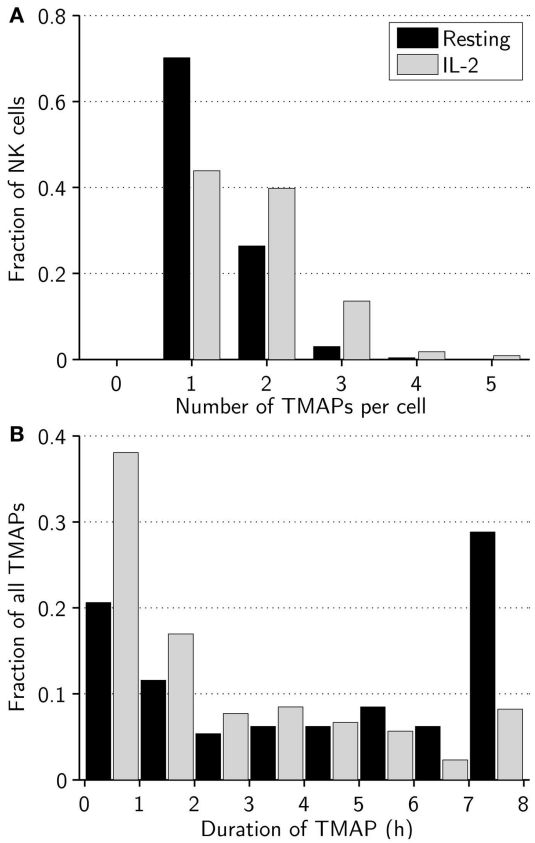

FIGURE 2 | Activated NK cells alter between different modes of migration more frequently than resting NK cells. **(A)** Distribution of number of TMAPs recorded for individual resting (black bars) and activated (gray bars) NK cells. The number of TMAPs detected was 354 for resting and 389 for activated NK cells. **(B)** Distribution of duration of individual TMAPs for resting (black bars) and activated (gray bars) NK cells.

STRIKING DIFFERENCES IN CONTACT FORMATION BETWEEN RESTING AND ACTIVATED NK CELLS

Next, we compared the ability of activated and resting cells to form contacts with target cells. Activated NK cells were found to form up to eight contacts during the assay while the maximum was five for resting NK cells (**Figure 3A**). Strikingly, on average, activated NK cells formed more than twice as many contacts as resting NK cells (1.7 vs. 0.8, $p < 0.005$).

We next scored the duration of conjugation, where the NK cell assumes a rounded morphology with membrane flattening at the site of intercellular contact, and attachment, where the NK cell remains attached to the target cell but visibly seeks to migrate away (24). Both conjugation (**Figure 3B**) and attachment (**Figure 3C**) phases were significantly longer for activated NK cells than resting NK cells. On average, activated NK cells remained conjugated to target cells twice as long (65 vs. 29 min, $p < 0.005$) and attached nearly eight times as long (110 vs. 14 min, $p < 0.005$) as resting NK cells. A considerably larger fraction of resting NK cells completely lacked an attachment phase compared to activated NK cells (44 vs. 27%). These data imply that resting NK cells do not form as "strong" contacts as activated NK cells and are less likely to remain attached to target cells during termination of cell–cell contact.

DONOR-TO-DONOR VARIATIONS CAN PARTLY EXPLAIN THE OBSERVED DIFFERENCES IN MIGRATION – BUT NOT CONTACT DYNAMICS

Cells from distinct donors were used in the majority of experiments and to assess if any of the observed differences in migration and contact dynamics could be related to donor-to-donor differences rather than IL-2 activation, data for each donor was studied separately (Figure S3 in Supplementary Material). Statistical analysis revealed significant differences in terms of migration dynamics within resting and activated conditions and also overlapping distributions of mean values for the two experimental conditions. In contrast, separate analysis of contact and attachment times showed clearly that although some variations were detected within conditions, the mean values formed non-overlapping groups (Figure S3 in Supplementary Material). Thus, this suggests that donor-related

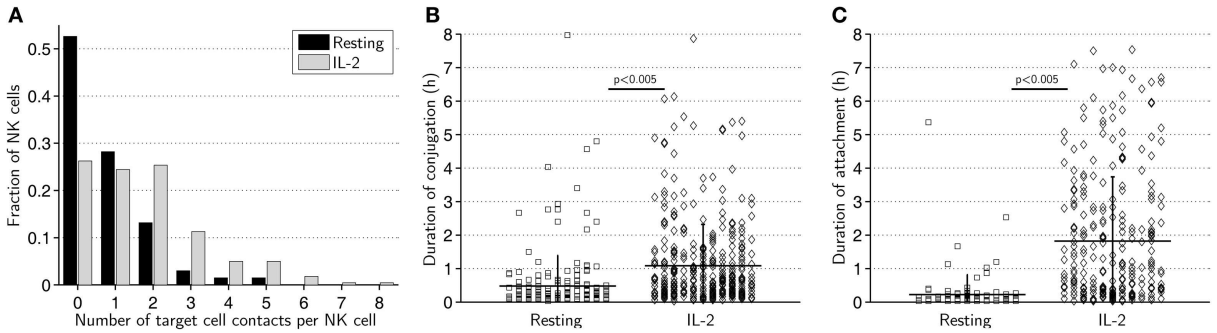

FIGURE 3 | Resting NK cells form fewer and shorter contacts compared to IL-2-activated NK cells. **(A)** Distribution of number of contacts formed by resting (black bars) and activated (gray bars) NK cells. The total number of contacts recorded was 205 for resting and 379 for activated NK cells. **(B)** Duration of conjugation periods for resting (squares, left) and activated (diamonds, right) NK cells. **(C)** Duration of attachment periods for resting (squares, left) and activated (diamonds, right) NK cells.

effects cannot be ignored for the migration parameters measured. In terms of contact dynamics, however, IL-2-activation is clearly the dominant effect.

For one set of experiments, cells isolated from one donor was first studied under resting conditions and then again after 7 days in IL-2 culture ($n_{\text{resting}} = 55$ and $n_{\text{activated}} = 48$ in resting and activated conditions, respectively; Figure S4 in Supplementary Material). These data followed the same trend as the pooled data with smaller differences in migration properties (Figures S4A–C in Supplementary Material) but dramatic differences in contact times (Figures S4D,E in Supplementary Material).

MOTILE SCANNING AND SPEED IN CONTACT

When in conjugation with target cells, resting and activated NK cells had comparable average mean speeds ($\pm 0.9\,\mu$m/min for resting NK cells and $\pm 0.7\,\mu$m/min for activated NK cells, $p < 0.005$) (**Figure 4A**). The average speed in attachment was slightly higher ($\pm 0.8\,\mu$m/min for resting NK cells and $\pm 0.9\,\mu$m/min for activated NK cells) consistent with a more migratory morphology (**Figure 4B**). During attachment periods, in particular for activated NK cells, it was occasionally observed that NK cells dragged target cells along after termination of the conjugation phase (data not shown). While the difference in mean attachment speed was not statistically significant ($p = 0.23$), the difference in mean conjugation speed was $p < 0.005$. This could be explained by differences in the distribution of measured speeds, and indeed, some NK cells were observed to move at a considerable speed while in conjugation with target cells.

During tracking we had observed that some NK cells remained highly motile, moving across the surface of target cells during conjugation (**Figures 5A,B**). In an attempt to investigate such "motile scanning" further, we identified three criteria to isolate subsets of conjugates consistent with this behavior. Those criteria were: (1) The total distance moved while in conjugation should be $>100\,\mu$m. (2) The area covered by the NK cell during the conjugation period should be $<900\,\mu\text{m}^2$, corresponding to a rectangle with sides approximately similar to the diameter of up to three target cells ($30\,\mu$m) and determined by the max and min values for x and y coordinates during conjugation. (3) The distance from the starting point of the conjugate to the endpoint should be $<30\,\mu$m. The conjugation periods identified by these three criteria can be seen in a plot of area covered vs. total distance (**Figure 5C**). Among the colored markers used to denote conjugates exhibiting motile scanning also some unfilled symbols can be seen that were excluded based on the displacement criterion. The conjugates isolated by these criteria were not associated with increased speeds as shown in plots of total distance or area covered vs. mean speeds (**Figures 5D,E**). These criteria were satisfied by 25% of conjugates from the activated subset and 8% of the conjugates from the resting subset, suggesting that motile scanning is more common among activated NK cells.

ACTIVATED NK CELLS HAVE MIGRATORY MORPHOLOGY AND SPREAD ACROSS THE TARGET CELL WHILE RESTING CELLS REMAIN ROUND DURING SYNAPSE FORMATION

Morphologically, resting cells appeared to be relatively small and round when migrating freely, and to maintain their shape upon target-cell contact (**Figure 6A**). By contrast, activated NK cells generally had more elongated and irregular shapes which, upon encounter with target cells, became more rounded with significant spreading across the target-cell surface, creating an intercellular contact that was flat and relatively large (**Figure 6B**). These observations are consistent with recent imaging studies of murine NK cells (12). Measurements of roundness and area of isolated resting ($n = 106$) and activated ($n = 68$) NK cells confirmed that resting NK cells generally were rounder and smaller (Figure S2 in Supplementary Material). Roundness and area were also measured for time sequences involving phases of free migration and synapse formation for the cells displayed in **Figures 6A,B** (**Figures 6C,D**) and a randomly selected subset of NK cells ($n = 4$ for each condition, Figure S5 in Supplementary Material). Transitions between different phases were reflected in changes in roundness and area for IL-2-activated but not resting NK cells. Thus, under the experimental conditions used here, freely migrating activated NK cells had an elongated, irregular shape that was altered to a rounded shape upon target-cell contact. In contrast, resting NK cells were smaller and appeared to maintain a similar morphology throughout the different phases of migration and target-cell contact.

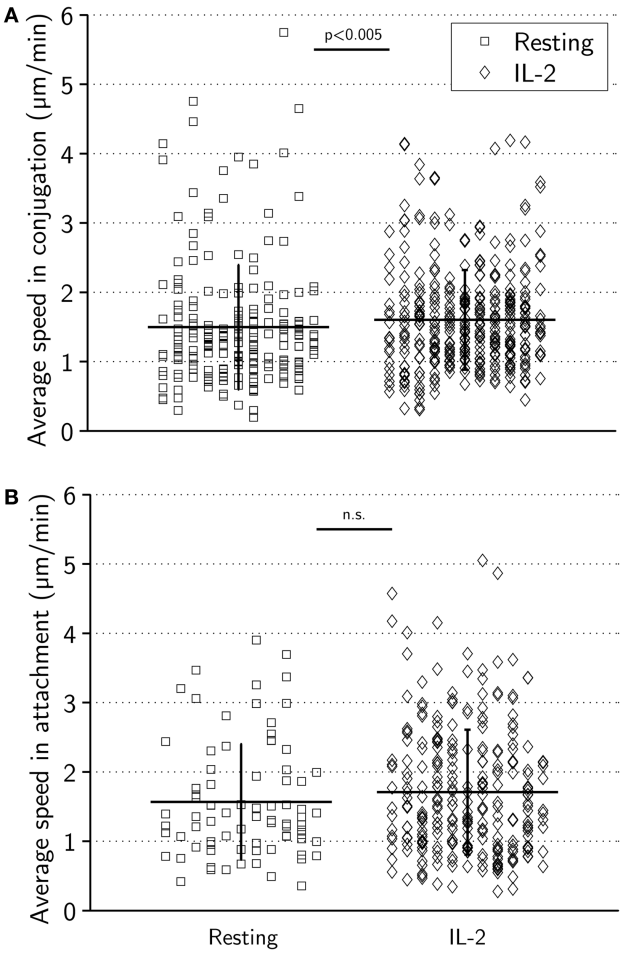

FIGURE 4 | Average speeds during target-cell contact are similar for resting and activated NK cells. **(A)** Average speed in conjugation for resting (left) and activated (right) NK cells. Mean ± SD was 1.5 ± 0.9 μm/min for resting and 1.6 ± 0.7 μm/min for activated cells. **(B)** Average speed in attachment for resting (squares, left) and activated (diamonds, right) NK cells. Mean ± SD was 1.6 ± 0.8 μm/min for resting and 1.7 ± 0.9 μm/min for activated cells.

MARKED DIFFERENCE IN CYTOTOXICITY OF RESTING AND ACTIVATED NK CELLS TOWARD HEK293T CELLS

Finally, single-cell cytolytic activity was compared for resting and activated NK cells. Both resting and activated NK cells displayed cytolytic activity against HEK 293T cells by standard chromium release, although resting NK cell lytic ability was reduced [(24) and data not shown]. A detailed analysis of single-cell level cytotoxicity of activated NK cells has been presented elsewhere (24). During the 8-h analysis, here, activated NK cells killed a total of 240 target cells, an average of 1.1 kills/NK cell. In sharp contrast, only two cytolytic events resulted from the 205 contacts made by the 265 resting NK cells analyzed. Thus, in our assay, resting NK cells showed a significantly lower ability to kill HEK293T cells than activated cells.

Recently, it was suggested by mathematical modeling (27), and later measured experimentally (28), that NK cells become more efficient killers after initial contact with target cells. In light of that, we analyzed the time to lytic hit, i.e., the time from initiation of conjugate to delivery of the lytic hit (24), for a subset of activated NK cells that performed more than one kill in direct sequence. A majority (59%) of NK cells delivered the lytic hit faster for the second conjugate compared to the first but statistical analysis could not confirm that this was significant ($n = 54$, $p = 0.19$).

DISCUSSION

Here, we have compared resting and IL-2-activated NK cells using a microchip-based method with single-cell resolution for resolving migration and NK–target-cell contact dynamics over extended periods of time. Although the difference in average migration speed was small between resting and activated NK cells, a more detailed analysis revealed that resting NK cells were less likely than activated cells to transiently switch between different modes of migration. Overall, activated NK cells were more dynamic with a broader range of morphologies consistent with alterations between migration, stopping, and immune synapse formation. These differences in dynamics are in line with previous imaging studies of NK cells *in vitro* with and without IL-2 activation (12), *in situ* under steady state and inflammatory conditions (21) and during tumor surveillance (29). Thus, it appears that activated NK cells are more dynamic independently of their means of stimulation.

Resting NK cells generally formed shorter contacts and, in contrast to activated cells, rapidly terminated conjugation without attaching to the target cell for prolonged periods of time. This is consistent with previous reports stating that IL-2 leads to upregulation of adhesion molecules and stronger conjugate formation (12, 15). Interestingly, it was recently shown that NK cell education/licensing also leads to increased formation of stable conjugates, emphasizing its importance for efficient cytotoxicity (30). However, the effect observed was not due to a general upregulation of the integrin LFA-1 in educated NK cells, but due to increased inside-out signaling triggered by activating receptors leading to increased expression of high-affinity LFA-1.

Activated NK cells more frequently formed dynamic contacts where the NK cell appeared to scan the surface of the target cell. This motile scanning could be related to "kinapses" described for T cells, a process believed to optimize the search for ligands on the target cell (31). At the same time, it is known that NK cells receiving sufficient activation signals will stop and spread out (32). Thus, it may seem paradoxical that motile scanning was more frequently observed for IL-2-activated NK cells, where conjugation and killing was much more efficient. However, it is known that some integrin interactions can promote migration, e.g., LFA-1/ICAM-1 (32). So considering that adhesion molecules are upregulated upon interleukin-2 stimulation is conceivable that motile scanning could indeed be more common among activated NK cells. More thorough studies are needed to better characterize motile scanning and to resolve its significance. It is for example possible that motile NK cells can integrate the signals generated by encountering spatially separated ligands, possibly expressed on separate cells. This is supported by data recently reported by us where a microwell array for ultrasonic manipulation was used to form clusters of single NK cells and varying numbers of target cells

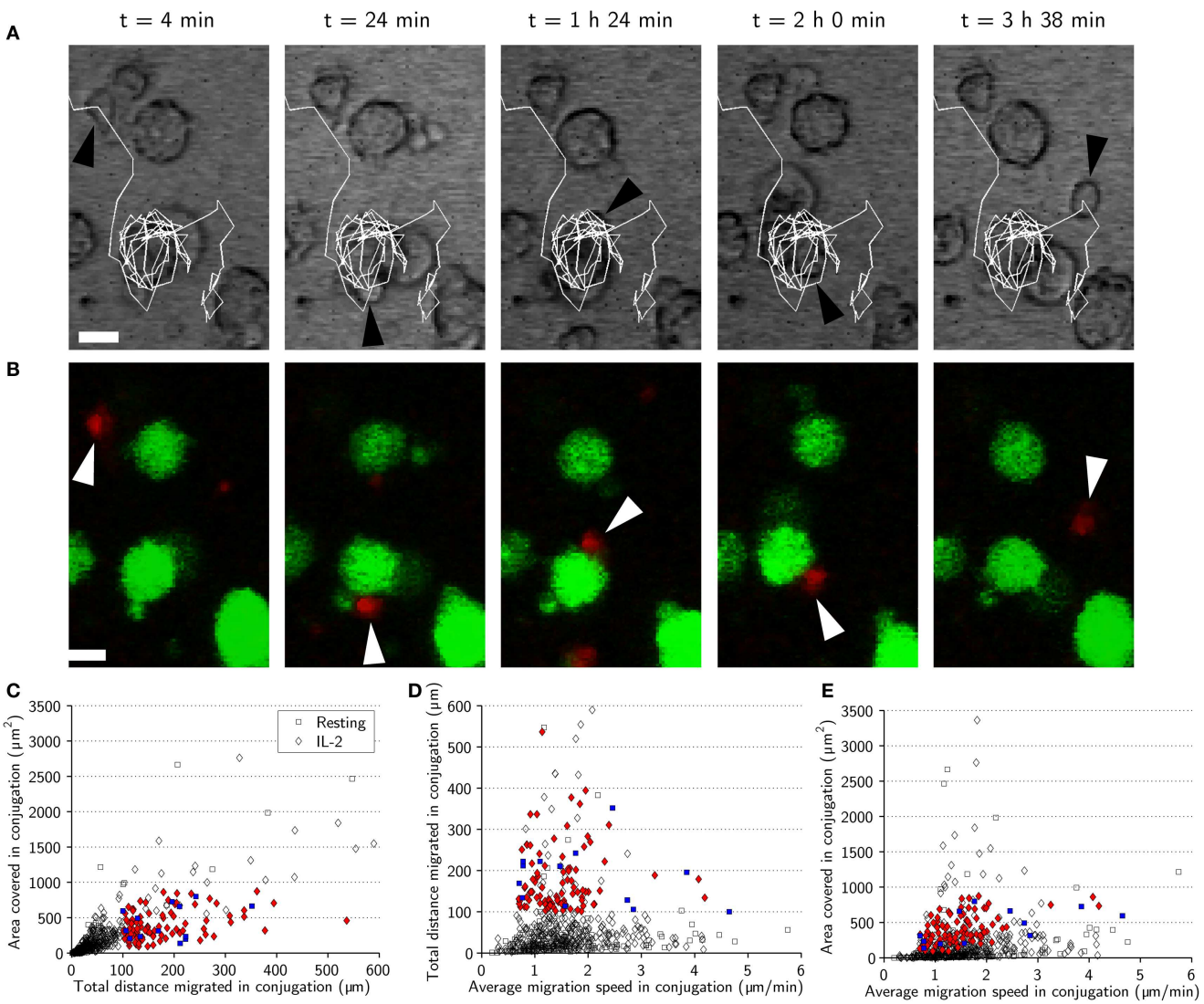

FIGURE 5 | Natural killer cell motile scanning of the target-cell surface. **(A)** Time-lapse sequence showing an IL-2-activated NK cell (black arrowhead) scanning the surface of a target cell. The images are overlaid with a trace of the NK cell trajectory (white) showing that the NK cell moves several laps around the target cell before detaching. Scale bars indicate 10 μm. **(B)** Fluorescence images corresponding to **(A)** showing the fluorescently labeled NK cell (red, white arrowhead) and target cells (green). Both transmitted-light and fluorescence images have been resampled. **(C)** Total distance migrated in conjugation plotted vs. area covered in conjugation for resting (squares) and activated (diamonds) NK cells indicating the conjugation periods fulfilling the three criteria (blue squares and red diamonds for resting and activated NK cells, respectively). **(D)** Average migration speed in conjugation plotted vs. total distance migrated in conjugation for resting (squares) and IL-2-activated (diamonds) indicating motile scanning behavior (colored symbols). **(E)** Average migration speed in conjugation plotted vs. area covered in conjugation indicating motile scanning behavior (colored symbols).

(23). Interestingly, NK cells surrounded by two or more target cells were more likely to kill compared to those in contact with only one target cell. Integration of signals from several targets could also be linked to "burst kinetics," where NK cells were observed to kill faster after initial contact with target cells (28). In our data, there was a tendency of the second and third killing events committed by individual NK cells being faster than the first, but the effect could not be verified statistically. Of note is that we used a different cell system and that target cells were more spread out in the assay leading to subsequent killing events that could be separated both in space and in time, i.e., some NK cells had to detach from the first target and migrate to the next before killing could take place. Thus, simultaneous contact with several target cells was not the case for all NK cells and killing events in our assay.

The data presented here demonstrate that there are significant differences in migration and contact dynamics between resting and IL-2-activated NK cells. At the same time, there were also significant overlaps between the two populations reflecting inherent heterogeneity among peripheral blood NK cells and a varying response to activation. Importantly, differences in migration

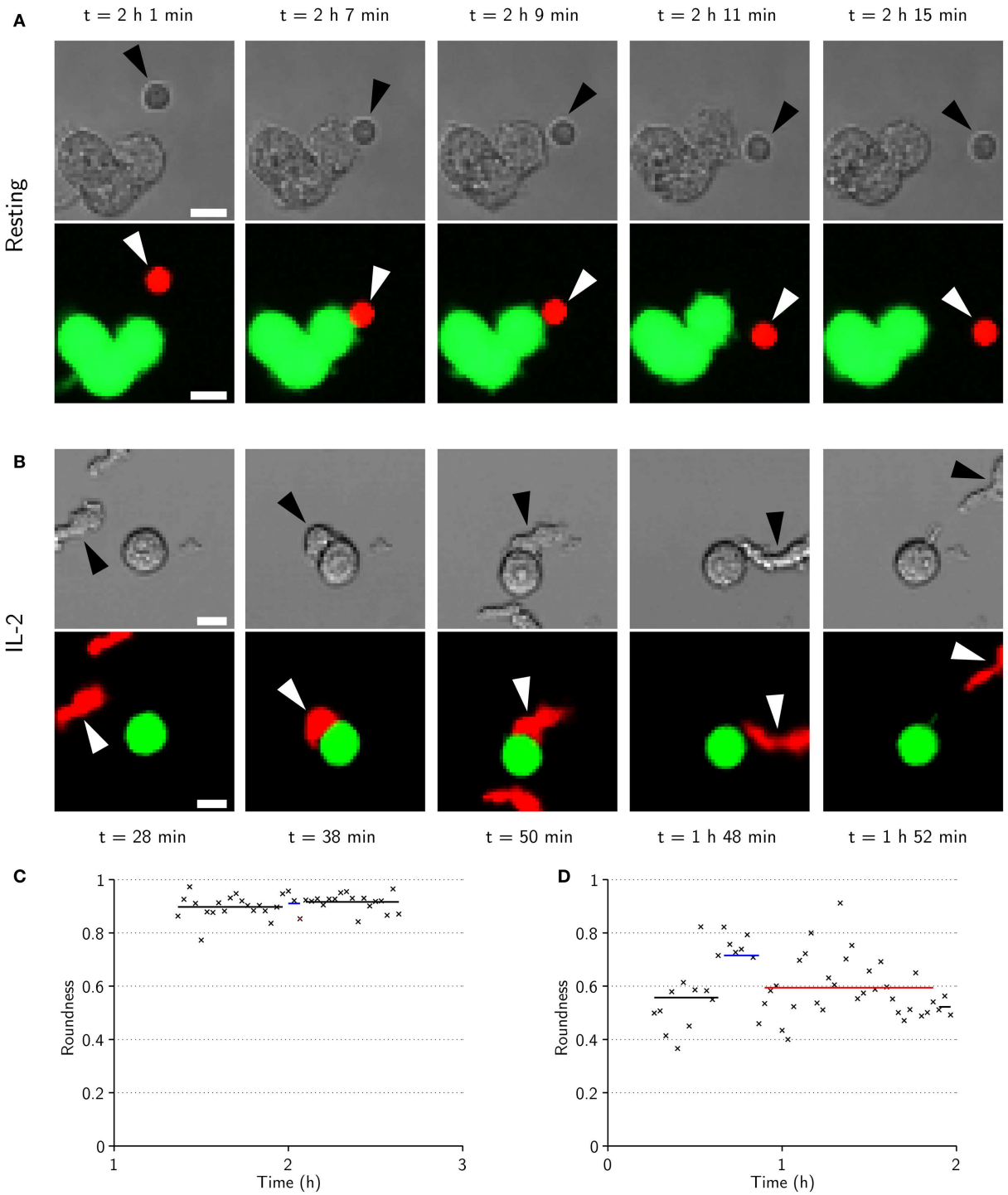

FIGURE 6 | IL-2-activated NK cells are larger, have elongated shapes during migration, and spread across the target cell during conjugation. **(A)** Time-lapse sequence showing transmitted-light (top) and fluorescence (bottom) images of a resting NK cell (arrowheads) approaching ($t = 2$ h 1 min) a cluster of three target cells (green in fluorescence images). The NK cell is briefly conjugated to the target cell ($t = 2$ h 7 min), before it appears to end its commitment to the target and enter an attachment phase ($t = 2$ h 9 min) that ends as the NK cell detaches ($t = 2$ h 11 min) and resumes free migration ($t = 2$ h 15 min). Scale bars indicate 10 μm. **(B)** Same as in **(A)** but for an activated NK cell. The NK cell approaches a target cell ($t = 28$ min) and forms a conjugate ($t = 38$ min) that ends as the NK cell assumes a migratory morphology ($t = 50$ min). The NK cell remains attached to the target cell for almost an hour before it detaches ($t = 1$ h 48 min) and migrates away from the target cell ($t = 1$ h 52 min). **(C)** NK cell roundness vs. time for the sequence shown in **(A)**. **(D)** NK cell roundness vs. time for sequence shown in **(B)**. Shown are also mean roundness in conjugation (blue lines), attachment (red lines), and free migration (black lines).

dynamics may be overlooked if analysis of transient behavior is not performed. The type of analysis performed here is only possible by studying the migration and contact history of all individual cells in a population over extended periods of time. Although this arguably could be achieved by conventional wide-field imaging, it is greatly facilitated by microchip-based approaches like the one used here. While there are several reports of heterogeneity of NK cells in terms of receptor expression and how that is related to activation, maturation or level of education, little is currently known about how this is reflected in migration dynamics, conjugate formation, and killing at the single-cell level (33). We foresee that methods allowing studies of single cells over time will become more widespread and help bridge the existing gaps in understanding of the behavior of individual cells and the function of cell populations.

AUTHOR CONTRIBUTIONS

Per E. Olofsson designed experiments, carried out experiments, analyzed data, and wrote the manuscript. Elin Forslund designed experiments, carried out experiments, and contributed to writing the manuscript. Bruno Vanherberghen designed experiments, carried out experiments, analyzed data, and contributed to writing the manuscript. Ksenia Chechet, Oscar Mickelin, Alexander Rivera Ahlin, and Tobias Everhorn analyzed data. Björn Önfelt designed experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00080/abstract

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

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Erratum: Distinct migration and contact dynamics of resting and IL-2-activated human natural killer cells

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An erratum on

Distinct migration and contact dynamics of resting and IL-2-activated human natural killer cells
by Olofsson PE, Forslund E, Vanherberghen B, Chechet K, Mickelin O, Ahlin AR, Everhorn T and Önfelt B. Front Immunol (2014) 5:80. doi:10.3389/fimmu.2014.00080

REASON FOR ERRATUM

In the Section "Results" of the article, several numbers in the text were misinterpreted due to a typesetting error. This mistake does not change the scientific conclusions of the article in any way. The publisher apologizes for this error and the correct versions of the relevant paragraphs appear below.

The corrected values are in bold.

RESULTS

ACTIVATED NK CELLS EXHIBIT MORE DYNAMIC MIGRATION, WHICH IS RELATED TO MODES OF MIGRATION

The average mean migration speed of the resting NK cells over the 8-h assay was $\mathbf{1.6 \pm 0.6}$ μm/min, while it was $\mathbf{1.7 \pm 0.7}$ μm/min for activated NK cells ($p < 0.005$). A histogram of mean migration speeds revealed substantial differences in the mean speeds of individual cells within subsets and that fast-migrating NK cells were more common in the activated subset (Figure 1A).

We then set out to investigate if there were any detectable differences in the migration behavior of activated and resting NK cells. To this end, we applied a previously developed method to subdivide cell trajectories into three distinct modes of migration, i.e., TMAPs, directed migration, or random movement (21). Overall, a majority of NK cells spent considerable time in TMAPs with the average fractions of time 89% for resting and 71% for activated NK cells (Figure 1B). Strikingly, approximately 72% of resting NK cells spent between 90 and 100% of the assay in TMAPs compared to 38% for the activated NK cells (Figure 1C). Thus, a large fraction of the resting cells displayed low motility while activated NK cells were significantly more motile ($p < 0.005$).

Examining directionally persistent migration showed that all resting NK cells (with the exception of one cell) spent little time in directed migration (<40% of the time), while a few activated NK cells spent >40% of the assay in directed migration (Figure 1D). The average times spent in directed migration were 2% for resting NK cells and 12% for activated cells (Figure 1B). Thus, in this assay, resting NK cells almost completely lacked directionally persistent migration. The rest of the time, on average, 9% for resting NK cells and 17% for activated NK cells, was spent in random movement (Figures 1B,E). Analysis showed that differences between resting and activated cells in the fractions of time spent in different modes of migration were statistically significant ($p < 0.005$).

Next, we compared the mean migration speeds of cells in different modes of migration and, as expected, the average mean speeds in TMAPs were considerably lower, $\mathbf{1.7 \pm 0.8}$ μm/min for resting and $\mathbf{1.7 \pm 0.8}$ μm/min for IL-2-activated NK cells ($p < 0.05$) compared to other modes of migration. In directed migration, the average mean speeds were $\mathbf{3.0 \pm 1.1}$ and $\mathbf{2.5 \pm 0.7}$ μm/min for resting and activated NK cells, respectively ($p < 0.005$). The random movement periods had average mean speeds of $\mathbf{2.6 \pm 1.1}$ μm/min for resting and $\mathbf{2.4 \pm 1.0}$ μm/min for activated NK cells (n.s; $p = 0.09$, Figure S1 in Supplementary Material). Thus, resting NK cells had higher average mean speeds in both directed migration and random movement and, yet, had a lower overall average mean speed.

Taken together, the observed shift in the distribution of migration modes (Figure 1B) for resting and activated NK cells shows that IL-2 gives the NK cells a more migratory phenotype. This difference was reflected in a slight skewing of the distribution toward higher mean speeds for activated cells but even more pronounced when looking at transient migration behavior.

MOTILE SCANNING AND SPEED IN CONTACT

When in conjugation with target cells, resting and activated NK cells had comparable average mean speeds ($\mathbf{1.5 \pm 0.9}$ μm/min for resting NK cells and $\mathbf{1.6 \pm 0.7}$ μm/min for activated NK cells, $p < 0.005$) (Figure 4A). The average speed in attachment was slightly higher ($\mathbf{1.6 \pm 0.8}$ μm/min for resting NK cells and $\mathbf{1.7 \pm 0.9}$ μm/min for activated NK cells) consistent with a more migratory morphology (Figure 4B). During attachment periods, in particular for activated NK cells, it was occasionally observed that NK cells dragged target cells along after termination of the conjugation phase (data not shown). While the difference in mean attachment speed was not statistically significant ($p = 0.23$), the difference in mean conjugation speed was $p < 0.005$. This could be explained by differences in the distribution of measured speeds, and indeed, some NK cells were observed to move at a considerable speed while in conjugation with target cells.

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Activation of lymphocyte cytolytic machinery: where are we?

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Target cell recognition by cytotoxic lymphocytes implies the simultaneous engagement and clustering of adhesion and activating receptors followed by the activation of an array of signal transduction pathways. The cytotoxic immune synapse represents the highly specialized dynamic interface formed between the cytolytic effector and its target that allows temporal and spatial integration of signals responsible for a defined sequence of processes culminating with the polarized secretion of lytic granules. Over the last decades, much attention has been given to the molecular signals coupling receptor ligation to the activation of cytolytic machinery. Moreover, in the last 10 years the discovery of genetic defects affecting cytotoxic responses greatly boosted our knowledge on the molecular effectors involved in the regulation of discrete phases of cytotoxic process at post-receptor levels. More recently, the use of super resolution and total internal reflection fluorescence imaging technologies added new insights on the dynamic reorganization of receptor and signaling molecules at lytic synapse as well as on the relationship between granule dynamics and cytoskeleton remodeling. To date we have a solid knowledge of the molecular mechanisms governing granule movement and secretion, being not yet fully unraveled the machinery that couples early receptor signaling to the late stage of synapse remodeling and granule dynamics. Here we highlight recent advances in our understanding of the molecular mechanisms acting in the activation of cytolytic machinery, also discussing similarities and differences between Natural killer cells and cytotoxic $CD8^+$ T cells.

Keywords: NK cell, CTL, cytotoxicity, cytolytic synapse, signal transduction

INTRODUCTION

Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) are major actors in immune protection against viral infections and cell transformation, and also mediate, in certain conditions, the killing of autologous or allogeneic un-diseased cells (1, 2). Target cell killing can occur upon the polarized secretion of cytotoxic mediators, such as perforin and granzymes, stored in specialized secretory lysosomes termed lytic granules (3).

While CTLs are activated by specific antigen recognition, the activation of NK cells is regulated by a balance of activating and inhibitory signals through a multitude of germ-line encoded receptors following the recognition of ligands expressed on the surface of target cells (4).

Based on recent acquisitions, this review attempts to draw a comprehensive picture on the coupling of receptor proximal signals to the late stages of synapse remodeling and granule dynamics; rather than covering how signals from discrete activation receptors cooperate to control NK-cell activation, a topic which has been extensively addressed in recent excellent reviews (5), we would try to recapitulate for every individual phase of the cytolytic process how the molecular signals arising upon receptor ligation are coupled to the distal molecular effectors responsible for the activation of cytolytic machinery, also highlighting the differences between CTLs and NK cells.

CYTOLYTIC SYNAPSE FORMATION

The cytotoxic event is a well defined multistep process starting with the formation of a cell–cell contact specialized area called cytolytic synapse (3, 6) devoted to the polarized secretion of cytotoxic molecules.

Upon target recognition, receptors and signaling molecules rapidly segregate in the cytolytic synapse forming a supramolecular activation cluster (SMAC) that can be divided into concentrical zones: the central (cSMAC) and the peripheral (pSMAC) SMAC that is thought to be the focal point for the exocytosis of secretory lysosomes.

The formation of a mature synapse is not always essential for cell lysis by CTLs (7, 8), but it is believed to increase the efficiency of lytic granule polarization and target cell killing (9). Indeed, intra-vital imaging of the behavior of individual CTL or NK-cell infiltrating solid tumors in a mouse model has revealed that while CTLs tend to form more stable contacts with tumor cells, NK cells establish dynamic contacts (10).

An early stage in the commitment to cytolytic synapse formation is actin reorganization. As shown by 3-D confocal microscopy studies, actin rapidly polymerizes at the synapse periphery of both CTLs and NK cells to arrange a dense ring of cortical F-actin surrounding a central area through which lytic granules are secreted (6, 11).

Recently, the model of NK cells secreting lytic granules through a central region devoid of F-actin has been exceeded. A couple of companion papers (12, 13), both using very high-resolution imaging techniques, reveal that F-actin forms a pervasive network at the synapse, and that following activating receptor engagement, lytic granules are secreted through the filamentous network by accessing minimally sufficient sized clearances instead of a large-scale clearing of actin filaments. Such remodeling of cortical actin occurs within the central region of the synapse establishing secretory domain where lytic granules dock.

Strictly dependent on actin dynamics, activating signals are initiated by the formation of receptor micro-clusters at the periphery of the synapse in CTLs (14) and NK cells (15) undergoing a centripetal migration toward the synapse center. This movement is directed by actin depolymerization flow from an actin-rich periphery into an actin-poor area as shown by total internal reflection fluorescence microscopy (TIRF)-based studies in live T cells on lipid bilayer (16, 17).

Although, LFA1 ligation by ICAM-1 can signal on its own in NK cells (18), the formation of a stable and symmetric F-actin ring at cytolytic synapse requires integrin and NKG2D activating receptor co-ligation (12, 19). Similarly, in T cells, T cell receptor (TCR) and LFA1 co-aggregation is needed for the efficient synapse formation (20) (**Figure 1A**).

Downstream to LFA1, Cdc42 becomes active (21) and exhibits an oscillatory activation behavior at NK synapse (22); its molecular effector, Wiskott–Aldrich syndrome protein (WASp) is directly responsible for actin polymerization through the activation of the actin nucleator Arp2/3 complex. Accordingly, in the absence of WASp, as it occurs in the immune disorder WAS, or in the presence of actin inhibitors, F-actin accumulation at the synapse and the ability to kill is reduced in both NK cells (23–25) and CTLs (26) (**Figure 1A**). WASp activation strictly depends on phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP2) that is rapidly consumed at the cytolytic synapse (27). In this context the role of the actin binding protein talin has been clarified: its binding to the cytoplasmic tail of beta2 integrin mediates the recruitment of Arp2/3 which initiates actin polymerization upon LFA1 ligation (28).

SECRETORY APPARATUS POLARIZATION

The activation of cytolytic machinery is achieved through a strong cell polarization driven by the reorganization of microtubule and actin cytoskeleton allowing the polarized secretion of lytic granules (**Figure 1B**).

Lytic granule journey starts with a retrograde minus-end transport on microtubules toward the centrosome or microtubule-organizing center (MTOC), followed by movement of the MTOC with clustered granules toward the edge of cSMAC both in CTLs and NK cells (29, 30). Microtubule-based molecular motor dynein has been implicated in the retrograde transport of granules to the MTOC and the subsequent movement of the MTOC toward the immune synapse in an actin-independent manner (31, 32). While in T cells the MTOC was believed to associate closely with the synapse to directly deliver lytic granule without the need of additional plus-ended granule motors (29), a recent report demonstrates that the microtubule motor protein kinesin-1 complexed to the small G protein Rab27a and synaptotagmin-like protein (slp)-3, acts in the terminal anterograde transport of cytotoxic granules close to the plasma membrane in CTLs (33). Whether kinesin-1 is required for the final microtubule transport to position lytic granule in NK cells needs further studies.

Recently, describing a spatio-temporal dissociation of the MTOC with lytic granules, the question of the requirement of MTOC polarization for efficient lethal hit delivery in CTLs has been raised (34). Beside microtubules, the movement of the MTOC/lytic granule complex toward the synapse also involves actin dynamics (6, 32). Indeed, in a pre-final step, the motor protein non-muscle myosin IIA mediates F-actin association with lytic granules (35, 36) and drives the final transit through the minimal clearance in the F-actin network across the cytolytic synapse, thus allowing granule approximation to synaptic membrane in NK cells. Furthermore, also in CTLs the two motor complexes dynein and myosin II has been described to work in a collaborative manner (37). The mutation of the gene encoding myosin IIA leads to May–Hegglin anomaly implying a reduced NK cytotoxicity (38).

In human NK cells signals for granule polarization can be uncoupled from degranulation: LFA1 engagement by ICAM-1 is sufficient to induce granule redistribution (18) thus featuring the minimal requirement for secretory lysosome polarization.

PLC gamma is regarded as a major factor in driving microtubule polarity and granule redistribution. Recently, LFA1-dependent Syk phosphorylation has been linked to the activation of PLC gamma-protein kinase C (PKC)-dependent pathway required for granule polarization (39) in NK cells. Additionally, Src kinases downstream to activating receptors, have been also implicated in the repositioning of MTOC and lytic granule both in CTL and NK cells (40, 41). Moreover, beta2 integrin-dependent phosphorylation of the molecular scaffold paxillin that associates with the tyrosine kinase Pyk2 has been shown to participate in directing MTOC polarization at NK synapse (42, 43). In CTLs the combined signal LFA1 and TCR is required for paxillin recruitment (44) to the site of integrin engagement.

PLC gamma defines a critical polarization pathway mediating the localized accumulation of second messenger diacylglycerol (DAG), which in turn promotes the recruitment of dynein at the MTOC/granule clusters (45). Notably, in agreement with the failure of integrin receptors to be coupled to Ca^{2+}-dependent pathways, polarization resulted unaffected in the absence of calcium signaling in NK cells (39, 41). Differently in CTLs, variation in the Ca^{2+} concentration is thought to determine the kinetics of granule recruitment to the MTOC (9, 46).

Downstream to DAG, MTOC dynamics and polarization is driven by PKC isozymes recruited to the synaptic membrane. In CTLs, PKCη, PKCε, and PKCθ function redundantly to regulate the two motor complexes dynein and myosin II in driving MTOC polarization. Recent findings clarified how TCR signaling is coupled to the force-generating machinery demonstrating that PKCs activity controls myosin II localization directly by phosphorylating inhibitory sites within the myosin regulatory light chain; concurrently, Rho kinase (ROCK), mediating the phosphorylation of distinct sites within myosin regulatory light chain, induces myosin II clustering behind the MTOC (37). Additionally, PKC

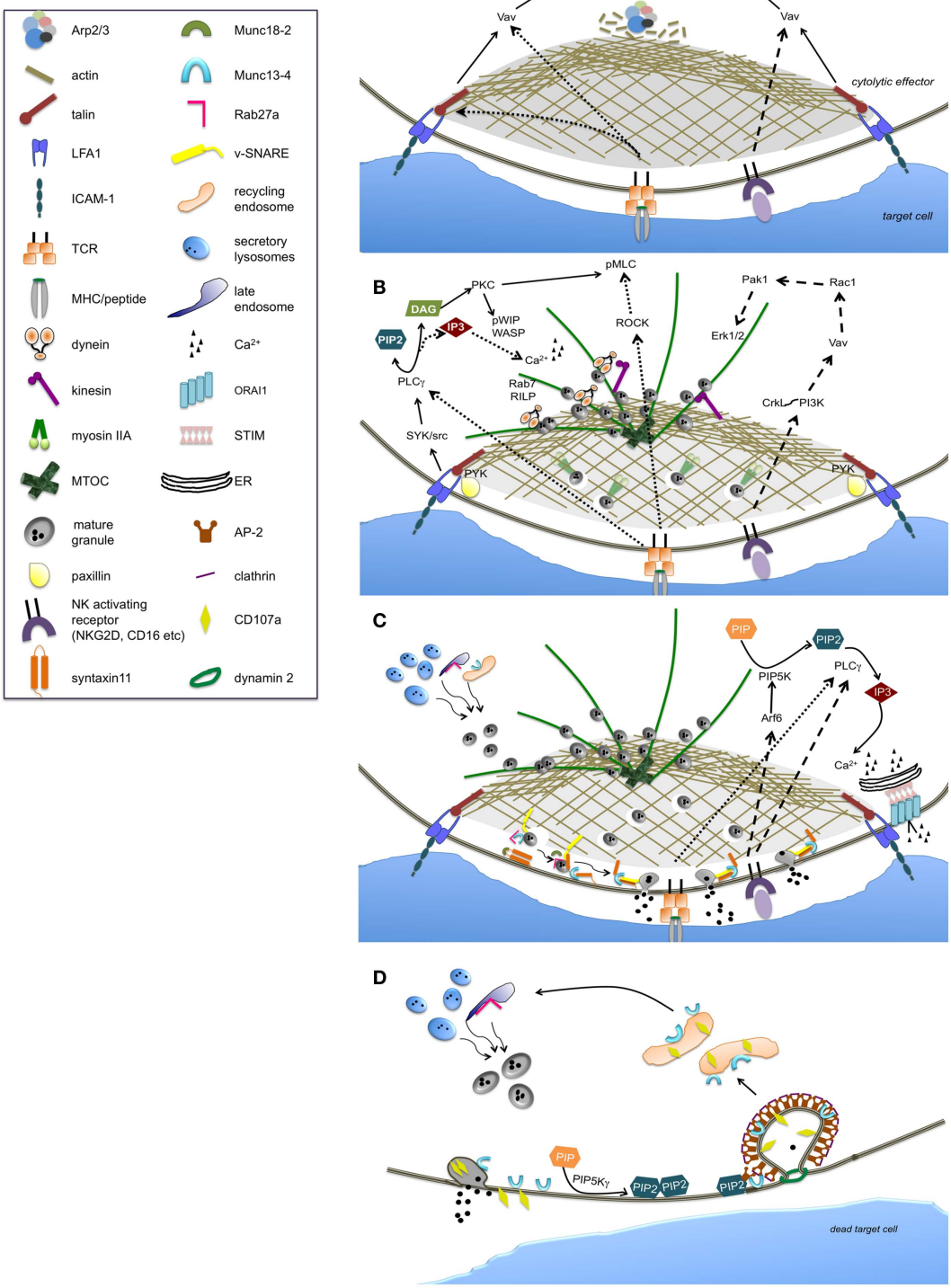

FIGURE 1 | Main signaling pathways and molecular effectors implicated in the regulation of individual phases of the cytolytic process. The sequence of steps leading to the activation of cytolytic machinery are represented as: lytic synapse formation **(A)**, secretory apparatus polarization **(B)**, lytic granule secretion **(C)**, and lytic granule trafficking and retrieval **(D)**. The continuous line arrows indicate the signaling pathway likely common to NK cells and CTLs. Large-dashed arrows indicate the exclusive pathways of NK cells, while fine-dashed arrows indicate the exclusive pathways of CTLs.

delta has been shown to co-localize with polarized granules in T cells (47).

In NK cells PKCθ is required for the WASp interacting protein WIP activation and association with secretory lysosomes at cytolytic synapse; the subsequent interaction with F-actin and myosin IIA (48, 49) links lytic granules to the actomyosin-dependent movement.

Another protein that plays a role in lytic granule movement in CTLs, is the small GTPase Rab7 acting by recruiting dynein-dynactin motor complex to secretory lysosomes through its molecular effector Rab interacting lysosomal protein, RILP, (50). Rab7 is recruited to the WASp-WIP-F-actin complex at the NK lytic synapse (48).

Cdc42-dependent signals have also been implicated in MTOC polarization through CDC42-interacting protein (CIP4), which contributes to anchor MTOC to the synapse by interacting with WASp and tubulin (51).

Proximal signals required for MTOC polarization in NK cells also include the extracellular regulated kinases, Erk1/2. The well-characterized phosphatidylinositol 3-kinase (PI3K)-dependent Rac1 → p21-activated kinase 1 (Pak1) → MEK-ERK1/2 pathway has been long referred as critical for lytic granule polarization in NK cells (43, 52, 53). On the opposite, in CTLs Erk activity is dispensable for MTOC reorientation (44). Erk activation has been uncoupled from integrin receptors while it is turned on downstream to activating receptors. Following NKG2D ligation for instance, PI3K-dependent pathway involving the adaptor CrkL and the small GTPase Rap1, has been shown to be required for MTOC polarization (54).

Despite multiple evidences, the involvement of Erk in microtubule remodeling is not fully understood. Erk2 was found to co-localize with microtubules (55), whereas paxillin phosphorylation has been linked to the PI3K-Erk pathway (56).

The final part of granule journey involves actin remodeling which occurs independently from LFA1 co-ligation (57), and defines a further "checkpoint" in the cytotoxic process: recent evidences demonstrate that NK-cell activation through several activating receptors including CD16 and NKG2D, leads to the remodeling of the cortical actin mesh at the synapse center to produce discrete nanometer-scale domains, as above mentioned (12). Contextually, micro-clusters of Grb2 and Vav1 signaling molecules rapidly reorganize to form a ring-shaped structure at the synapse center. Indeed, the pathway Vav1 → Rac1 → Pak1 is activated following CD16 and NKG2D ligation (58, 59) and regulates actin and microtubule dynamics (53) (**Figure 1B**). Whether remodeling of cortical actin within the central region of the NK-cell synapse is also controlled by this pathway, requires further investigation.

LYTIC GRANULE SECRETION

Once lytic granules have reached the plasma membrane they are highly dynamic and mobile (60) and, contextually, docking and priming occur. Rab27a GTPase plays a critical role in granule docking; ultrastructural analysis evidenced that polarized granules fail to dock at the plasma membrane in response to TCR stimulation (61). The gene encoding Rab27a is mutated in the immunodeficiency Griscelli syndrome 2 (GS2) and in Ashen mice, resulting in severely reduced cytotoxic activity in CTLs (62, 63). In CTLs Rab27a binds to the synaptotagmin-like proteins Slp1 and Slp2 facilitating granule tethering (64, 65). In NK cells a Rab27a-independent pathway for CD16-mediated killing has also been reported (66). The finding of a residual degranulation in NK cells from GS2 patients is thought to indicate a relative redundancy of Rab protein activity (67).

Upon granule docking, the Rab27a binding partner, Munc13-4, mediates the priming of lytic granule in CTLs and NK cells (68, 69). Munc13-4 is mutated in familial hemophagocytic lymphohistiocytosis type 3 (FHL3); in the absence of Munc13-4, cytotoxic granules dock at the site of secretion but cannot fuse with the plasma membrane, thus leading to a severe reduction of cytotoxic activity. Munc13-4 has been postulated to open the conformation of the target (t-)SNARE syntaxin11 by removal of Munc18-2 which is required for syntaxin11 stabilization in NK cells (70). Indeed, Munc18-2 mutation causes FHL5 resulting in reduced granule exocytosis in NK cells (71). Moreover, mutations of syntaxin11 in FHL4 (72, 73) implicate a partial impairment of granule exocytosis both in NK cells and CTLs; the observation that the defect can be partially restored by IL-2 stimulation suggests the redundancy of syntaxin isoforms in the secretory mechanisms. Functional studies have shown that deficiency in vesicle (v-)SNAREs, VAMP7, VAMP8, and VAMP4 results in defective granule exocytosis (74–76) (**Figure 1C**).

An additional role of Munc13-4 in enabling the maturation of perforin-containing granules has been highlighted in CTLs: a seminal study demonstrated that close to the cytolytic synapse, Munc13-4 promotes the coalescence of a pool of endosomal/recycling vesicles carrying effectors of cytolytic machinery such as Rab27a and Munc13-4, with perforin-containing granules, leading to the formation of a unique mature "exocytic vesicle" (77). The observation that in NK cells neither Rab27 or Munc13-4 are present on the lytic granule surface but associate with them upon receptor engagement, suggests a similar role; moreover, separate signaling routes are used in NK cells to direct Munc13-4 and Rab27a to lytic granule (67).

While the nature of the signals that couples activating receptors to secretory machine remains partially unknown, the strict calcium-dependence remains the hallmark for lytic granule secretion (78). The essential role of PLCgamma2 in granule exocytosis has been demonstrated in knock out mice (79). Downstream to TCR or NK activating receptors, the activation of PLC gamma results in the hydrolysis of PIP2 to generate the second messenger inositol-1,4,5-trisphosphate (IP3) which triggers the mobilization of intracellular Ca^{2+} from endoplasmic reticulum; the resulting depletion of Ca^{2+} stores and aggregation of endoplasmic reticulum Ca^{2+} sensor, STIM, trans-activate the plasma membrane Ca^{2+} release-activated Ca^{2+} channel ORAI1, leading to the store operated Ca^{2+} entry (SOCE). Patients with mutation in either STIM1 or ORAI1 exhibit a defect in secretion, whereas lytic granule polarization results unaffected (80). Phosphatidylinositol-4-phosphate 5-kinase (PIP5K)alpha and gamma isoforms are the enzymes mainly responsible for PIP2 synthesis in NK cells; they synergistically act in the control IP3/Ca^{2+} levels required for lytic granule exocytosis (27); in contrast, PIP5Ks isoenzymes behave redundantly in the control of granule polarization which is in line with

the lack of Ca^{2+}-dependence, as above mentioned. Downstream to CD16, PIP5Kalpha activation is regulated by small G protein Arf6; accordingly, interfering with Arf6 function leads to reduced lytic granule exocytosis (81).

The Ca^{2+}-dependent factors required for lytic granule exocytosis are largely unknown. The high-affinity Ca^{2+} binding protein synaptotagmin is a possible candidate; recently, synaptotagmin VII has been implicated in exocytosis of lytic granules (82) (**Figure 1C**).

Because Munc13-4 contains two C2, Ca^{2+} binding domains, it also might represent a Ca^{2+} sensor for exocytosis. Actually, the translocation of Munc13-4 to membrane rafts, indicating granule fusion with plasma membrane, occurs in Ca^{2+}-independent manner (83).

LYTIC GRANULE TRAFFICKING AND RETRIEVAL

The demonstration that Rab11+ recycling endosome polarizes at cytolytic synapse along with secretory apparatus in order to allow Munc13-4-dependent granule maturation into a fusion-competent lytic organelle (77), have raised the disrupting concept that the cytolytic synapse behaves as a focal point for both exocytosis and endocytosis. Indeed, a bidirectional trafficking of lytic granule proteins exposed at the plasma membrane on degranulation has been demonstrated; both lysosome-associated membrane protein-1 (LAMP-1, also known as CD107a) (84) and Munc13-4 (83) undergo a rapid endocytosis, leading to the hypothesis that granule exocytosis is coordinated with the retrieval of cytolytic machinery components. Additionally, cytolytic mediators are also recaptured into early endosomes of NK cells via a clathrin-dependent route after target cell stimulation thus contributing to the cytolytic potential (85).

In this framework, the ability of cytolytic effectors to execute multiple killing cycles in a short time period (86–88) is thought to depend both on the release of a fraction of lytic granules (77) and on a continuum refilling of the granule store through newly synthesized cytotoxic mediators (89). Whether secretory lysosome retrieval could facilitate recycling and reusing of cytotoxic machinery components thus contributing to the serial killing potential, is not fully understood.

The molecular signals controlling the endocytic traffic at cytolytic synapse have begun to be clarified. Recent findings reported that a constitutive PIP5Kgamma-dependent PIP2 pool is involved in the control of Munc13-4 re-internalization through a clathrin/AP2 dependent endocytic route, which is functional to ensure the full serial killing potential in NK cells (83). Such findings strengthen the analogy between neuronal and cytolytic synapse where PIP5K gamma also triggers the clathrin-mediated retrieval of synaptic vesicles (90) (**Figure 1D**).

An additional effector molecule involved in granule recapture during exocytosis can be the fission factor dynamin 2 which have been shown to be required for cytotoxicity in NK cells (91).

PERSPECTIVES

One of the aspects that remains enigmatic in the biology of lymphocyte cytotoxicity is the serial killing potential. The ability of NK cells and CTLs to mediate the sequential attack of successive targets by a single effector was suggested in early observations; only recently, however, intra-vital and real-time imaging of a single cell behavior have shed light on a marked heterogeneity in the cytotoxic potential and on relevant differences in the contact dynamic with target cells (87, 88): CTLs forming stable independent synapse are able to simultaneously bind and attack multiple targets, while NK cells forming short-lived synapse, allows the detachment and the subsequent engagement of other targets. Intriguingly, NK cells are proposed to integrate signals arising form previous and current targets resulting in a continuous signaling that persist until formation of a new synapse (87). A considerable challenge for the future will be the understanding of the spatial and temporal coordination of molecular signals which may account for different qualities of cytolytic responses. In such framework the knowledge on how NK-cell contact with target is terminated and the molecular basis of retrieval processes also represent future challenges.

Also we need to learn more on NK-cell education: in particular, while the molecular basis of MHC-I-dependent licensing have began to be clarified (92, 93), completely unknown remains the molecular basis of NK hypo-responsiveness that follows the sustained stimulation of certain activating receptors.

The unraveling of NK functional plasticity would have a major impact in NK-cell-based immunotherapeutic approaches and could drive a renewed interest in signal transduction processes.

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Late steps in secretory lysosome exocytosis in cytotoxic lymphocytes

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Natural Killer cells are a subset of cytotoxic lymphocytes that are important in host defense against infections and transformed cells. They exert this function through recognition of target cells by cell surface receptors, which triggers a signaling program that results in a re-orientation of the microtubule organizing center and secretory lysosomes toward the target cell. Upon movement of secretory lysosomes to the plasma membrane and subsequent fusion, toxic proteins are released by secretory lysosomes in the immunological synapse which then enter and kill the target cell. In this minireview we highlight recent progress in our knowledge of late steps in this specialized secretion pathway and address important open questions.

Keywords: lytic granules, secretory lysosomes, maturation, endosomes, degranulation

Natural Killer (NK) cells and cytotoxic T lymphocytes (CTL) are essential effectors of innate and adaptive immunity against infected or nascent cancer cells (1). The cytotoxic process is initiated by recognition of target cells via immune receptors and accessory plasma membrane proteins (2). CTL are activated via the T cell receptor (TCR) that interacts with MHC class I molecules and cognate antigenic peptides derived from target cells. NK cytotoxicity occurs via ligation of activating receptors and is kept in check by recognition of self MHC class I molecules. Cytotoxic lymphocytes use two non-redundant pathways to execute their effector functions. The first of which involves the interaction of Fas ligand on CTL and NK cells with Fas receptor on the target cell, which causes receptor oligomerization and apoptosis of the target cell (3). The second pathway also known as the lytic granule pathway represents a rapid and efficient exocytic transport route of vesicular structures containing the lytic molecules perforin and granzymes and is the focus of this minireview.

The lytic granule pathway involves the vectorial and polarized trafficking of the granules toward the immunological synapse and the re-organization of the microtubule organizing center toward the target cell and subsequent release of cytotoxic proteins. Besides the cytotoxic proteins, the granules also contain lysosomal enzymes, an acidic proteoglycan core, and lysosome-associated membrane proteins. Since this content is released by outside in signaling, lytic granules represent a hybrid organelle with shared properties of lysosomes and secretory granules and are therefore also called secretory lysosomes or lysosome related organelles (4, 5).

An essential driver for the development of our understanding of the lytic pathway was the presentation of patients with Familial Hemophagocytic Lymphohistiocytosis (FHL), whose cytotoxic lymphocytes fail to kill target cells. As a consequence of which patients suffer from uncontrolled and massive lymphocyte and macrophage proliferation. A group of genes has been discovered in which mutations causes strongly impaired lytic capacity. These include *RAB27A* (Griscelli syndrome type 2, GS2), *UNC13D* (FHL3), *STX11* (FHL4), and *STX-BP2* (FHL5), which encode proteins of conserved families that regulate membrane trafficking (4). It is thought that the related disease phenotype of the cytotoxic lymphocytes, reflects the functional organization of the wild type forms of these proteins in a network that cooperatively regulates the degranulation pathway. Although this is a widely held belief, our understanding of the molecular mechanisms coupling their function in regulating the degranulation pathway and how they are controlled by upstream signaling is limited.

Even though NK cells and CTL functions belong to distinct arms of immunity, mutations in genes causing FHL produce dramatic yet remarkably related cytoarchitectural and immunodeficiency phenotypes. In addition, primary CTL and NK cell responses reveal similar molecular requirements (6, 7). Collectively this supports the notion of a common principle for granule exocytosis in cytotoxic lymphocytes on which cell-type specific layers of signaling are superimposed. We therefore will not distinguish between the lytic granule pathway in CTL and NK in our discussion.

MATURATION OF SECRETORY LYSOSOMES

Careful electronmicroscopy studies in CTL, mast cells, and melanocytes revealed a heterogenous appearance of secretory lysosomes in terms of cytoplasmic localization, size, and electron dense luminal matrix (8–10). Kinetic tracer uptake experiments and antibody labeling on ultrathin sections suggested the presence of three or more classes of secretory lysosomes, that likely represent different stages in the formation of mature

secretory lysosomes. Secretory lysosomes are formed from precursor organelles through a series of distinct membrane transport steps that continuously deliver house-keeping content and specific effector molecules from endosomes, the trans Golgi network, and the cytoplasm (11). How the pathways between them and with other granules are regulated is only recently being explored (12–15). An early stage in the formation of secretory lysosomes involves the merger of recycling endosomes containing rab11 and munc13-4 with late endosomes characterized by the presence of rab27a and rab7, to a so called exocytic endosome (**Figure 1**). The coalescence of the two endocytic structures might serve to bring exocytic traffic regulatory proteins that are needed at later stages of the secretory lysosome release. Since rab11 does not bind munc13-4 (13), other small GTPases could provide a link between munc13-4 and the endosomal system. Rab15 an endosome-localized rab (16) might take on this task as it binds to munc13-4 and coregulates with rab27 the exocytosis of von Willebrandt factor from Weibel–Palade bodies (17), the secretory lysosomes of endothelial cells.

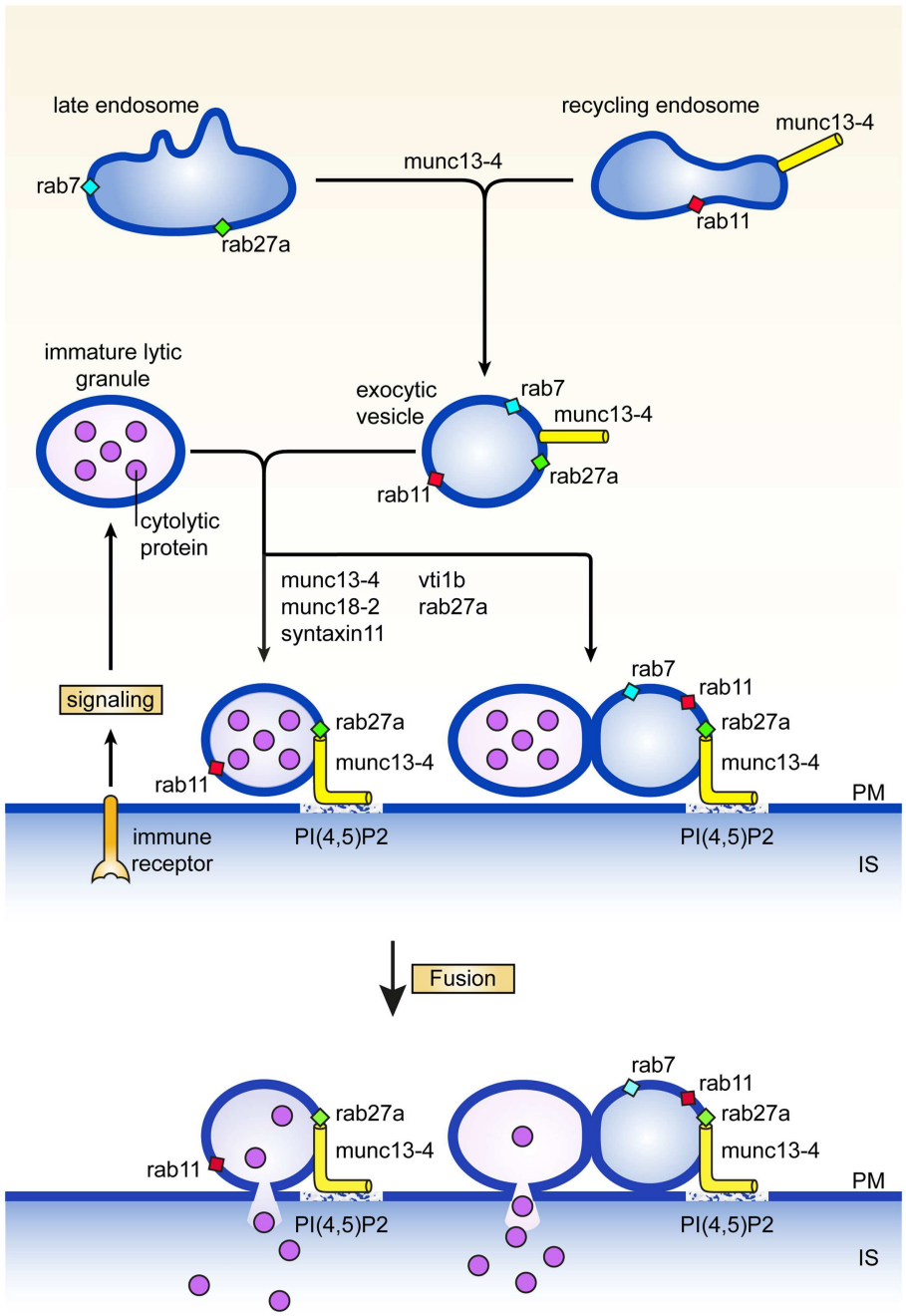

FIGURE 1 | Maturation and fusion. Pathways and proteins regulating various aspects of the maturation and fusion of secretory lysosomes (see text for details). PM denotes plasma membrane, IS immunological synapse.

The tetrameric adaptor complex AP-1 and kinesin KIF13A represent two other membrane traffic regulators through recycling endosomes that have been implicated in the function of secretory lysosomes (18). A complex of AP-1 and KIF13A is thought to define a recycling endosomal domain partially overlapping with rab11 (18) that could facilitate cargo sorting. The AP-1*KIF13A complex positions endosomes close to secretory lysosomes in the cell periphery thereby facilitating interorganellar connections.

TETHERING SECRETORY LYSOSOMES AT THE PLASMA MEMBRANE

A second step in secretory lysosome maturation occurs when the cytotoxic lymphocyte engages itself in an interaction with a target cell during which the exocytic endosomes and associated exocytic machinery move to the immunological synapse in parallel with organelles containing LAMPs, perforin, and granzymes. At least two scenarios are compatible with the available data for the next stage in the secretory lysosome maturation pathway. The two organelles may fuse with each other to generate a structure with lytic and exocytic potential at the immunological synapse. Alternatively, tethering of the exocytic endosome equipped with munc13-4, rab27, and rab11, to the lytic precursor organelle with perforin and granzyme (without actual fusion) is already sufficient to assemble and concentrate the protein machinery for efficient docking and fusion at the plasma membrane (**Figure 1**). Enhanced tethering of secretory lysosomes that are paired with TCR-containing endosomes has been observed at the immunological synapse of CTL. In effect tethering prolongs the dwelling time of the organelle at the immunological synapse which facilitates the subsequent fusion step with the plasma membrane, and release of lytic content (19). In support of this notion, we uncovered a second role for munc13-4 in tethering secretory lysosomes at the cell surface that is regulated independently from the upstream function of munc13-4 on recycling endosomes. Total Internal Reflection Fluorescence microscopy of cells expressing munc13-4 point mutants defective in rab27a-binding revealed that secretory lysosomes are severely impaired in the characteristic stalling behavior seen upon initiation of immune receptor signaling, showing that a complex between munc13-4 and rab27a is required for the tethering role of munc13-4 at the plasma membrane (12). In neutrophils, munc13-4 was also found to limit mobilization of rab27a positive granules after lipopolysaccharide stimulation and to restrict them at the plasma membrane (20), suggesting a more general role of the rab27*munc13-4 complex in immune cells.

Molecular insight into the question as to how munc13-4 could serve as a tethering molecule derived from computational structure predictions which revealed very weak homology between the MUN domain and subunits of intracellular membrane tethering complexes (21). The MUN domain is the central region of munc13-4 that is interspaced between the C2B and C2C domains (**Figure 2**). It contains two conserved munc homology domain sequences (22) that constitute an autonomously folding MUN domain. The MUN domain is conserved in all munc13's and related proteins and is principally involved in the priming function. The subsequent structure of the MUN domain (23) revealed that it folds into a two-stacked helical bundle, characteristic for the exocyst subunit sec6 and other members of the CATCHR family (complex associated with tethering containing helical rods) of tethering factors. Additional subunits of the oligomeric exocyst, GARP, COG, and Dsl1 tethering complex complexes (24) also fold in this structure as do the cargo binding sites of yeast type V myosins (25). Another common denominator in these complexes is their propensity to bind traffic regulators like rho and rab GTPases, SNAREs, and phospholipids.

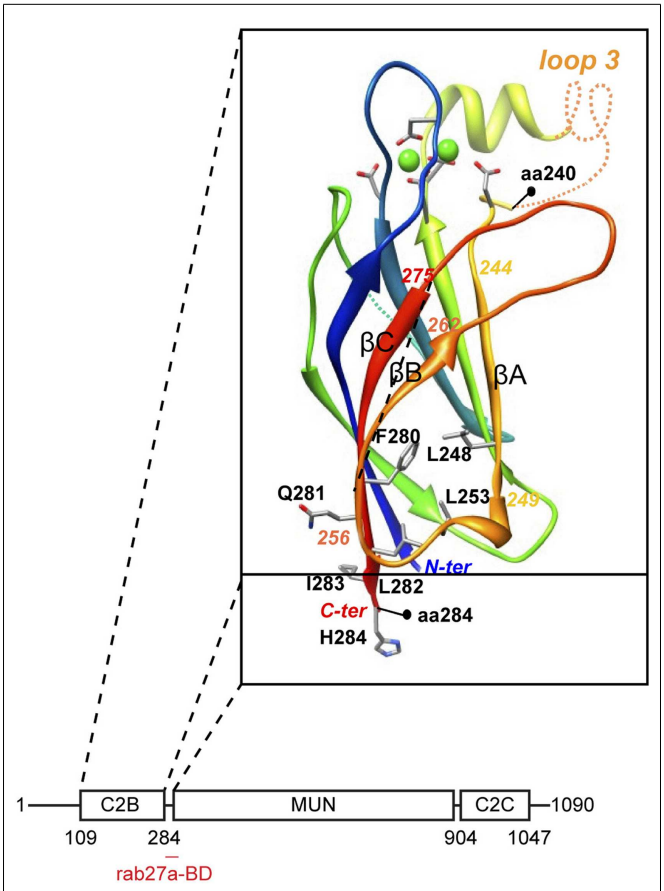

FIGURE 2 | Domain architecture of munc13-4. The C2B domain was modeled on the munc13-2 C2B domain structure (26). Note the rab27-binding domain at the junction between C2B and MUN domain.

We recently characterized the requirements for rab27-binding to munc13-4, and mapped the rab27-binding site to a short sequence just at the junction of the MUN and C2B domains (12) (**Figure 2**). Since the munc13 C2B domain engages in Ca^{2+}-dependent PI(4,5)P2 binding and the C2C domain might constitutively bind membranes, munc13-4 could bridge two membranes via this multiplicity of interactions. CTL and NK cells express two other rab27a effectors, slp1 and slp2-a. Both are non-essential for secretory lysosome release, but they could assist in tethering since their C2 domains are important for membrane binding and they focus tightly at the immunological synapse (27, 28). Since the MUN domain of neuronal munc13 interacts with syntaxin-1 (26), we anticipate that munc13-4 will also bind to a (yet to be identified) syntaxin. In that vein munc13-4 mediated tethering

could control specificity in SNARE reactions needed for secretory lysosome fusion.

In contrast to molecules on the secretory lysosome that facilitate tethering and docking at the immunological synapse, the molecular cue(s) on the partnering inner leaflet of the plasma membrane have not yet been defined. Given the analogies between cytokinesis, polarized exocytosis, and degranulation (29) we speculate that exocyst complex subunits could play a role in this pathway. Several lines of evidence are in accordance with this notion. The exocyst subunit sec15 binds directly to rab27 (30), while the capture of secretory granules depends on the rab27a effector MyRIP that interacts directly with exocyst subunits sec6 and sec8 (31, 32). Finally, munc13-4 localizes via its C2B domain to PI(4,5)P2-enriched regions on the plasma membrane (33), while PI(4,5)P2 is also key to localizing exocyst subunits sec3 (34) and exo70 (35). Thus molecules important for polarized secretion in other cell types, are implicated in the degranulation pathway through putative interactions with rab27 and munc13-4.

FUSION OF SECRETORY LYSOSOMES WITH THE PLASMA MEMBRANE

Tethering of munc13-4, rab27, and rab11 structures with LAMP containing organelles can be considered as a pre-requisite for late endosome-lysosome fusion. In CTL and macrophages this heterotypic fusion event has been shown to involve the Qb SNARE vti1b, which is required for release of secretory lysosome content (19, 36, 37) (**Figure 1**). Interestingly, vti1b interacts with the Qa SNARE syntaxin-11 in macrophages, where silencing of syntaxin-11 causes formation of enlarged late endosomes and inhibition of late endosome-lysosome fusion. In accordance with this function, syntaxin-11 associates predominantly with compartments of the late endosome-lysosome system in a variety of immune cells. In NK cells it colocalizes with CD-MPR in late endosomes, relocates to the immunological synapse, but is not found on lytic granules (38). In CTL syntaxin-11 localizes to an organelle close to but not on lytic granules (39), while it is found mainly in LAMP-1 and LBPA containing late endosomes in macrophages (37). Loss of syntaxin-11 function causes FHL4 in man (40, 41) and hemophagocytosis in mice (42–44). NK cells and CTL of FHL4 patients normally polarize their secretory lysosomes to the immunological synapse, but fail to release their content. This phenotype is very similar to munc13-4 deficiency in FHL3 patients and strongly argues that syntaxin-11 acts in the same pathway as munc13-4. Unlike for Griscelli type 2 and FHL3 patients, ultrastructural data are not yet available for cytotoxic lymphocytes of FHL4 patients or the recently established syntaxin-11 knock-out mice (42–44). Information on such features as size, position, content, number, etc., of endosomal structures and secretory lysosomes will be required for the evaluation at what stage the lytic pathway is blocked and where syntaxin-11 function is localized with respect of rab27a and munc13-4 activities.

In hemopoietic cells, syntaxin-11 interacts munc18-2 (45, 46), a member of the small family of SM proteins that regulate the activity of SNARE proteins for membrane fusion in time and in space (47). The localization of munc18-2 has been addressed in mast cells where it is associated with late endocytic organelles, just beneath the plasma membrane (48). Mutations in munc18-2 cause FHL5 and also reduce the amount of its partner syntaxin-11, suggesting that munc18-2 acts as a chaperone that stabilizes syntaxin-11 (45, 46). Initially, inactivating munc18-2 mutations appeared to affect degranulation in CTL, NK cells, and neutrophils (49). More recent observations however show that patient mutations of munc18-2 do not only affect cytotoxic lymphocytes but also cause changes in the intestinal and renal epithelium resulting in severe diarrhea and renal proximal tubular dysfunction (50). The fact that additional tissues are affected in FHL5, likely reflects the more ubiquitous expression of munc18-2 compared to munc13-4, and syntaxin-11. Many of the proteins that are described above, act upstream of the final fusion event of secretory lysosomes with the plasma membrane. The implication is that the secretory lysosome pathway might interface with more general fusogenic machinery at or close to the final step. Recent observations of Rettig's lab support this idea and suggest that munc13-1 (51) and synaptobrevin 2 (52), two mediators of neuronal secretion, act at the final stage of secretory lysosome exocytosis. The daunting challenge now is to piece together which of the SNAREs and associated proteins cooperate in the final stage to fuse the secretory lysosomes with the plasma membrane.

A breakthrough in our understanding of munc13 and munc18 function came from *in vitro* reconstitution assays. Rizo's lab recently developed an *in vitro* liposome fusion assay in which they could show for the first time a dependency of fusion on munc18 and munc13 (53). Although this was accomplished in combination with SNAREs involved in synaptic vesicle fusion, it will likely provide a general explanation for the role of munc13 and munc18 proteins in membrane fusion. Ma et al. discovered that the closed form of syntaxin is clamped in a tight complex with munc18 and cannot bind other SNAREs. Munc13 can extract syntaxin through a interaction of the MUN domain with syntaxin and possibly munc18, thereby catalyzing the formation of the full syntaxin-SNAP-25-synaptobrevin complex and fusion (26, 53).

COUPLING FUSION OF SECRETORY LYSOSOMES WITH ENDOCYTIC RETRIEVAL

The trafficking proteins that regulate degranulation of secretory lysosomes are long lived. After exocytic release of content, these are in principle available for re-utiliatization in a next encounter with a target cell. A seminal paper from Eric Long's lab showed that lytic granules of NK cells undergo both complete and incomplete fusion with the plasma membrane, and suggests that incomplete fusion may promote efficient recycling of lytic granule membrane and proteins (54). SNAREs involved in fusion with the plasma membrane are returned by retrograde endocytic transport (55, 56).

It is less well understood how cytoplasmic proteins like rab27 and munc13-4 are retrieved. After fusion they can either undergo two fates, one of which involves dissociation and return through the cytoplasm via non-vesicular transport. Alternatively they may diffuse out in the plasma membrane or remain trapped in the vicinity of the fusion site and be efficiently re-internalized. Fluorescence recovery after photobleaching experiments with munc13-4 and rab27a showed that rab27a is relatively stably associated with membranes (57) and predominantly in the GTP bond form (58). The turnover of munc13-4 on secretory lysosome membrane is

also relatively slow and decreased ∼twofold after immune receptor signaling in rat basophil leukemia cells (59), suggesting that munc13-4 might be retrieved by coupling degranulation with endocytic re-uptake. In accord with this notion, Galandrini et al. recently found that munc13-4 was recycled back from PI(4,5)P2-enriched domains at the immunological synapse by AP-2 dependent endocytosis (33). The mechanism via which this occurs is remarkably similar to synaptic vesicle protein retrieval in presynaptic neurons. CD16 stimulation of human NK cells causes transient activation of Arf6 (60) which is essential for the recruitment of PIP5K-alpha and PIP5K-gamma to the plasma membrane, the formation of PI(4,5)P2 pools and secretion of secretory lysosomes (61). Upon fusion, granule membrane delivers munc13-4 to the PI(4,5)P2 pool generated by PIP5K-gamma (33). Presumably the association of munc13-4 depends on the C2B domain that has the ability to bind PI(4,5)P2 (62), which in turn serves as a recruitment hub for proteins regulating clathrin coated pit formation (63). Subsequent sorting processes in the endosomal system might then control delivery of re-internalized munc13-4 to its steady state localization in recycling endosomes (12, 13, 64).

POSTTRANSLATIONAL MODIFICATIONS OF TRAFFICKING PROTEINS REGULATING DEGRANULATION

A central question is how the signals generated by cross-linking surface receptors with the corresponding ligands on target cells are transduced to the proteins that regulate transport and fusion of secretory lysosomes and how this modulates their function. SNAREs and their partners, and rabs play an essential role in degranulation, yet the mechanisms that determine the spatiotemporal control of their assembly in complexes for transport and fusion are incompletely understood. As protein kinases serve critical roles in reversible regulation of membrane transport (65), it is expected that phosphorylation of traffic proteins may modulate degranulation. In secretory lysosome exocytosis in mast cells, cross-linking of the high-affinity FcepsilonRI receptor leads to phosphorylation of SNAP-23 by IkappaB kinase 2, through a upstream PKC signaling pathway that is conserved in lymphocytes (66). Phosphorylation of SNAP-23 is essential for degranulation as ectopic expression of phospho-mimetic SNAP-23 mutant partially rescued the impaired IgE-mediated degranulation in IkappaB kinase 2-deficient mast cells (67, 68). Signaling cascades in platelets also cause phosphorylation of SNAP-23, which occurs on Ser95 and is a positive regulator of SNAP-23 dependent membrane fusion *in vitro* and platelet release *in vivo* (69). The gain in function of phosphorylated SNAP-23 correlates with its propensity to increase SNARE complex formation with syntaxin-11 and the R-SNARE VAMP8 that is critical for lytic granule exocytosis and cytotoxicity (36). Even though not yet shown in secretory lysosome release in immune cells, munc18-2 becomes phosphorylated during stimulatory conditions for regulated secretion in epithelial cells. As for SNAP-23, phosphorylation of munc18-2 enhances the assembly of a membrane fusion complex which serves as positive regulatory mechanism for fusion (70). Given the wide spread expression of munc18-2 (71), we anticipate that threonine/serine phosphorylation of munc18-2 and SNAP-23 has a general augmenting role in membrane fusion underlying secretory lysosome release (71).

PERSPECTIVE

The polarized secretion of lytic granule content in immune cells involves integration of major cytoarchitectural changes with precisely timed vesicular transport to the immunological synapse. Many proteins are known now that play a role in this traffic route and are important in degranulation. A major question is to understand how the signals that are initially generated upon encountering a target cell, control the proteins involved in membrane fission and fusion processes of this pathway. It is essentially unknown for instance if guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) for rab27a, and thereby the activity of the small GTPase are controlled upon signaling. This information could contribute insight in the order with which different rab27a effectors are recruited within a cell. We also need to improve our understanding where the proteins that control membrane traffic during degranulation are localized. Progress on this question will come from the application of powerful new imaging technologies that are increasingly being employed in immunological research. Ultimately such information will be crucial to annotate which step is controlled by which protein and how the network of traffic regulators cooperates in degranulation.

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In the crosshairs: investigating lytic granules by high-resolution microscopy and electrophysiology

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Cytotoxic T lymphocytes (CTLs) form an integral part of the adaptive immune system. Their main function is to eliminate bacteria- and virus-infected target cells by releasing perforin and granzymes (the lethal hit) contained within lytic granules (LGs), at the CTL-target-cell interface [the immunological synapse (IS)]. The formation of the IS as well as the final events at the IS leading to target-cell death are both highly complex and dynamic processes. In this review we highlight and discuss three high-resolution techniques that have proven invaluable in the effort to decipher key features of the mechanism of CTL effector function and in particular lytic granule maturation and fusion. Correlative light and electron microscopy allows the correlation between organelle morphology and localization of particular proteins, while total internal reflection fluorescence microscopy (TIRFM) enables the study of lytic granule dynamics at the IS in real time. The combination of TIRFM with patch-clamp membrane capacitance measurements finally provides a tool to quantify the size of fusing LGs at the IS.

Keywords: cytotoxic T cells, lytic granules, TIRFM, structured illumination microscopy, correlative light and electron microscopy, SNARE proteins

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are a part of our body's defense system. They eliminate bacterially and virally infected cells after coming in close contact with them to form an immunological synapse (IS). An IS is initiated when the antigen presented by MHC class 1 molecules on the infected cell forms a complex with the T cell receptors (TCR) on the CTL membrane. At least three antigenic peptide-MHC (p-MHC) complexes are needed to initiate the signaling cascade in CTLs that result in target-cell death (1). So, following p-MHC-induced TCR triggering, several morphological and functional changes occur within the CTL. Multiple receptor types and adhesion proteins aggregate at the contact zone to form supramolecular activation clusters [SMACs; (2)]. A central SMAC, composed of TCRs, Lck, ZAP70, and a host of other signaling molecules, is surrounded by a peripheral SMAC (pSMAC) that contains of adhesion molecules such as LFA1 and Talin, which are essential for stabilizing the contact between the CTL and the target-cell. The pSMAC is supported by an F-actin ring, formed after polar local actin polymerization and microfilament reorganization. The actin cytoskeleton supports the translocation of the microtubule organizing center (MTOC) toward the IS. The cascade of signaling events induced at the IS includes local and global calcium influx and ultimately leads to the MTOC-aided polarization of the lytic granules (LGs) to the IS (3).

Lytic granules are specialized lysosomes that contain cytotoxic molecules such as perforin and granzymes that induce target-cell death. They undergo a complex and still ill-defined set of maturation stages (see below) and are uniquely organized such that they are protected from the cytotoxic effects of the molecules they contain. Their acidic pH renders the pore-forming molecule perforin inactive. Following fusion at the IS, perforin is exposed to a neutral pH which results in its activation. Perforin is then bound to the target-cell membrane via its C2 lipid-binding domain and generates pores after oligomerization (4). Perforin also binds to the membrane of the CTL, but is cleaved by cathepsin B, which is incorporated into the CTL membrane when LGs fuse at the IS (5). Thus, perforin is only active in the membrane of the target-cell. CTLs are also protected from granzyme B (a serine protease) mediated killing by the expression of serpins (serine protease inhibitors) on their plasma membrane (6).

Lytic granules are the principal effectors of killer-cell function. Therefore, understanding the mechanism of their action and regulation is of great interest. Following polarization to the IS, LGs dock, prime, and finally fuse to release the perforin and granzymes at the IS. The docking, priming, and fusion steps of LGs at the IS are all tightly regulated and mediated by specific SNARE and SNARE-associated proteins (7). Several molecules essential for lytic granule exocytosis have been identified. Mutations within the genes of Rab27a, hMunc13-4, Syntaxin11, and Munc18-2 lead to defective CTL and NK cell function resulting in often lethal immune disorders: Griscelli syndrome (Rab27a) and FHL type-3, -4, and -5 (hMunc13-4, Syntaxin11, and Munc18-2, respectively). In the case of Rab27a and Munc13-4, in-depth studies using confocal imaging and high-resolution electron microscopy (EM) identified their precise function in LG fusion (8, 9). In addition, new insight on LG maturation was gained by elucidating the requirement of Munc13-4 for this process (10). Munc13-4 is an effector of Rab27a (11), and later studies using high-resolution total internal reflection fluorescence microscopy (TIRFM) showed that the Munc13-4 Rab27 complex is required for tethering LG at the

plasma membrane (12). Detailed EM revealed that in patients suffering from Chediak–Higashi Syndrome (CHS), only lysosomes and not late multi-vesicular endosomes are enlarged (13). CHS is an autosomal recessive disease that results in defective T- and NK cell cytotoxicity, due to mutations in the *lyst* gene encoding for LYST protein. The accurate analysis from the EM studies in combination with confocal immunofluorescence imaging provided an elegant demonstration of the function of LYST and the molecular mishap behind the disease. Similarly, to investigate the precise function of Synaxin11 and Munc18-2 in CTLs, the molecular mechanism behind FHL-4 and 5 and to determine if Syntaxin11 is indeed the t-SNARE for the fusion of LG at the IS as has been hypothesized in several reports, TIRFM and EM would be the ideal methods of choice. Therefore, microscopic methods with high-resolution are essential in order to understand these spatially and temporally restricted processes at the IS. Furthermore, highly specific marker proteins for the different organelles involved, in particular LGs, are needed.

In this review we highlight a toolbox of techniques and molecules that should enable the quantitative analysis of LG biogenesis and fusion in CTLs.

INVESTIGATING GRANULE MATURATION, ITS TYPES AND CONTENT THROUGH ELECTRON MICROSCOPY AND CORRELATIVE LIGHT AND ELECTRON MICROSCOPY

Only fully mature LGs fuse at the IS, but surprisingly little is known about the biogenesis of these LGs. Mature LGs contain many proteins, for example CD63 and the lysosomal-associated membrane proteins LAMP1, LAMP2, and LAMP3, that are also found on lysosomes (14, 15, 16). Therefore, they are also called secretory lysosomes (17) or lysosome-related organelles [LRO; (18)]. However, it remains unclear whether LGs are derived from lysosomes or whether they share a common precursor from which the two organelles mature independently (**Figure 1A**). Since they are only synthesized upon activation of the CTL, the presence of the lytic components perforin and granzymes seems to be a reliable indicator for the identification of mature LGs and their precursors. EM of cryosections revealed that perforin and granzymes are always colocalized in a homogenous population of LGs in mouse CTLs (15). As expected for the regulated secretory pathway, traces of the proteins can be found in the rough endoplasmic reticulum and in the trans-Golgi network (TGN), but not in endosomal compartments containing the mannose-6-phosphate receptor. These data indicate that at least the dense-core of LGs is derived directly from the TGN with no involvement of endosomal compartments. Interestingly, while in human CTLs the vast majority of perforin immunostaining was found in the dense-core of LGs, in mouse CTLs both perforin and granzyme B were preferentially detected in small internal vesicles surrounding the dense-core. It is currently unknown whether these small internal vesicles in LGs originate from fusion of immature LGs with late endosomes and/or multivesicular bodies (10, 18) or whether these vesicles fuse with the dense-core to add more lytic components. As shown in **Figure 1B**, high pressure freezing EM yields excellent preservation of intracellular organelles, but also reveals many different organelles which resemble LGs. Therefore, it is impossible to follow the maturation of LGs to the fully mature, fusogenic LGs from EM alone.

Immunogold EM has been the method of choice to verify the localization of proteins on structures such as LGs. However, it suffers from two serious drawbacks. First, it cannot be used in conjunction with osmium tetroxide, resulting in a very poor contrast of the images. Second, it relies on the availability of highly specific antibodies, which, for the majority of proteins, are still lacking. A recently developed alternative is correlative light and EM [CLEM; (20)]. Although it can be used with classical antibody-based immunostaining as well, its major advantage is that it allows the use of proteins tagged with green fluorescent protein (GFP) or derivatives for localization studies. These proteins can either be introduced into CTLs by overexpression or by the genetic replacement of the endogenous protein by a fusion protein in transgenic mice (knock in). An example for the latter strategy is shown in **Figure 1C**. Synaptobrevin2 is a vesicular SNARE protein that mediates the fusion of LGs with the plasma membrane at the IS (19). We genetically replaced the endogenous synaptobrevin2 gene by a synaptobrevin2-mRFP (monomeric red fluorescent protein) fusion protein. As a result, the purified primary CTLs from this knock in mouse contain red LGs. Importantly, since synaptobrevin2 is essential for the final step of LG function the red LGs from this mouse represent by definition fully mature LGs. It is interesting to note that although vesicles might look indistinguishable in electron micrographs, only a fraction of them are fully mature, i.e., fusogenic, as indicated by the red fluorescence (**Figure 1C**, lower right panel). Thus, it appears that CLEM is an excellent choice to identify the maturation steps and the associated morphologies of LGs.

Apart from LG biogenesis and maturation, there are still several open questions relating to some of the key components of LGs such as perforin. At least 50 different mutations in perforin are linked to familial hemophagocytic lymphohistiocytosis type-2 (FHL-2), emphasizing the importance of this protein in CTLs and NK cells. Perforin is synthesized as a 65 kDa precursor in the ER. Both the N-terminus and the C-terminus of perforin are distinct in their functional contributions. The N-terminus contains the signal peptide and the amino-terminal membrane attack complex perforin-like (MACPF)/cholesterol dependent cytolysin (CDC) domain. The C-terminal contains the C2-domain needed to bind membranes in a calcium-dependent manner. X-ray crystal structure of monomeric murine perforin and cryo-EM reconstruction of the entire perforin pore revealed new insights and flexibility into the mechanism of pore formation (21). This study showed, using EM, that the perforin MACPF domain in the pore is inside-out relative to the subunit arrangement in CDCs, thus raising new possibilities in the mechanism of perforin pore formation. The extreme C-terminus of perforin is needed for the transport of the protein from the ER to the Golgi. However, a mechanism of perforin sorting from the TGN has been postulated (22) and could be resolved using EM.

VISUALIZING AND QUANTIFYING LYTIC GRANULES AT THE IS BY TIRFM

After having matured, the LG has to fulfill its central function to release its cytotoxic content into the synaptic cleft. To this end, LGs dock, prime, and then fuse (exocytose) with the plasma membrane. TIRFM has been widely used to study exocytic and

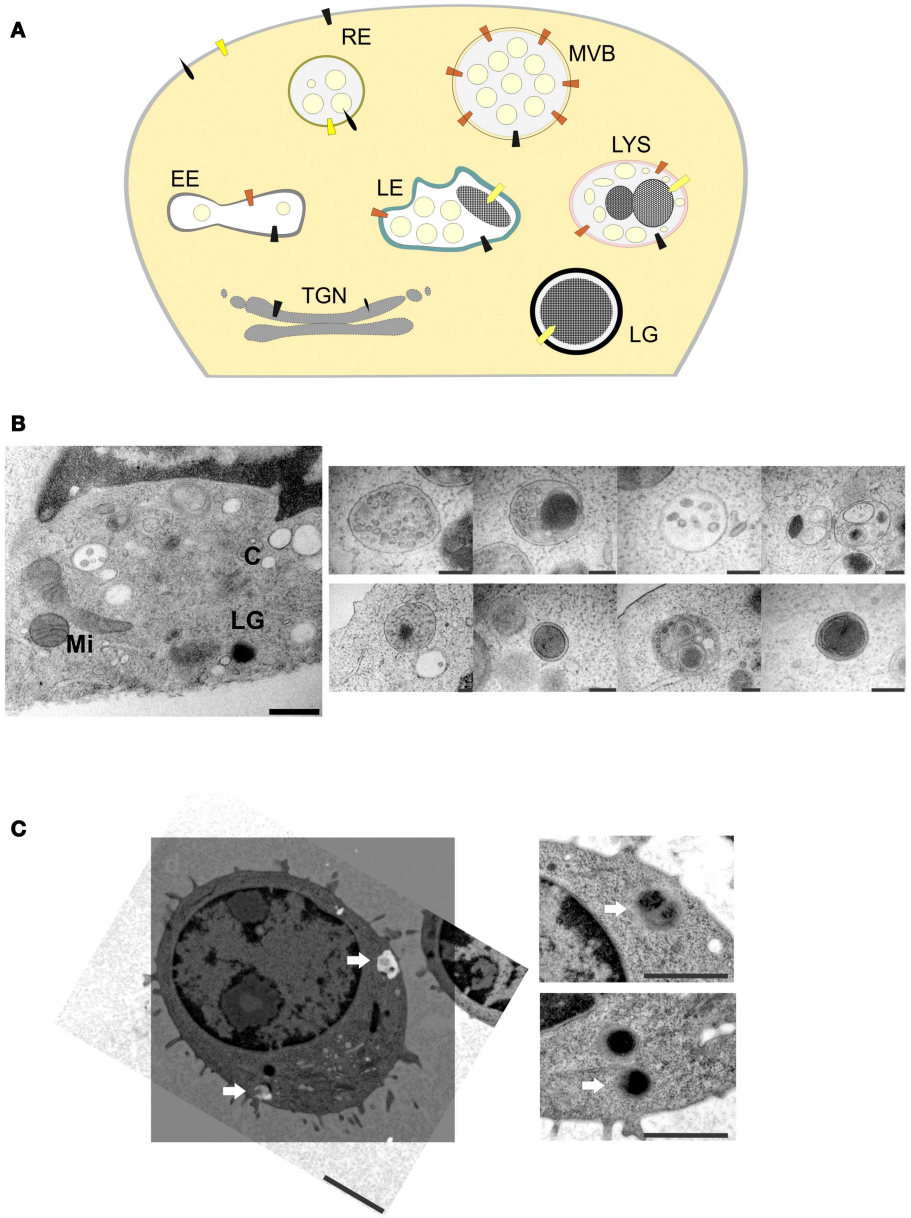

FIGURE 1 | (A) Model of LG biogenesis in CTLs. RE, recycling endosomes; EE, early endosomes; TGN, trans-Golgi network; LG, lytic granule; LE, late endosomes; LYS, lysosomes; MVB, multi-vesicular bodies. **(B)** Left, ultrastructure of an immunological synapse of a mouse CTL formed after contact with anti-CD3/CD28 coated sapphire, mimicking the target-cell (scale bar: 500 nm). Right, EM micrographs of different organelles of unknown nature present at an immunological synapse (scale bar: 200 nm). LG, lytic granule; C, centriole; Mi, mitochondria; N, nucleus. **(C)** Representative correlative fluorescence electron microscopy (CLEM) image of a primary mouse CTL obtained from synaptobrevin2-mRFP knock in mice (19). Left, EM micrograph (ultrathin section of 80 nm) of a mouse CTL with the corresponding processed SIM-image. SIM-image was taken with a 63× Plan-Apochromat N. A. 1.52 with excitation light of 561 nm wavelength, z-stack of 0.2 μm step size were used to scan a 500 nm thick section. Arrows indicate synaptobrevin2-positive lytic granules (scale bar: 2 μm). Right, enlarged areas with the corresponding organelles (scale bar: 1 μm).

endocytic events at the plasma membrane (23). TIRFM is an optical technique based on Snell's law which states that excitation light is totally internally reflected at the interface between areas with different refractive indices, when the angle of incidence exceeds the critical angle. In a biological context, the media with different refractive indices are the cells ($n \approx 1.37$) in aqueous solution plated on a coverslip ($n \approx 1.8$). Upon total reflection, the photons slightly penetrate the aqueous medium thereby generating an electromagnetic field, which propagates parallel to the interface. This so-called evanescent wave exponentially decays with distance from the interface, therefore only fluorescent molecules close to the interface are efficiently excited. Depending on the angle of incidence, the

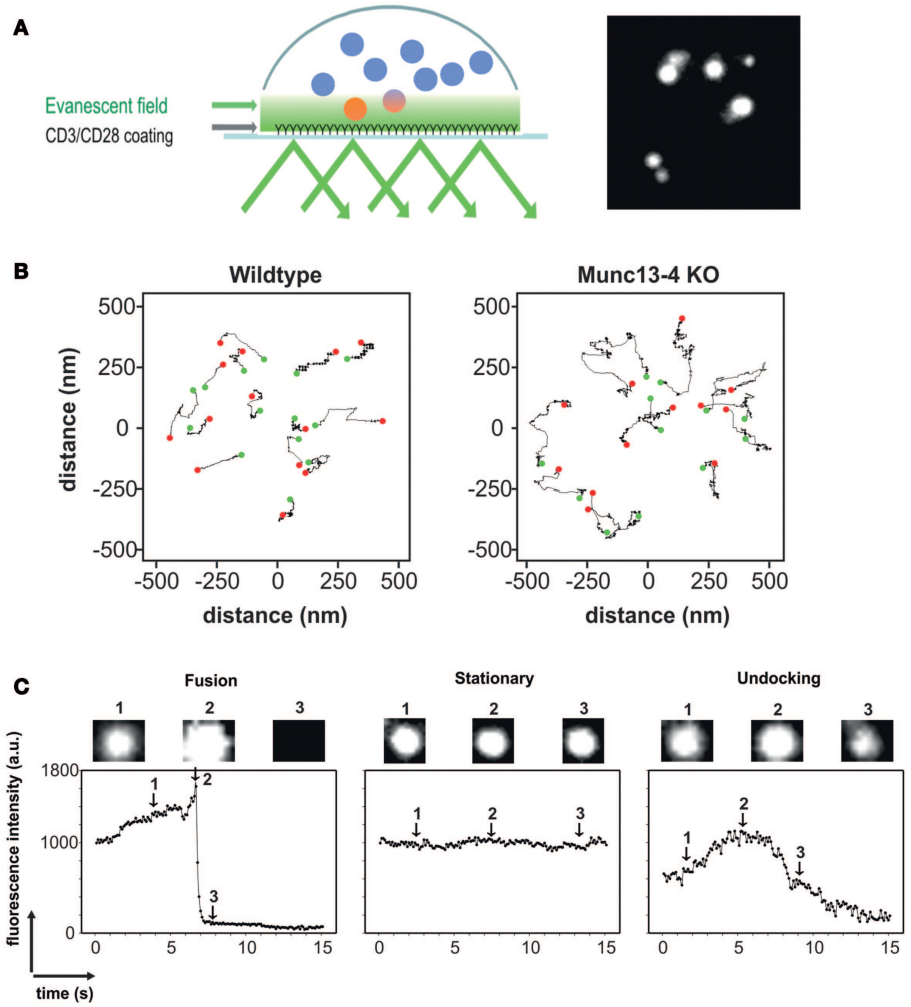

FIGURE 2 | (A) Left, cartoon depicting the methodology of TIRFM in CTLs using anti-CD3/anti-CD28 antibody-coated coverslips to mimic an IS. Only LGs that are within the evanescent field (close to the IS; see Text for details) are fluorescent. Right, TIRFM image of CTLs transfected with granzyme B-TFP to specifically label LGs. **(B)** Representative trajectories of vesicles from WT (left) and Munc13-4 KO (right) CTLs to display their mobility. The green dot represents the starting position of the LG and the red dot represents the end position of the LG. The absence of the priming factor Munc13-4 leads to a significant increase in vesicle mobility in TIRFM (28). **(C)** Fluorescence profile of a fusing LG (left), a stationary LG (middle), and an LG that is undocking (right) as seen in TIRFM. Acquisition frequency was 10 Hz. Granzyme B-TFP was used to label LGs.

excitation wavelength and the numerical aperture of the objective, the thickness of the evanescent field is usually within 150–200 nm from the glass-liquid interface. Fluorescence excitation by this evanescent wave results in images with very low background fluorescence (more than 2000-fold lower than in images collected by normal epifluorescence microscopy). This results in a high signal to-noise ratio, because virtually no out-of-focus fluorescence is collected.

For this reason, TIRFM has become increasingly popular for the observation of LG exocytosis at the IS in CTLs and NK cells. An IS suitable for TIRFM has been induced in these cells in a variety of ways. One method consists of using planar lipid bilayers that carry ligands for activating specific receptors on the membrane of CTL or NK cells (24). Another method uses antibody-coated glass coverslips [**Figure 2A**; (25, 26)]. Imaging of CTLs transfected with F-actin that settled on antibody-coated glass coverslips showed the clustering and spreading of F-actin at the periphery of the cell, a typical characteristic of an early IS. Both methods successfully induce formation of synapses ideally suited for TIRFM imaging. Though LAMP1 is widely used as an endogenous marker for lytic granules, it also labels lysosomes. The debate on the maturation of lytic granules and their distinction from lysosomes is still ongoing (see above). Thus the use of markers specific for lytic granules such as perforin and granzyme B for studying their behavior and kinetics at the IS is preferable. Perforin goes through several stages of processing before becoming fully mature (see above). Therefore generating recombinant perforin appeared challenging. After perforin is delivered to LGs, its last 20 amino acids are cleaved by Cathepsin-L to generate fully functional perforin (27). This was demonstrated by the use of Cathepsin inhibitors to significantly

decrease target-cell killing. Interestingly, target-cell killing by CTLs and NK cells from Cathepsin-L deficient mice is not diminished despite a reduction in the amount of processed perforin in the killer cells. This could indicate that other Cathepsins can compensate for the absence of Cathepsin-L. While Cathepsin-L is active only in the acidic pH of the granules, other cathepsins can function at a neutral pH and might also cleave perforin at the C-terminus after release from the LG. Human perforin from CTLs was indeed successfully cloned as a fusion construct with mCherry at the C-terminus (25). While it remains possible that the mCherry could indeed hinder Cathepsin-L from cleaving the last 20 amino acids at the C-terminus of perforin, this will not affect the validity of using perforin-mCherry as an LG marker. Granzyme B or FasL tagged with various fluorophores such as RFP, mTFP, mCherry, and pHluorin have been used as specific markers for LG studies using TIRFM. Co-transfection of perforin-mCherry and granzyme B-mTFP in human CTLs showed complete colocalization as expected and co-secretion at the TIRF plane mimicking an IS (data not shown). Our recent work in CTLs isolated from a Synaptobrevin2-mRFP knock in mouse demonstrated that Synaptobrevin2 mediates the final fusion step of lytic granules in CTLs (19). Synaptobrevin2 perfectly colocalized with mouse granzyme B and both were present at fusing LGs at the IS, thus providing an endogenous marker for lytic granules in mouse CTLs (see **Figure 1C**).

An additional advantage of TIRFM is that one can carefully distinguish and quantify pre-fusion events such as docking and priming. Docking is defined as the tethering of vesicles to the plasma membrane. The time that a vesicle resides in the TIRFM plane, referred to as the dwell time, is therefore a good measure of vesicle docking. The axial mobility of vesicles has also been used to quantify docking (29). Vesicle docking is followed by priming, which is defined molecularly as the formation of the trimeric SNARE complex, resulting in restricted mobility of the vesicle. Analyzing the mobility of vesicles in TIRFM can be used to characterize and differentiate the molecular states of priming and docking (30). An example of the quantification of the mobility of LGs from CTLs of wildtype and Munc13-4 knockout mice is shown in **Figure 2B**. Munc13-4 is considered to be a priming factor for LGs. A clear difference in the mobility of the vesicles could be seen, thereby establishing the correlation between less mobility and the state of priming. Analyses of the mean square displacement (MSD) over the increment of time or analyzing the caging diameter (CD) are well-established methods for analyzing the mobility of vesicles. However, the CD analysis is preferred since it also allows the quantification of the dynamic changes in the mobility itself, over time.

The fusion of lytic granules can be visualized in real time and quantified by TIRFM. Fast acquisition allows the analysis of the fluorescence intensity of a vesicle over time, to clearly distinguish between a vesicle that undergoes fusion, a stationary vesicle and a vesicle that is moving away from the plasma membrane (undocking, **Figure 2C**). In addition, the modes of fusion such as complete and incomplete fusion can also be analyzed by visualizing fusion pore opening (31). Their work implicated the existence of both fusion modes for LGs in primary NK cells. Incomplete fusion, also called "kiss and run," also occurs in neuroendocrine cells and in several neuronal synapses as studied by imaging and combined patch-clamp and amperometric recordings (32).

Total internal reflection fluorescence microscopy has therefore been invaluable not only to specify the role of key players in LG release, but also to define the sequence of events that lead up to the final lethal hit. Actin, which is considered a marker for the early IS, was recently shown by TIRFM to also play a role in LG degranulation itself (33).

EXAMINING LYTIC GRANULE FUSION BY COMBINED TIRFM AND PATCH-CLAMP MEASUREMENTS

The final step of LG maturation is the fusion of LGs with the plasma membrane at the IS. As discussed above TIRFM is the method of choice to visualize LG dynamics at the IS as it enables the quantitative analysis of membrane-bound pre-fusion events like docking and priming. Furthermore it allows the real-time visualization and quantification of fusion by studying the kinetics of the loss of the fluorophore. However, TIRFM does not allow an estimate of the size of a docked, primed, or fusing LGs due to resolution limits defined by Abbe's law (34). Immunogold labeling with perforin- and/or granzyme B-antibodies in human and mouse CTLs revealed ∼25 granules/cell with an average diameter of 700 nm, but yielded only static snapshots with unknown physiological relevance (15). Therefore, a method, which measures the size of a fusing LG, would be ideal.

The lipid bilayer of biological membranes separates the intracellular space from the extracellular solution surrounding the cell and acts as an electrical insulator between two conducting mediums. From a physical standpoint the cell membrane thus acts as a capacitor, and its specific capacitance has been calculated as 1 μF/cm^2 (35). The membrane capacitance is proportional to the surface area of the cell. If an intracellular vesicle fuses with the plasma membrane, it increases the membrane capacitance proportional to the surface area of the vesicle. Conversely, if endocytosis occurs, the membrane capacitance decreases. Membrane capacitance measurements, as a highly quantitative readout for exocytosis of chromaffin granules, was introduced 30 years ago (36). Fusion of an individual chromaffin granule from mouse results in a capacitance increase of 1.3 fF (37) which translates to a diameter of about 200 nm. This value is in excellent agreement with morphological EM data of chromaffin granules (38).

The measurement of membrane capacitance increases upon LG fusion from CTLs is complicated for two reasons. First, stimulated CTLs are highly mobile cells that tend to move through culture dishes while searching for potential target cells. This mobility makes patch-clamp recordings with a glass electrode technically quite challenging. Second, CTLs continuously add and retrieve proteins to and from the plasma membrane through exo- and endocytosis, mostly through recycling endosomes. For example, upon target-cell recognition TCR are integrated into the plasma membrane to form the cSMAC long before LGs arrive and fuse at the IS (39). Therefore, in order to distinguish between the fusion of LGs and other organelles, patch-clamp membrane capacitance measurements must be combined with TIRFM in order to visually identify LG fusion events that occur simultaneously with step-like capacitance increases [**Figure 3A**; (40)]. An exemplary trace of an LG fusion event is shown in **Figures 3B,C**. A sudden drop in

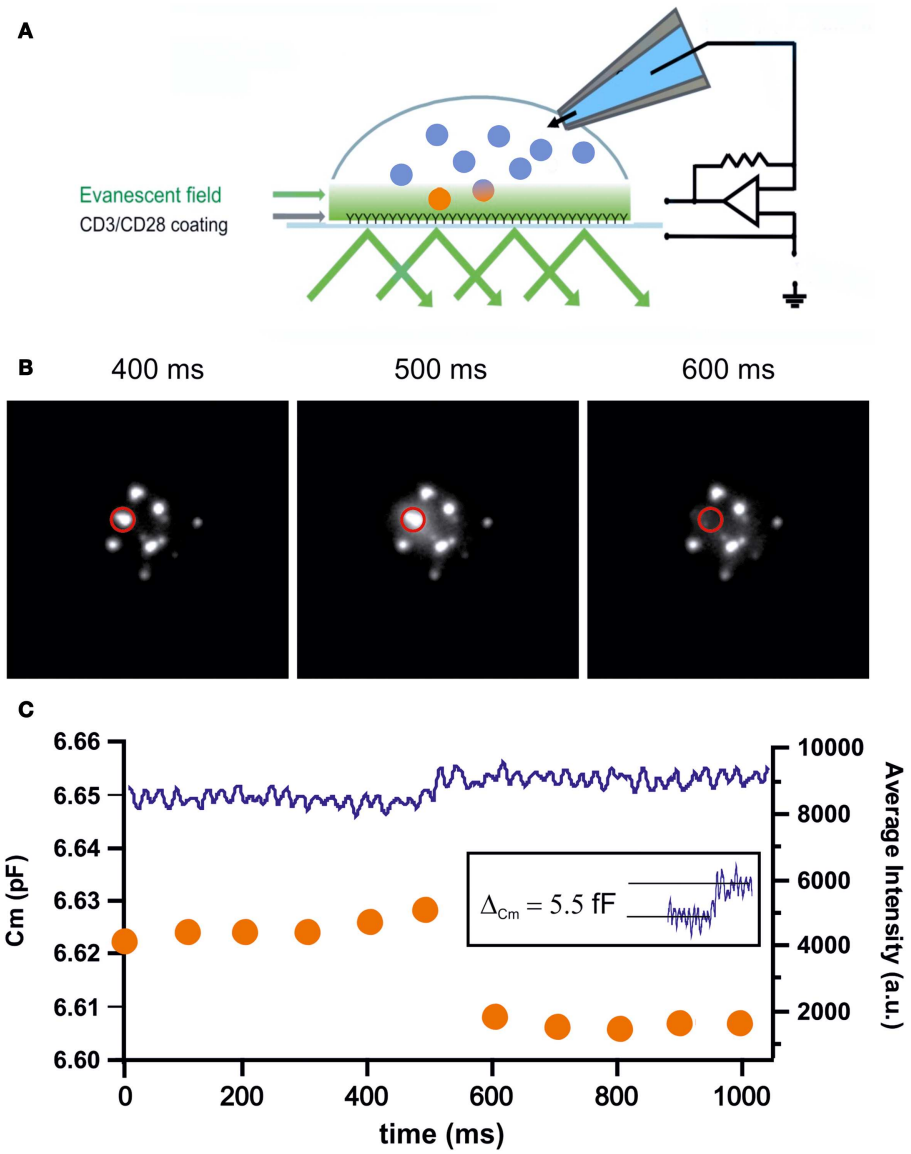

FIGURE 3 | (A) Cartoon depicting the methodology of combined TIRFM/patch-clamp experiments. The coverslip was coated with anti-CD3/anti-CD28 antibodies to mimic an IS. CTLs were transfected with granzyme B-TFP to visualize LGs in TIRFM. Simultaneously, CTLs were patched with a pipet containing iso-osmolar solution containing 2 μM Ca^{2+} to trigger secretion. Capacitance measurements were performed using the Lindau–Neher technique implemented as the "sine + dc" mode of the software lock-in extension of the PULSE software (41). **(B)** Exemplary TIRFM images showing the fusion of an LG within one frame. The middle panel shows the typical "halo" that originates from dequenching and diffusion of the fluorophore. **(C)** Graph showing the simultaneous measurement of membrane capacitance (left axis) and fluorescence intensity (right axis) of the LG shown in **(B)**. Upon fusion the fluorescence steeply declines within one frame while in parallel the capacitance increases step-like. The capacitance increase of the fusing vesicle (5.5 fF; inset) corresponds to an LG diameter of 418 nm.

fluorescence (orange trace) is accompanied by an increase in membrane capacitance (blue trace). The measured capacitance step of 5.5 fF relates to an LG diameter of 418 nm, considerably smaller than the estimate from EM data (15).

Besides the accurate determination of the diameter of fusogenic LGs, combined TIRFM/patch-clamp measurements enables the researcher to address other, important features of LG release. The unrestricted access to the interior of the cell through the patch pipette allows, for example, the manipulation of the intracellular calcium concentration to investigate whether the LG fusion process itself is calcium-dependent and, if yes, quantify the kinetics of this coupling.

OUTLOOK-FUTURE DIRECTION

We have described three high-resolution methods that enable the quantitative analysis of CTL function. Besides these techniques,

novel microscopy techniques such as structured illumination microscopy [SIM (42)], stimulated emission depletion [STED (43)] and direct stochastic optical reconstruction microscopy [dSTORM (44)] have recently become available. The combination of these techniques will allow the investigation of CTL and NK cell function with unparalleled detail and resolution, promising a deeper understanding of this fundamental, immunological process.

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Inhibitory receptor signaling destabilizes immunological synapse formation in primary NK cells

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Upon engagement of their cognate class I major histocompatibility complex ligands, receptors containing immunotyrosine-based inhibitory motifs (ITIMs) transduce signals that block cytolytic and inflammatory responses. In this manner, ITIM-coupled receptors play a crucial role in maintaining natural killer (NK) cell tolerance toward normal, healthy tissue. A number of studies, mostly using immortalized NK cell lines, have demonstrated that ITIM signaling functions by disrupting the cytolytic immunological synapse formed between an NK cell and its target. However, more recent imaging experiments using primary NK cells have suggested that inhibitory receptor engagement does not antagonize contact formation, casting doubt on the hypothesis that ITIM signals destabilize the synapse. To resolve this issue, we analyzed primary NK cell activation and contact formation on supported lipid bilayers containing controlled combinations of activating and inhibitory ligands. Under these conditions, we observed that ITIM signaling clearly inhibited adhesion, cell arrest, and calcium influx, three hallmarks of synapse formation. These results are consistent with previous reports showing that inhibitory receptors deliver a "reverse stop" signal, and confirm that ITIM signaling functions at least in part by destabilizing cytolytic synapse formation.

Keywords: NK cell, signal transduction, immunological synapses, ITIM, imaging

INTRODUCTION

Natural killer (NK) lymphocytes play an important role in antiviral and anti-tumor responses by specifically eliminating cells that bear signs of infection or transformation. Target cell recognition triggers the formation of a stereotyped junction between the NK cell and the target known as an immunological synapse (IS) (1, 2). This is followed by directional release of cytolytic perforins and granzymes into the synaptic space, leading to target cell death by apoptosis. By mediating adhesion and focusing secretion in this manner, the IS promotes target cell killing while limiting damage to surrounding healthy tissue.

Natural killer cell cytotoxicity is governed by a number of distinct activating and inhibitory cell-surface receptors (3, 4). Activating receptors induce IS formation, target cell killing, and the release of inflammatory cytokines such as interferon-γ, while inhibitory receptors transduce signals that block these activating responses. Activating receptors are quite structurally diverse, and bind to ligands that are indicative of infection, transformation, or immune targeting. The C-type lectin NKG2D, for example, recognizes a set of proteins (including the MIC and ULBP families) that are upregulated in response to cellular stress. CD16, by contrast, is a low affinity Fc receptor that enables engagement of antibody-coated targets. Inhibitory NK receptors, for their part, almost exclusively recognize class I major histocompatibility complex (MHC), which is highly expressed in normal, healthy tissue. This leads to the phosphorylation of immunotyrosine-based inhibitory motifs (ITIMs) located in the cytoplasmic tail of the receptor. Phosphorylated ITIMs recruit the tyrosine phosphatases SHP-1 and -2, which are thought to dephosphorylate signaling proteins required for NK cell activation.

Precisely how inhibitory receptor engagement blocks activating responses in NK cells remains an area of intense interest. SHP-1 has been shown to dephosphorylate Vav-1 (5), a large scaffolding protein and guanine nucleotide exchange factor involved in multiple activating pathways. ITIM-receptor signaling has also been linked to the phosphorylation of the adaptor protein Crk and its dissociation from activating signaling complexes (6, 7). Translating these biochemical events into a cellular response, however, has been challenging. To address this deficiency, a number of groups have employed videomicroscopy approaches in which individual NK cells are imaged on surfaces containing defined mixtures of activating and inhibitory NK receptor ligands (6, 8, 9). If sufficient activating ligand is present, NK cells form stable, symmetric contacts on these surfaces that bear the structural hallmarks of an IS. Because these contacts are positioned at the cell-surface interface in an orientation perpendicular to the axis of illumination, it is possible to image them using high-resolution modalities such as total internal reflection fluorescence (TIRF) microscopy.

To facilitate day-to-day experimentation, most studies using this approach have employed human cell lines such as NKL, which was derived from an NK cell leukemia. Initial efforts focused on the effects of NKG2A, a C-type lectin that binds to the nonclassical MHC HLA-E. NKG2A engagement strongly inhibited IS formation on stimulatory glass surfaces, inducing instead an active migratory phenotype (9). These results suggested that NKG2A delivers a "reverse stop" signal that antagonizes stable contact with

the target cell. Subsequently, our lab analyzed inhibitory signaling from the killer immunoglobulin receptor KIR2DL2 using a supported lipid bilayer system that allows free diffusion of activating and inhibitory ligands (8). Stimulation of KIR2DL2 with its cognate ligand HLA-Cw3 essentially blocked IS formation in these experiments. Using a photostimulation approach, we also demonstrated that KIR2DL2 signaling induced the retraction of preexisting synapses. These results lent further support to the idea that inhibitory receptor engagement destabilizes the IS. They were also consistent with previous studies indicating that ITIM signaling disrupts NK cell-target cell adhesion (10, 11).

More recently, the stimulatory bilayer approach was employed to examine inhibitory signaling in primary human NK cells (6). Surprisingly, contacts were observed on bilayers containing HLA-E, implying that NKG2A signaling does not disrupt IS formation. The authors of this study attributed discrepancies between their data and previous results to differences between primary NK cells and the NKL cell line. However, the relative strength of the observed inhibitory contacts was not assessed relative to activating synapses, and the relative motility of NK cells on activating and inhibitory bilayers was not quantified. To address these issues, we have profiled the activation status and migratory behavior of primary human NK cells on both activating and inhibitory bilayers. Our results indicate that ITIM-receptors do indeed destabilize cytolytic IS formation and promote migration by delivering a reverse stop signal.

RESULTS

ACTIVATION AND INHIBITION OF PRIMARY NK CELLS ON LIPID BILAYERS

Supported lipid bilayers containing purified activating and inhibitory ligands are increasingly used as target cell proxies in imaging-based studies of NK cell signaling. A number of strategies exist for incorporating ligands into the bilayer. For our experiments, we used a 1:10 mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and the biotinylated lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl). Treatment of these bilayers with streptavidin followed by controlled amounts of biotinylated NK receptor ligands results in orientated incorporation of these ligands into the membrane.

We obtained primary human NK cells expressing NKG2A or KIR2DL3 from peripheral blood of a group A haplotype donor. To establish the stimulus requirements for the activation and inhibition of these cells, we monitored calcium (Ca^{2+}) responses in highly pure (>95% $CD3^-CD56^+$) NK cell preparations using the Ca^{2+} sensitive dye Fura-2 (**Figure 1A**). Ca^{2+} flux is a common feature of activating NK receptor signaling pathways and is required for cytolytic degranulation responses (3, 12–14). Hence, it serves as a reliable index of activation status at a single cell level. NK cells were stimulated by simultaneous engagement of NKG2D and the SLAM family receptor 2B4 with a mixture of their respective cognate ligands, ULBP3 and CD48. We also activated NK cells through CD16 using an anti-CD16 antibody, which we found to be more stimulatory than IgG, the cognate CD16 ligand (**Figure 1B**). Activating ligands were presented to NK cells both in the presence and the absence of the adhesion molecule ICAM-1, which binds to the $\alpha_L\beta_2$ integrin LFA-1. In this manner, we were able to assess the importance of integrin coengagement for NK cell activation. Finally, purified HLA-Cw3 and HLA-E were used to inhibit activating signals in $KIR2DL3^+$ and $NKG2A^+$ NK cells, respectively.

Ca^{2+} responses were visualized by ratiometric Fura-2 imaging at low magnification, enabling quantification over many cells. Activation at a population level was defined as elevation of intracellular Ca^{2+} levels above background during the sustained phase of the response (between 8 and 16 min after the start of the experiment). Remarkably, coengagement of activating receptors together with LFA-1 was a prerequisite for NK cell activation under these conditions (**Figure 1B**). Neither anti-CD16 nor the combination of ULBP3 and CD48 induced appreciable activation in the absence of ICAM-1. Similarly, ICAM-1 alone was incapable of driving Ca^{2+} flux without activating receptor stimulation. Addition of HLA-Cw3 and HLA-E robustly inhibited activating responses in $KIR2DL3^+$ and $NKG2A^+$ NK cells, respectively, often restoring intracellular Ca^{2+} to background levels (**Figure 1B**). These data were consistent with previous reports demonstrating the potential of ITIM-derived signals to override activating pathways at a very early stage (15–19). Although we initially used IL-2-cultured NK cells for these experiments, we observed a similar pattern of results using resting NK cells (**Figure 1C**); ICAM-1 was required for robust Ca^{2+} influx, and this response was largely blocked by inhibitory receptor engagement. We conclude that LFA-1 stimulation is crucial for primary NK cell activation on lipid bilayers, and that this activation is reversed by inhibitory receptor engagement.

ITIM-RECEPTORS DELIVER A REVERSE STOP SIGNAL IN IL-2-CULTURED NK CELLS

Stable cytolytic IS formation is associated with an arrest in cell motility, and signals from inhibitory receptors have been proposed to overcome this arrest by delivering a reverse stop signal (9). To assess the validity of this model in our system, we quantified the movement of $KIR2DL3^+$ and $NKG2A^+$ primary IL-2-cultured NK cells on lipid bilayers containing various combinations of activating and inhibitory ligands. Cells were stained with the membrane dye PKH26 and imaged by TIRF microcopy, which enabled us to focus on the behavior of cells in contact with the bilayer.

Single cell tracking analysis revealed that NK cell activation and inhibition were associated with dramatic differences in cell motility (**Figures 2A,B**). Under conditions of simultaneous activating receptor and LFA-1 stimulation, cells formed stable, largely stationary contacts characterized by a mean instantaneous velocity between 10 and 20 nm/s (**Figures 2A,C**). By contrast, coengagement of either KIR2DL3 or NKG2A increased mean velocity to between 50 and 100 nm/s, similar to the motility exhibited by NK cells on bilayers containing ICAM alone (**Figures 2B,C**). ITIM-receptor signaling appeared to have a weaker effect on cells activated via NKG2D and 2B4 than on cells activated via CD16 (**Figure 2C**). However, activation through NKG2D/2B4 also resulted in more pronounced cell arrest in our hands. Indeed, after accounting for differences in the degree of activation, we found that inhibitory receptor stimulation induced a four- to fivefold increase in motility under all stimulus conditions (**Figure 2C**). Taken together, these data demonstrate that engagement of KIR2DL3 or NKG2A relieves activation-induced

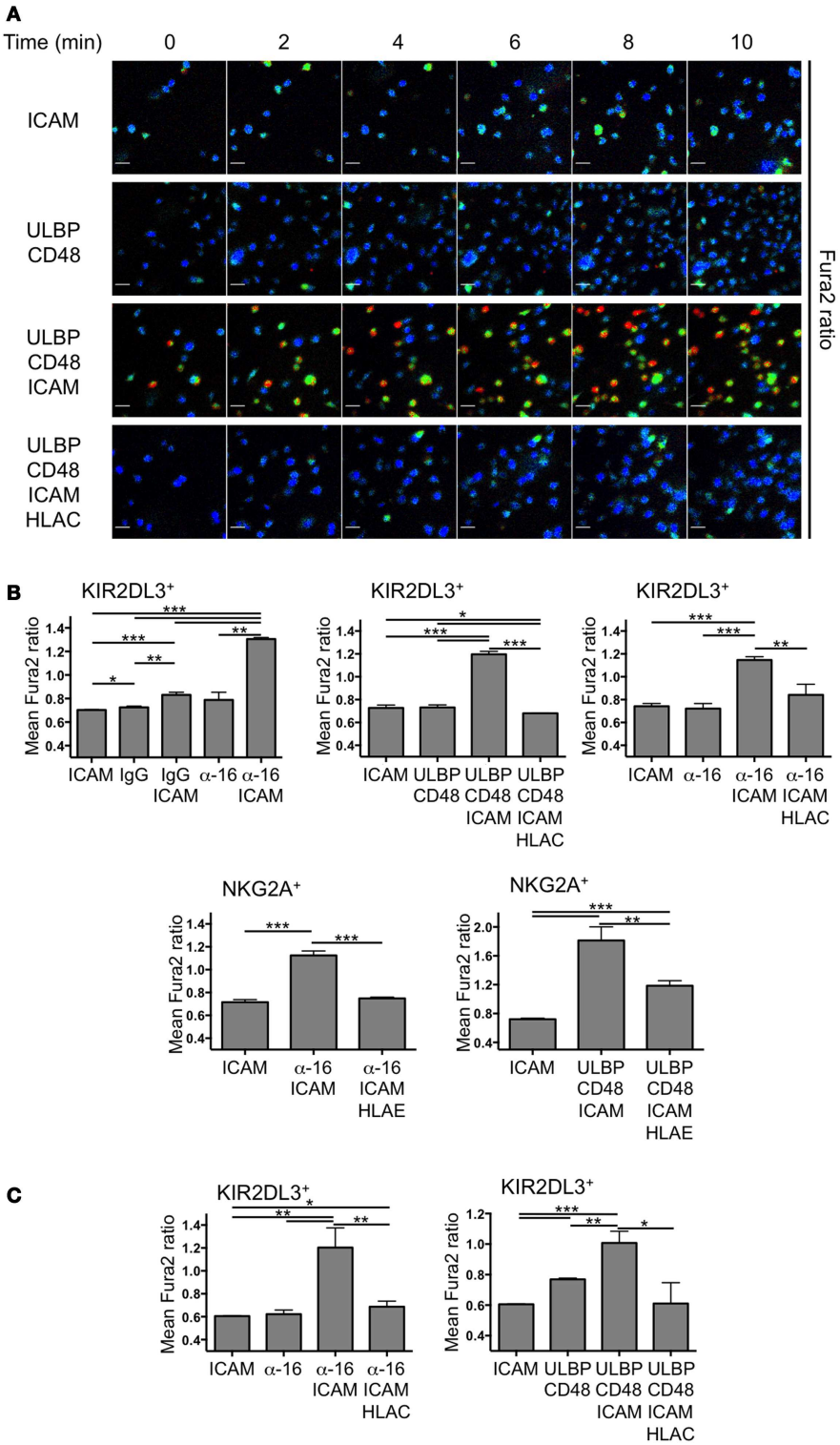

FIGURE 1 | Primary NK cell activation on stimulatory lipid bilayers. IL-2-cultured and resting primary human NK cells were sorted into KIR2DL3$^+$ or NKG2A$^+$ populations, loaded with Fura2-AM and imaged on supported lipid bilayers. **(A)** Time-lapse montages from a representative experiment showing single cell Ca^{2+} responses from KIR2DL3$^+$ cells on bilayers containing the indicated activating and inhibitory NK receptor ligands. Fura2 ratio is pseudocolored with cooler and warmer colors indicating low and high intracellular Ca^{2+} concentration, respectively. **(B,C)** Ca^{2+} influx was quantified for IL-2-cultured **(B)** or resting **(C)** KIR2DL3$^+$ or NKG2A$^+$ cells as indicated by calculating the average Fura-2 ratio for all cells in the imaging field during the plateau phase of the global response (see Materials and Methods). Error bars denote standard error of the mean (SEM). *P*-values were calculated using Student's *t*-test, with $***P < 0.001$, $**P < 0.01$, and $*P \leq 0.05$. HLAC = HLA-Cw3; HLA-E = HLA-E; ULBP = ULBP3; α-16 = anti-CD16.

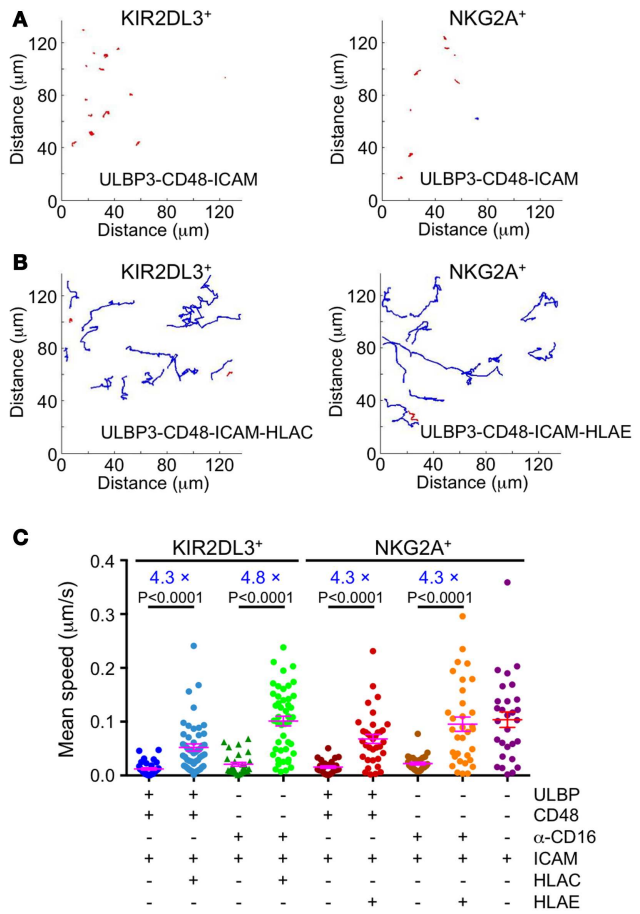

FIGURE 2 | Immunotyrosine-based inhibitory motif-receptor signaling induces a reverse stop signal. IL-2-cultured primary human NK cells were labeled with the membrane dye PKH26 and imaged by TIRF microscopy on supported lipid bilayers containing activating and inhibitory NK receptor ligands. **(A,B)** Representative trajectories for KIR2DL3$^+$ or NKG2A$^+$ NK cells on bilayers containing either activating ligands alone **(A)** or mixtures of activating and inhibitory ligands **(B)**. Blue trajectories denote average speed ≥30 nm/s, while red trajectories denote average speed <30 nm/s. **(C)** Average speed of KIR2DL3$^+$ or NKG2A$^+$ NK cells on bilayers containing the indicated activating and inhibitory ligands ($n \geq 28$ cells per condition). Factors relating average speed in the presence or absence of inhibitory ligand for each activating condition are shown in blue. Magenta lines and error bars indicate mean and SEM. *P*-values were calculated using the Mann–Whitney test.

arrest in primary NK cells, consistent with the model whereby ITIM-receptors deliver a reverse stop signal.

ITIM-RECEPTOR SIGNALING REDUCES ADHESION IN RESTING NK CELLS

Next, we sought to extend our studies of synaptic stability to resting NK cells. However, under conditions where IL-2-cultured NK cells were highly dynamic (e.g., ICAM-1 alone), resting NK cells exhibited little to no motion. This made it difficult to use motility to assess the quality of synapses formed by these cells. We did notice however, that fewer cells became immobilized on bilayers containing inhibitory ligands. Accordingly, we implemented an imaging-based adhesion assay to quantify the number of cells making strong contact under both activating and inhibitory conditions. Cells were incubated with bilayers for 10 min, followed by a round of washing with PBS. Then, the remaining cells in contact with the bilayer were quantified by epifluorescence imaging at low resolution (see Materials and Methods). Both KIR2DL3$^+$ and NKG2A$^+$ resting NK cells exhibited basal adhesion on bilayers containing ICAM-1 alone (**Figure 3**). Addition of activating receptor ligands enhanced this adhesion two- to threefold, indicative of activating IS formation. Importantly, stimulation of either KIR2DL3 or NKG2A reduced adhesiveness to background levels (**Figure 3**). Indeed, often fewer cells were observed on bilayers containing combined activating and inhibitory ligands than on bilayers presenting ICAM-1 alone. We conclude that ITIM-receptor signaling profoundly destabilizes cytolytic synapses in resting NK cells.

DISCUSSION

The idea that ITIM-receptor signaling undermines NK cell-target cell interactions initially grew out of seminal experiments from Long and colleagues demonstrating that engagement of inhibitory KIRs disrupted conjugates formed by the NK cell line YTS (11). Subsequently, it was shown that stimulation of NKG2A blocked the inside-out upregulation of integrin affinity in primary NK cells (10). Single cell imaging studies with NKL cells confirmed and extended these results by demonstrating that inhibitory signals antagonized IS formation, providing a reverse stop signal that encouraged migration rather than focused cytolysis (8, 9). Here, we demonstrate that engagement of ITIM-receptors disrupts IS formation in both resting and IL-2-cultured NK cells, which is largely consistent with these previous studies.

Interestingly, whereas inhibitory stimulation promoted migration in IL-2-cultured NK cells, it induced detachment in resting NK cells. Both increased motility and membrane detachment, however, are manifestations of a destabilized IS. As we have suggested (2), the behavior exhibited by NK cells under inhibitory conditions likely depends not only on the amount of inhibitory stimulation but also on the basal levels of adhesiveness and motility in each cell type. IL-2-cultured NK cells display robust motility under non-stimulatory conditions, while resting NK cells do not. Hence, in both cases inhibitory stimulation causes the NK cells to revert to their unperturbed state, consistent with the idea that ITIM-receptors specifically target activating signals and leave other aspects of NK cell physiology intact.

Previous studies have demonstrated an important role for LFA-1-ICAM interactions in the regulation of NK cell activation. LFA-1 can drive cytoskeletal polarization toward a target cell in the absence of activating receptor stimulation (20), and it also appears to be required for establishing radially symmetric organization within the NK cell IS (21). Here, we demonstrate that LFA-1 engagement is required for IS formation and Ca^{2+} flux on supported lipid bilayers. Importantly, these activating responses could not be induced by LFA-1 alone, but only by combining LFA-1 engagement with stimulation of CD16 or NKG2D/2B4. Hence, LFA-1 appears to be behaving as an obligate costimulatory receptor in this context. These results were somewhat surprising given previous reports that stimulation of certain activating

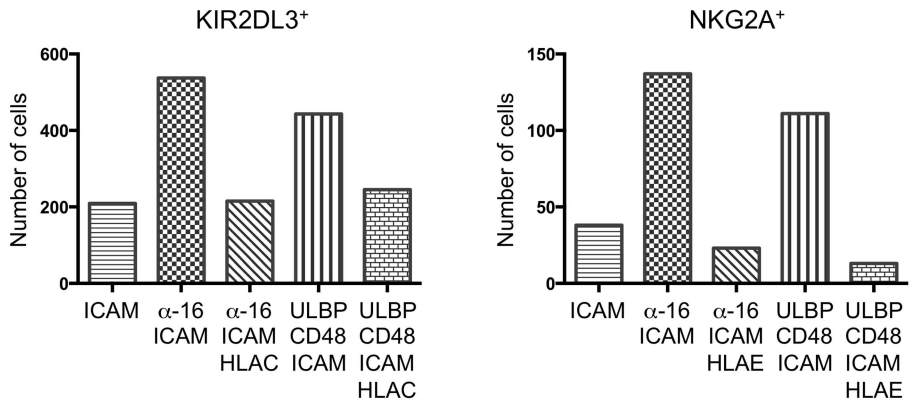

FIGURE 3 | Immunotyrosine-based inhibitory motif-receptor signaling inhibits attachment in resting NK cells. Resting KIR2DL3$^+$ (left) or NKG2A$^+$ (right) NK cells were stained with PKH26 and plated on bilayers containing the indicated activating and inhibitory NK receptor ligands. After washing, attached cells were fixed and counted. A representative experiment is shown.

receptors, such as CD16, can drive Ca^{2+} flux in resting NK cells in the absence of LFA-1 engagement (22) (Bryceson, personal communication). In these previous studies, NK cells were activated either by antibody crosslinking in suspension or by coincubation with S2 cells expressing selected activating ligands. These differences in experimental approach could potentially explain the apparent contradiction with our work. On supported bilayers, ICAM-1 may be required for promoting close apposition of the NK cell membrane with the surface, a prerequisite for activating receptor stimulation. This sort of adhesive function would be irrelevant for antibody crosslinking experiments, and in NK cell-S2 conjugates, tight intermembrane spacing could be established by another receptor-ligand pair. Further investigation of when, where, and how LFA-1-ICAM interactions contribute to NK cell IS formation is clearly warranted.

The importance of LFA-1 for NK cell activation on bilayers might also explain the discrepancies between our results and those of (6), who found that stimulation of NKG2A did not alter IS stability. The bilayers used in this previous study contained no ICAM-1, only human Fc (to engage CD16), which based on our results would not be expected to induce bona fide IS formation on its own. Using this stimulatory regime to assess the effects of NKG2A stimulation on IS stability is therefore somewhat problematic. Recent studies have suggested that NK cells can kill targets by forming transient interactions accompanied by weak Ca^{2+} flux (23). T cell biologists have coined the term "kinapse" to describe contacts like this because they contain certain elements of activating synapses without the associated cell arrest (24). It is conceivable that the interactions visualized by Liu et al. are more akin to kinapses, and as such they are not strictly comparable to the stable, radially symmetric contacts documented here. Moving forward, it may be worthwhile for the field to establish certain objective criteria, based on architecture, stability, or associated signaling, for what qualifies as an "immunological synapse." This could facilitate the reconciliation of observations made under different experimental conditions. Our present results, however, when taken together with previous studies, leave little doubt that inhibitory contacts are substantially less stable than activating synapses, and that ITIM-receptors induce this destabilization.

MATERIALS AND METHODS

NK CELLS

Human NK cells were isolated from peripheral blood of a healthy donor by negative selection (Miltenyi Biotec). The procedure typically yielded >95% $CD3^-CD56^+$ cells. KIR2DL3$^+$ and NKG2A$^+$ subsets were isolated from this purified population by FACS. Resting NK cells were resuspended in CellGenix GMP SCGM medium supplemented with 10% human serum and were used within 2 days. IL-2-cultured NK cells were prepared by culturing purified resting NK cells in CellGenix GMP SCGM medium supplemented with 500 IU/ml IL-2 for at least 2 weeks. IL-2-cultured NK cells were used for experiments between 14 and 23 days after initial isolation.

LIPID BILAYERS

Supported lipid bilayers containing a 10:1 mixture of DOPC and biotinyl cap phosphoethanolamine (both obtained from Avanti Polar Lipids) were prepared in eight-well chamber slides (Fisher) as described previously (8). ULBP3 (a.a. 24–207), human CD48 (a.a. 27–219), mouse ICAM-1 (a.a. 28–485), HLA-Cw3 (a.a. 25–302), and HLA-E (a.a. 22–296) were expressed with C-terminal BirA recognition sequences to enable site specific biotinylation. Recombinant proteins were purified, refolded, and biotinylated as described previously (8, 25). Biotinylated proteins were incorporated into the bilayers at the following concentrations: ULBP3 (2 μg/ml), CD48 (2 μg/ml) ICAM-1 (1 μg/ml), anti-CD16 (3G8, BD Biosciences, 2 μg/ml), human IgG1 (Jackson ImmunoResearch, 2 μg/ml), HLA-Cw3 (1 μg/ml), and HLA-E (1 μg/ml). In cases where one or more proteins were left out, a non-stimulatory biotinylated mouse MHC molecule (either $I\text{-}E^k$ or $H\text{-}2D^b$) was added to keep the total protein concentration constant.

Ca^{2+} IMAGING

Primary NK cells were loaded with Fura-2-AM dye and imaged on lipid bilayers using a 20× objective lens (0.75 NA, Olympus).

Three positions within each well were imaged every 30 s for a total of 30 min. For each position, the average Fura-2 ratio for all cells was determined as a function of time. Values during the plateau phase of the response (8–16 min) were averaged over all positions and graphed for comparison.

CELL MOTILITY ASSAY

IL-2-cultured NK cells were stained with PKH26 and imaged by TIRF microscopy on lipid bilayers using a 60× objective lens (1.45 NA, Olympus). Three positions were imaged every 10 s for 25 min. Single cell motility was tracked manually. Trajectories and velocities were calculated for each cell using custom MATLAB (MathWorks) scripts.

CELL ADHESION ASSAY

Primary resting NK cells were stained with PKH26 and seeded into wells containing stimulatory bilayers. After 10 min at 37°C, the wells were washed with pre-warmed (37°C) PBS and then fixed with 4% PFA for 5 min. The total number of cells in each well was quantified at low resolution (10× objective), excluding cells that accumulated within 800 μm of the edge of the well.

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The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity

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Considerable evidence has been gathered over the last 10 years showing that the tumor microenvironment (TME) is not simply a passive recipient of immune cells, but an active participant in the establishment of immunosuppressive conditions. It is now well documented that hypoxia, within the TME, affects the functions of immune effectors including natural killer (NK) cells by multiple overlapping mechanisms. Indeed, each cell in the TME, irrespective of its transformation status, has the capacity to adapt to the hostile TME and produce immune modulatory signals or mediators affecting the function of immune cells either directly or through the stimulation of other cells present in the tumor site. This observation has led to intense research efforts focused mainly on tumor-derived factors. Notably, it has become increasingly clear that tumor cells secrete a number of environmental factors such as cytokines, growth factors, exosomes, and microRNAs impacting the immune cell response. Moreover, tumor cells in hostile microenvironments may activate their own intrinsic resistance mechanisms, such as autophagy, to escape the effective immune response. Such adaptive mechanisms may also include the ability of tumor cells to modify their metabolism and release several metabolites to impair the function of immune cells. In this review, we summarize the different mechanisms involved in the TME that affect the anti-tumor immune function of NK cells.

Keywords: hypoxia, natural killer cells, autophagy, tumor-derived exosomes, tumor microenvironment

INTRODUCTION

Natural killer (NK) cells are potent cytolytic lymphocytes belonging to the innate immune system. NK cells comprise up to 15% of all circulating lymphocytes and are also found in peripheral tissues including the liver, peritoneal cavity, and the placenta. Although resting NK cells circulate in the blood, they are capable of infiltrating most cancer tissues following activation by cytokines. NK cells can be rapidly activated in the periphery by NK cell stimulatory factors, such as interleukin (IL)-12, interferon (IFN)-α and -β, IL-15, or IL-2 (1). Regulation of NK cell activity depends on the repertoire of germline-encoded activating and inhibitory receptors. The activating receptors recognize stress-induced, pathogen-derived, or tumor-specific ligands, whereas the inhibitory receptors bind self-molecules presented on normal cells. Owing to a diversified set of inhibitory and activating receptors, NK cells are capable of recognizing and killing an array of tumor cells (2). Beyond innate activity, NK cells are important for the regulation of anti-tumor adaptive immunity (3, 4).

In addition to their well-described role in inhibiting the early stage of tumor formation, NK cells are able to eradicate large solid tumors. Such eradication depends on the massive infiltration of proliferating NK cells due to the release and the presentation of IL-15 by cancer cells in the tumor microenvironment (TME). It has been shown that infiltrating NK cells are strikingly similar morphologically to uterine NK cells (5).

Based on the fact that NK cells can eliminate cancer cells in experimental conditions, it has been proposed that NK cells can be used clinically in therapeutic settings against cancer. Importantly, data from haploidentical hematopoietic stem cell transplantation and NK cell-based adoptive immunotherapy support the clinical effects of NK cells (6). Based on our current knowledge of the molecular specificities that regulate NK cell functions, it is tempting to speculate that a design of tailored NK cell-based immunotherapeutic strategies against cancer might be possible.

Recent data confirm that NK cells are required for the induction of potent anti-tumor-specific cytotoxic T lymphocytes (T cells) responses, by a mechanism involving dendritic cell (DC) editing (7, 8). Furthermore, NK cells can recognize tumors that might evade T cell-mediated killing by aberrant human leukocyte antigen (HLA) expression (9), indicating that NK cells participate in tumor immunosurveillance.

A significant correlation between high intratumoral levels of NK cells and increased survival has been shown in several types of cancer (10). Indeed, high levels of NK-infiltrating tumors have been associated with a significant improvement of clinical outcomes in patients with head and neck squamous carcinoma (HNSCC). It has been reported by van Herpen et al. that CD56+ NK cells in lymph nodes produced considerable amounts of IFN-γ that subsequently lead to tumor regression in IL-12-treated HNSCC patients (11). A direct positive correlation between the density of CD57+ NK cells and a good prognosis has been reported for oral squamous carcinoma (12) and gastric carcinoma (GC) tumors (13). In addition, NK cell infiltration was found to also correlate with the depth of invasion, the clinical stage, and the

venous invasion. Therefore, the 5-year survival rate of GC patients with a high rate of NK infiltration was significantly better than that of patients with a low level of NK infiltration (13).

Natural killer-based immunotherapy is a promising strategy for solid and hematologic cancers and it can potentially be combined with chemotherapy, radiation, or monoclonal antibody therapy. For example, the proteasome inhibitor bortezomib (Velcade®), which is clinically approved for the treatment of refractory/relapsed myeloma, downregulates the expression of major histocompatibility complex (MHC) class I on the target cell surface and thereby shifts the balance toward NK cell activation and target cell killing (14). Therefore, such combination therapy has important therapeutic implications for multiple myeloma (MM) and NK cell-related malignancies in the context of adoptively transferred allogeneic and autologous NK cells (15). NK cell-based therapy can be combined with radiation therapy as irradiation-induced tissue injury increases the expression of NK-activating ligands (e.g., NKG2D ligands) on malignant cells, thereby rendering tumors more susceptible to NK cell cytotoxic activity (16) Another NK cell-based approach used in therapy is the antibody-dependent cellular cytotoxicity (ADCC). This approach is based on the ability of NK cells, expressing an activating Fc receptor, to kill tumor cells by recognizing the constant region of tumor-bound monoclonal antibodies (mAbs). Clinically, ADCC strategy has been used in CD20+ lymphoma patients treated with rituximab (Rituxan™) (17) or HER2/neu-expressing breast cancer patients treated with trastuzumab (Herceptin™) (18). It is important to note that the co-administration of immunomodulatory cytokines (e.g., IL-12) can enhance the effects of anti-tumor mAbs via the activation of NK cells *in vitro*. This effect has been observed in breast cancer patients overexpressing HER2/neu and treated with IL-12 and trastuzumab in a phase I trial (19).

Despite the progress made in the field of NK-based immunotherapy, there are still many obstacles to eliciting an effective immune response. One major impediment is the ability of tumor cells to activate several mechanisms that lead to tumor escape from NK-mediated killing. It has become increasingly clear that the TME plays a crucial role in the impairment of the immune response and in the development of many overlapping mechanisms that create an immunosuppressive microenvironment. It has been reported that tumor-associated NK cells display a modified phenotype, thereby supporting the notion that tumor-induced alterations of activating NK cell receptor expression may hamper immune surveillance and promote tumor progression (20). Decreased cytotoxic activity of NK cells infiltrating tumors was also observed in different types of human cancer such as lung carcinoma (21), indicating that the TME is a critical factor influencing NK-mediated killing of tumor cells. Hypoxia, a characteristic feature of advanced solid tumors resulting from defective vascularization and a subsequent insufficient oxygen supply, is considered one of the hallmarks of the TME (22). It is now well established that hypoxia contributes to malignant progression in cancer by inducing an invasive and metastatic phenotype of tumor cells and by activating resistance mechanisms to different anti-cancer therapies (23). Extensive efforts have been made in recent years to identify these mechanisms. We review here how the local microenvironment, in the particular context of hypoxia, impacts NK cell responsiveness and shapes the anti-tumor response (**Figure 1**).

TUMOR-DERIVED FACTORS CREATE AN IMMUNOSUPPRESSIVE MICROENVIRONMENT FOR NK CELL FUNCTIONS

IMPAIRMENT OF NK CELL FUNCTION BY CELLS FROM THE TUMOR MICROENVIRONMENT

Hypoxic tumor cells have the ability to activate resistance mechanisms to create an immunosuppressive microenvironment. Indeed, through their ability to produce cytokines such as tumor necrosis factor (TNF)-α and stromal cell-derived factor 1 (SDF-1), hypoxic tumor cells induce the homing of bone marrow-derived CD45+ myeloid cells to tumor areas (24). The invasion of myeloid cells in the TME is reported to be a highly immunosuppressive factor for NK cells (25). Myeloid-derived suppressor cells (MDSCs) are one of the major components of the immune-suppressive network responsible for the impairment of NK cell- and T cell-dependent anti-cancer immunity (26). The immunosuppressive function of MDSCs is related to their production of IL-10 that decreases the production by macrophages of IL-12, a pro-inflammatory cytokine involved in the activation of NK cells (27). It has also been shown that cancer-expanded MDSCs induce anergy of NK cells by inhibiting cytotoxicity, NKG2D expression, and IFN-γ production through membrane-bound transforming growth factor (TGF)-β (28). Furthermore, it has been demonstrated that hypoxia, via the induction of hypoxia-inducible factor (HIF) 1-α in MDSCs, is responsible for their differentiation to tumor-associated macrophages (TAMs) (29). Although macrophages contribute to tumor cell death in the early immune response to neoplasia, their presence in the TME correlates with a poor prognosis for patients with advanced stages of cancer (30, 31).

Macrophages constitute another major myeloid component of the infiltrated tumors and can comprise up to 80% of the cell mass in breast carcinoma (32). Hypoxic tumor secrete chemoattractants [e.g., colony-stimulating factor (CSF)-1, CC chemokine ligands (CCL) 2 and 5], resulting in the recruitment of monocytes from the blood to the tumor site. Infiltrated monocytes differentiate into CD206+ TAMs and accumulate in hypoxic areas of endometrial, breast, prostate, and ovarian cancers (30). This process is driven by tumor-secreted molecules such as endothelial monocyte-activating polypeptide (EMAP) II, endothelin 2, and vascular endothelial growth factor (VEGF) and also by the inhibition of the CC chemokine receptors (CCRs) 5 and 2 expression (33). Exposure of TAMs to tumor-derived cytokines such as IL-4 and IL-10 converts the TAMs into polarized type II or M2 macrophages owing to the immunosuppressive and pro-angiogenic activities. Subsequently, M2 macrophages establish an environment that skews CD4+ and CD8+ T cell immunity toward a tumor-promoting type 2 response (34). It has been also demonstrated that hypoxia upregulates the expression of the matrix metalloproteinase (MMP)-7 protein on the TAM surface, leading to the cleavage of Fas ligand from neighboring cancer cells, making them less responsive to NK cells and T cell-mediated lysis (35) (**Figure 2**).

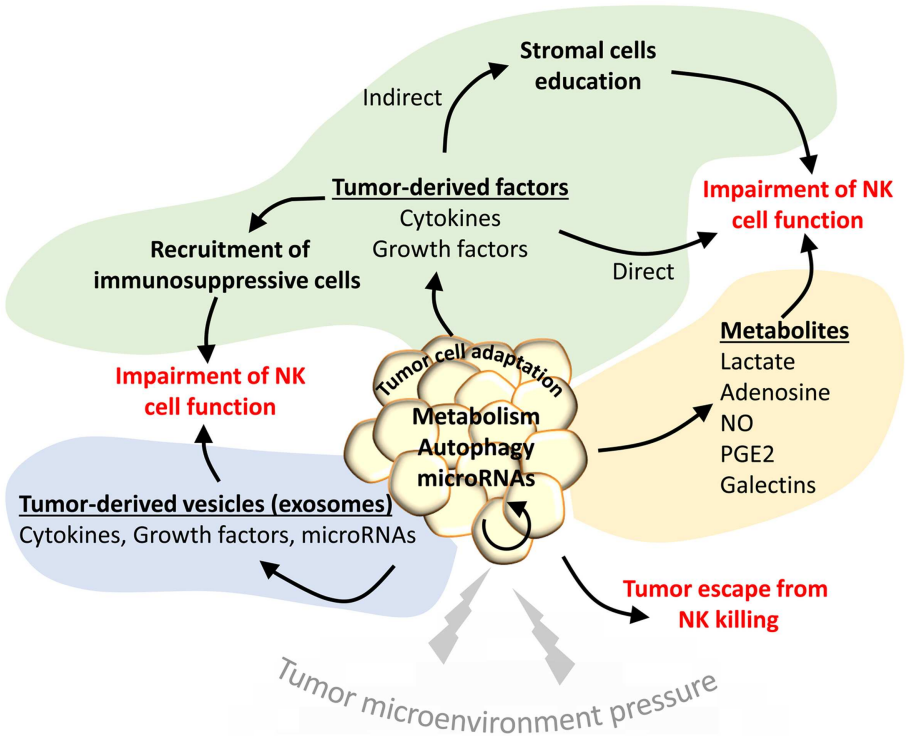

FIGURE 1 | The tumor microenvironment activates different mechanisms to impair the NK-mediated anti-tumor immunity. Under the pressure of the tumor microenvironment (TME), tumor cells adapt to such stress by activating intrinsic resistance mechanisms (autophagy) or by regulating their metabolism. Such regulation leads to the secretion of several metabolites that impair the function of NK cells in the tumor site (yellow area). Tumor cells under stress conditions may activate the release of tumor-derived vesicles containing cytokines, growth factors, or microRNAs to directly impact the NK functions (blue area). Such factors can be secreted directly in the TME to recruit immunosuppressive cells or to educate stromal cells involved in the impairment of NK cell functions (green area).

Recently, a link between tumor hypoxia and immune tolerance to NK cells through the recruitment of regulatory T (Treg) cells has been established. Hypoxia induces secretion of the immunosuppressive cytokine TGF-β from gastric cancer cells, which subsequently induces the proliferation and the accumulation of Treg cells in the TME (36). Moreover, human Treg cells induce anergy of NK cells through membrane-bound TGF-β and subsequently downregulate the activating receptor NKG2D on the surface of NK cells (37).

The immunosuppressive microenvironment can also be created through the ability of cancer cells to activate cancer-associated fibroblasts (CAFs) via the release of TGF-β or IL-6 (38, 39). CAFs have been shown to sharply interfere with NK cells cytotoxicity and cytokine production. Notably, it has been reported that CAFs are able to inhibit the IL-2-induced upregulation of the activating receptors NKp44, NKp30, and DNAX accessory molecule-1 (DNAM-1) at the NK cell surface. NKp44 and NKp30 expression is modulated by prostaglandin E2 (PGE2) released from CAFs, while DNAM-1 regulation requires cell-to-cell interaction. Such inhibition results in impaired NK cell-mediated killing of melanoma target cells (40). Likewise, CAFs directly impact cells of the TME and/or attract additional cells to the tumor site by secreting numerous factors including IL-6, TGF-β, VEGF, SDF-1, CXCL1/2, and IL-1β (41) (**Figure 2**).

Other mechanisms implicated in the establishment of immunesuppressive microenvironment are the expression of the immune checkpoint receptors, cytotoxic T-lymphocyte antigen (CTLA)-4, and the programed death receptor (PD)-1. Such receptors appear to play important roles in anti-tumor immunity and have been most actively studied in the context of clinical cancer immunotherapy. However, the effect of the TME on their regulation is poorly investigated. Nevertheless, the TME has been shown to mediate the induction of the PD-1 pathway (42). In line with this observation, NK cells from MM patients express PD-1, whereas normal NK cells do not. Anti-PD-1 antibody-based therapy enhances human NK cell function against autologous primary MM cells (43), highlighting the role of the PD-1/PD-L1 signaling axis in NK-mediated immune response against tumors. There is no direct evidence so far linking hypoxia and the induction of CTLA-4 expression and the PD-1/PD-L1 pathway. Further investigations are required to determine the precise role of the TME in the regulation of CTLA-4 and the PD-1/PD-L1 pathways.

INHIBITION OF NK CELLS BY TUMOR CELL-DERIVED FACTORS

The MHC class I chain-related (MIC) molecules, MICA and MICB, as well as the UL16-binding proteins (ULBPs), expressed on the surface of a broad range of carcinomas and some

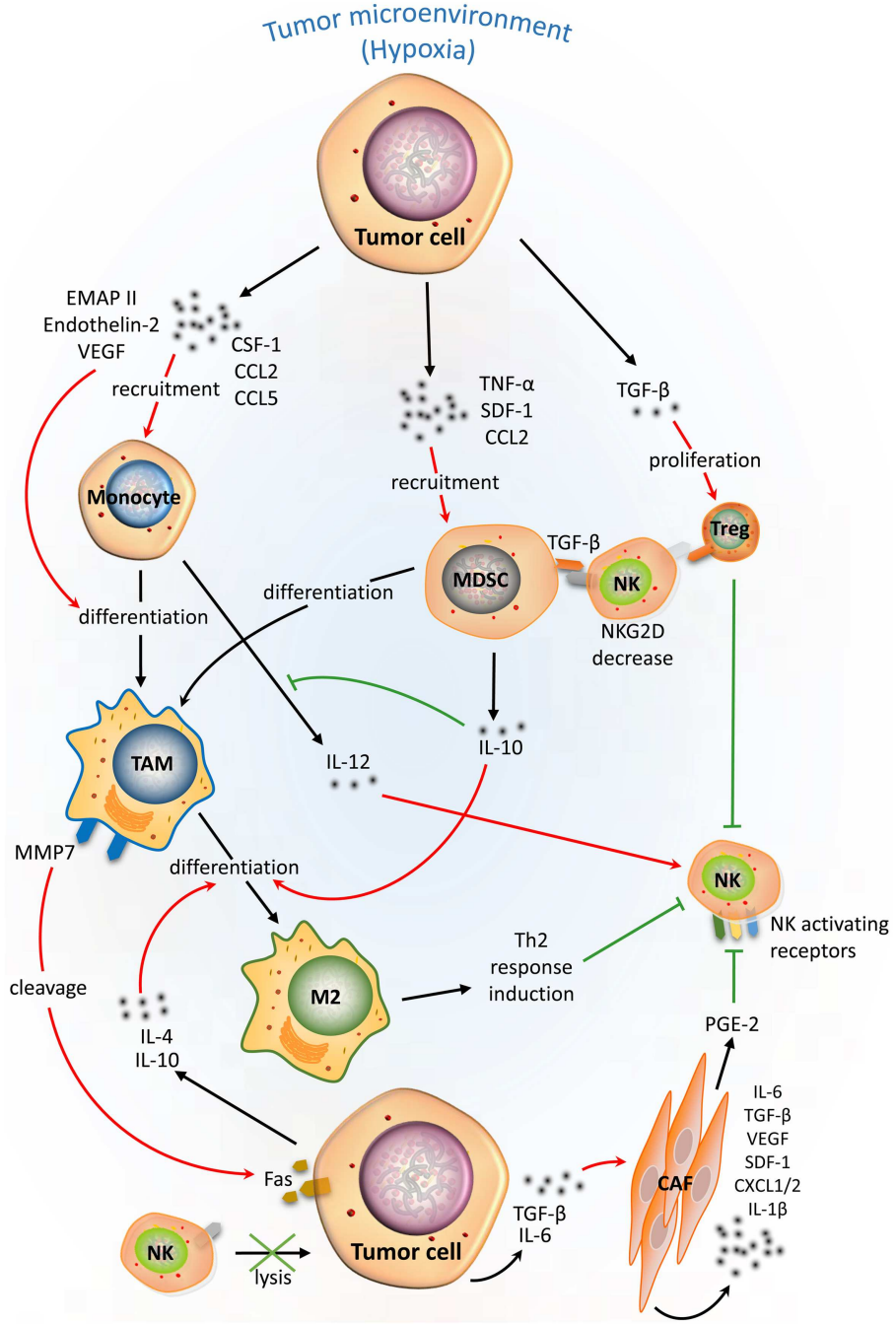

FIGURE 2 | Complex cellular interplay within the hypoxic tumor microenvironment inhibits NK-mediated killing. Tumor cells in a hypoxic tumor microenvironment (TME) secrete soluble factors that educate immune cells [e.g., monocytes, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Treg)], and stromal cells such as cancer-associated fibroblasts (CAFs). This scheme summarizes the effects of tumor-derived soluble factors on recruitment, differentiation, proliferation, and activation of tumor-associated cells (red arrows) in the hypoxic TME and their immunosuppressive activities (green lines) on NK-mediated lysis of tumor cells.

hematopoietic malignancies, play an important role in tumor surveillance by NK cells. The interaction of cell surface MIC molecules with NKG2D receptors on NK cells is critical to activate target cell killing. In this context, hypoxia has been reported to increase MICA shedding from the surface of cancer cells through the impairment of nitric oxide (NO) signaling and therefore affect the NK-mediated killing of target cells. Soluble MIC leads to a downregulated expression of NKG2D and CXC chemokine receptor (CXCR) 1 on the NK cell surface (44). This mechanism involves the HIF-1α-dependent upregulation of A disintegrin

and metalloproteinase domain-containing protein (ADAM) 10, which subsequently decreases the level of MICA on the tumor cell membrane (44, 45) (**Figure 3**).

In addition, hypoxic stress can induce the formation of dimers of the non-classical MHC class I molecule HLA-G at the surface of melanoma cells, thereby protecting tumor cells from NK-mediated killing. It appears that such induction is mediated by secretion of IFN-β and -γ and by direct interaction of HLA-G with NK cells (46).

Inhibiting the expression of activating NK cell receptors, including NKp30, NKp44, and NKG2D, has been shown to impair NK cell-mediated cytolytic activity in a model of melanoma (47). Although NK cells in the TME adapt and survive hypoxic stress by upregulating HIF-1α, they lose the ability to upregulate the surface expression of NKp46, NKp30, NKp44, and NKG2D receptors in response to IL-2 or other activating cytokines (e.g., IL-15, IL-12, and IL-21). However, it is important to note that hypoxia does not significantly alter the surface density and the function of the Fc-γ receptor CD16, thus allowing NK cells to maintain their capability of killing target cells via ADCC (48).

In addition to solid tumors, the immunosuppressive effect of the hypoxic TME has been also described in MM cells as hypoxia reduced NK cell killing of MM cell lines despite an unchanged NK cell degranulation level. In addition, hypoxia did not alter the surface expression of NK cell ligands (HLA-ABC and -E, MICA/B, and ULBP1-2) and receptors [killer cell Ig-like receptors (KIR), NKG2A/C, DNAM-1, natural cytotoxicity receptors (NCR), and 2B4], but decreased the expression of the activating NKG2D receptor and intracellular level of perforin and granzyme B. Pre-activation of NK cells by IL-2 removed the detrimental effects of hypoxia and increased NKG2D expression (49).

It is now well documented that the killing capacity of NK cells can be potentiated by cytokines such as IFN-γ and IL-2 (50, 51). Besides its effect of damping the cytotoxic activity of NK cells, hypoxia substantially decreases the ability of NK cells to be activated by IFN-γ through a mechanism that is not fully understood (52). Overall, it appears that manipulation of the TME will be an important consideration in achieving optimal NK-mediated, anti-tumor responses.

Since NKG2D ligand mRNAs are expressed in normal tissues, it has been proposed that their expression might be regulated at the post-transcriptional level by microRNAs (miRNAs) (53). Indeed, a subset of endogenous cellular miRNAs is proposed to repress MICA and MICB by targeting their 3′ UTR regions (54). Upon stress induction, the increase in MICA and MICB transcription might exceed the inhibitory function of miRNAs, whose expression remains constant, and result in an overexpression of MICA and MICB. Interestingly, among this subset of miRNAs, miR-17-5p, miR-20a, miR-93, miR-106b, miR-372, miR-373, and miR-520 have been shown to be overexpressed in various tumors and be involved in tumor progression and invasion. Therefore, a new function of these miRNAs has been proposed in the impairment of the immune response through the regulation of MICA and MICB expression (**Figure 1**). Based on these observations, a "miRNA-based immunoevasion" model has been described that highlights intracellular cancer-associated miRNAs as important factors able to impair immune recognition through the targeting of NK ligands (54). Furthermore, miR-10b, an important "metastamir," has been described to downregulate MICB and decrease the NKG2D-dependent cytotoxicity of NK cells (55). MiR-520b, an IFN-γ-induced miRNA, has been described to regulate MICA expression at both the transcriptional and post-transcriptional levels (56). It has also been proposed that viruses can take advantage of miRNA-based immunoevasion. Indeed, the hcmv-miR-UL112 encoded by the human cytomegalovirus impairs NK cell function during viral infection through the modulation of MICB expression (57). In addition, hcmv-miR-UL112 acts synergistically with the cellular miR-376a to induce escape from NK-mediated immune elimination (58). Together, these studies highlight the importance of miRNAs in the regulation of NKG2D ligand expression and tumor immune surveillance. Whether the expression of such miRNAs is regulated by hypoxia in the TME remains to be investigated.

TUMOR MICROENVIRONMENT-DEPENDENT MODULATION OF CANCER CELL METABOLISM AFFECTS NK CELL FUNCTIONS

Through the sensing of oxygen level and/or the transcriptional activity of HIF-1α, hypoxia plays a key role in the reprograming of cancer cell metabolism. Indeed, reduced O_2 availability induces HIF-1α, which regulates the transcription of a set of genes that encode proteins involved in various aspects of cancer biology (59). A well-known example is the shift of glucose and energy metabolism from oxidative to glycolytic metabolism that allows for the maintenance of redox homeostasis under conditions of prolonged

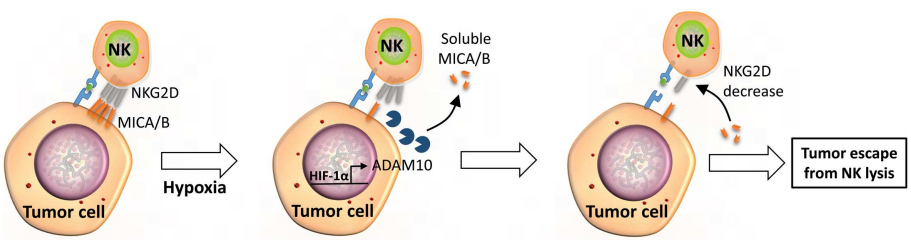

FIGURE 3 | Soluble MICA/B regulate NKG2D receptors on the surface of NK cells. Under hypoxic stress, tumor cells activate expression through HIF-1α and the release of ADAM10. Released ADAM10 cleaves MICA/B ligands on the surface of tumor cells and soluble MICA/B downregulates the expression of NKG2D on the surface of NK cells, leading to tumor escape from NK-mediated killing.

hypoxia (60). The effects of such metabolic adaptations evolved by hypoxic cancer cells have received particular attention in the establishment of immune tolerance. In this section, we will focus on the mechanisms involved in tumor metabolism adaptation that participate in shaping the NK cell anti-tumor response within a hypoxic microenvironment (**Figure 1**).

LACTATE

To adapt to oxygen deprivation, hypoxic cancer cells undergo a dramatic alteration of cellular glucose metabolism characterized by a high glycolytic activity. HIF-1α plays a central role in this metabolic switch by inducing the expression of multiple genes involved in glucose uptake (glucose transporters-1 and -3) and metabolism (i.e., hexokinases-1 and -2 and lactate dehydrogenase A) (61). In addition, HIF-1α regulates the expression of monocarboxylate transporter 4 and pyruvate dehydrogenase kinase 1, thereby inhibiting the conversion of pyruvate to acetyl CoA (62). The accumulation of pyruvate in cells prevents its metabolism through the tricarboxylic acid cycle in mitochondria. Pyruvate is subsequently reduced to lactate and finally released from the tumor cells. It has been recently reported that cancer cells escape immune response through the release of lactate in the microenvironment and the presence of a low extracellular pH, as a consequence of the "Warburg effect" induced under hypoxia. *In vivo* and *in vitro* evidence has been provided indicating that tumor-derived lactate directly and indirectly alters NK cell functions. The direct effect involves the impairment of the cytolytic activity of NK cells by downregulating NKp46 expression and reducing perforin/granzyme B production. Moreover, lactate affects the NK-mediated killing indirectly through the increased MDSCs generation from mouse bone marrow, thus creating an immunosuppressive microenvironment. Interestingly, these immunosuppressive effects were efficiently reverted in a lactate dehydrogenase A-depleted cancer model (63).

ADENOSINE

Hypoxia-driven accumulation of adenosine in the TME has been identified as another mechanism for immune modulation (64). It has been reported that the concentration of adenosine in the extracellular fluid of solid carcinomas may be increased up to 20-fold compared with normal tissues (65). The accumulation of adenosine is sustained, at least in part, by the hypoxia-mediated modulation of enzymes implicated in adenosine metabolism (i.e., adenosine kinase, endo-5′-nucleotidase). Moreover, the additional generation of extracellular adenosine from extracellular ATP occurs through the sequential enzymatic activity of the membrane-bound nucleotidases CD39 and CD73. It has been shown that CD73, involved in the dephosphorylation of AMP to adenosine, is upregulated by HIF-1α (66, 67). Once released in the extracellular environment, adenosine exerts various immunomodulatory effects via binding on adenosine receptors (i.e., A1, A2A, A2B, and A3) expressed on multiple immune subsets including NK cells.

In contrast to other immune cells such as macrophages and neutrophils, the effect of extracellular adenosine on NK cells is not fully known. Adenosine has been shown to inhibit TNF-α release from IL-2-stimulated NK cells and suppress their proliferation (68). Another study reported that adenosine inhibits cytotoxic granules exocytosis from murine NK cells via binding to an unidentified adenosine receptor (69). More recently, data support the fact that adenosine and its stable analog 2-chloroadenosine inhibit perforin- and Fas ligand-mediated cytotoxic activity as well as cytokines production (i.e., IFN-γ, macrophage inflammatory protein 1-α, TNF-α, and granulocyte-macrophage CSF) from activated NK cells. These inhibitory effects occur through the stimulation of the cyclic AMP/protein kinase A pathway following the binding of adenosine to A2A receptors on NK cells (70, 71). In this context, targeting the CD73-adenosine pathway has recently emerged as a potential clinical strategy for immunotherapy (66). *In vitro* data revealed that the inhibition of the CD39, CD73, or A2A adenosine receptor by siRNA, shRNA, or specific inhibitors resulted in a significant improvement of NK cell lytic activity against ovarian cancer cells (72). Furthermore, *in vivo* blocking of the A2A adenosine receptor enhanced NK cell activity in a perforin-dependent manner and reduced metastasis of CD73-overexpressing breast cancer cells (73).

As multiple immune competent cells express adenosine receptors, an additional level of immunomodulatory activity, via adenosine, needs to be considered. For example, several studies reported that adenosine interaction with other immune subsets impairs the cytotoxic activity, the pro-inflammatory cytokines production, and the proliferation of T cells. In addition, adenosine impairs the recruitment and the immunosuppressive activity of MDSCs in tumors, as well as the migration and the immunosuppressive function of Treg cells into the TME (74). Taken together, by sustaining the immunoregulatory activity of extracellular adenosine, all the mechanisms described above collaborate to impair the anti-tumor NK-mediated immunity.

NITRIC OXIDE

Accumulating evidence suggests that the exposure of cells to low oxygen levels results in a marked inhibition of NO production (75). NO is produced from L-arginine in a reaction catalyzed by the NO synthase (NOS) enzymes, in which oxygen is a required cofactor. Hypoxia has also been shown to increase arginase activity, thereby redirecting L-arginine into the urea cycle, away from the NO generation pathway (76). Siemens et al. provided evidence that hypoxia-mediated impairment of NO signaling in tumor cells contributes to tumor escape from NK immunosurveillance. They demonstrated that hypoxia-mediated shedding of MIC occurs through a mechanism involving impaired NO signaling in human prostate cancer. Such shedding can be blocked after reactivating NO signaling by the administration of NO mimetic agents (45). This work suggests that reactivation of NO could help to overcome hypoxia-driven tumor escape.

PROSTAGLANDIN E2

Several lines of evidence suggest that the deregulation of the cyclooxygenase (COX)-2/PGE2 pathway is a key factor in tumor evasion of the immune response (77). COX enzymes catalyze the formation of prostaglandins from arachidonic acid following sequential oxidation. Interestingly, COX-2 can be overexpressed in both adenoma and carcinoma cells under hypoxia via a mechanism dependent on HIF-1α. This upregulation is associated with PGE2 overproduction and secretion in the microenvironment (78). Early

studies showed that PGE2 suppresses the cytolytic activity of NK cells (79, 80) by a mechanism related to the inhibition of IFN-γ production (81, 82). Recently, Pietra et al. have shown that melanoma cells affect the function of NK cells by downregulating the surface expression of activating receptors, including NKp30, NKp44, and NKG2D. This impairment appears to be related, at least in part, to PGE2 production by melanoma cells as PGE2-specific inhibitor-restored NK cell functions (47). In addition to its direct effect on NK cells, more recent data reported that PGE2 can indirectly affect the NK cell function by promoting the establishment of an immunosuppressive microenvironment through the induction of Treg cells (83), macrophages (84), and MDSCs (27, 85) development.

GALECTINS

Galectins (Gal) are proteins belonging to the lectins family that participate in the delivery of signals after binding to glycoproteins and glycolipids on the cell surface of target cells. Using a proteomic approach, Le et al. have identified Gal-1 as a novel hypoxia-regulated protein (86). They proposed that tumor aggressiveness of HNSCC is dependent on hypoxia-mediated production and the secretion of Gal-1, which in turn negatively regulates the anti-tumor immune response. Additional studies have supported the contribution of Gal-1 in creating an immunosuppressive microenvironment at the sites of tumor growth by several mechanisms (87). Thus, it has been reported that recombinant Gal-1 is able to promote the differentiation of CD4+CD25+ Treg cells *in vitro* (88). Recently, Dalotto-Moreno et al. showed that tumor-derived Gal-1 increases the abundance and/or the expansion of peripheral Treg cells *in vivo* and modulates their suppressive capacity. Conversely, attenuation of Gal-1 reduces the frequency of Treg cells within tumors, lymph nodes, and spleen and removes the immunosuppressive function of Treg cells (89). More recently, Gal-3, another member of the galectin family regulated by HIF-1α (90), was reported to exert an immunosuppressive function in the TME. Tsuboi et al. provided evidence that cell surface Gal-3 on bladder tumor cells modulates MICA-NKG2D interactions by binding MICA through poly-*N*-acetyllactosamine, thereby severely impairing the NK cell activation and degranulation (91). The effect of Gal-9 is still debated as it may regulate both positively and negatively the NK cell response depending on the activation threshold and the expression of its receptor. Gleason et al. have shown that Gal-9 binding to the immune receptor T cell Ig and mucin-containing domain-3 (Tim-3) enhances the production of IFN-γ by NK cells (92). Conversely, higher doses of Gal-9 impair the cytotoxic function of NK cells in a Tim-3 independent manner (93).

REGULATION OF NK CELL-MEDIATED KILLING BY AUTOPHAGY

It has become increasingly clear that tumor cells activate key biochemical and cellular pathways under hypoxic stress that are important for tumor progression, survival, and metastasis. Several recent reports highlight autophagy as a critical process that modulates the anti-tumor immune response. Briefly, autophagy is a catabolic process in which a cell self-digests its own components. Autophagy can be activated in response to multiple stressors including hypoxia, nutrient starvation, growth factor withdrawal, and endoplasmic reticulum stress. Under stressful stimuli, autophagy activation serves as an adaptive response to provide nutrients and prevents accumulation of altered cell components (94).

To adapt to hypoxia, cells activate autophagy through both HIF-1α dependent and independent pathways, depending on the sensor activated (95). The role of autophagy in cancer immunity seems to be complex as hypoxia-induced autophagy occurs in target cells and in tumor-infiltrating immune cells. Although the role of autophagy induction in target cells is well documented, relatively little attention has been given to its role in immune cells. Therefore, understanding how autophagy modulates the tumor immune response represents a major challenge in the field of tumor immunotherapy. Recently, it has been reported that NK cells not only provide lytic signals to their target cancer cells, but also promote autophagy in the remaining un-killed target cells. Moreover, the NK-mediated autophagy induction in target cells was enhanced by provision of IL-2 and cell-cell interactions between NK cells and tumor cells. This study highlights autophagy induction in target cells as a cell mechanism of resistance to NK-mediated killing (96). More recently, we showed *in vitro* and *in vivo* that targeting autophagy under hypoxia restores NK-mediated lysis in breast cancer cells. In addition, we provided mechanistic evidence that the activation of autophagy under hypoxia led to the degradation of NK-derived granzyme B, making hypoxic tumor cells less sensitive to NK-mediated killing (**Figure 4**) (97).

TUMOR-DERIVED EXTRACELLULAR VESICLES INFLUENCE NK CELL ACTIVITY

Recent advances have led to the identification of an additional mechanism used by tumor cells to escape NK cell recognition and impair the NK-mediated immune response (98). Indeed, tumor cells release vesicle-bound molecules (cytokines, NKG2D ligands, and miRNAs) targeting and inhibiting NK cell functions (99).

Exosomes are 50–150 nm membrane vesicles derived from the multi-vesicular bodies that are secreted by all cell types [reviewed in Ref. (100)]. As a consequence, exosomes are found in many biological fluids such as urine, plasma, and saliva. As their content reflects the cells from which they are derived, exosomes represent, therefore, attractive biomarkers (101). Exosomes and other types of extracellular vesicles are well-known mediators of intercellular communication and play a crucial role in the development of aggressive and metastatic tumors (102, 103).

CANCER CELL-DERIVED EXOSOMES

The production of NKG2D ligand-bearing exosomes has been proposed as a mechanism for tumor cell escape from immune recognition (99, 104, 105). Indeed, it has been demonstrated that, in contrast to ULPB2, released ULBP3 is included into exosomes. Remarkably, ULBP3-containing exosomes have been shown to be more potent downregulators of the NKG2D receptor than the soluble form of ULBP2 proteins released by the metalloproteinases ADAM10 and 17. Pre-incubation of NK cells with ULBP3-containing exosomes induced a dramatic reduction of NKG2D-mediated lysis of MICA-expressing cells (106). Tumor-derived exosomes (TDEs) are rapidly taken up by NK cells and

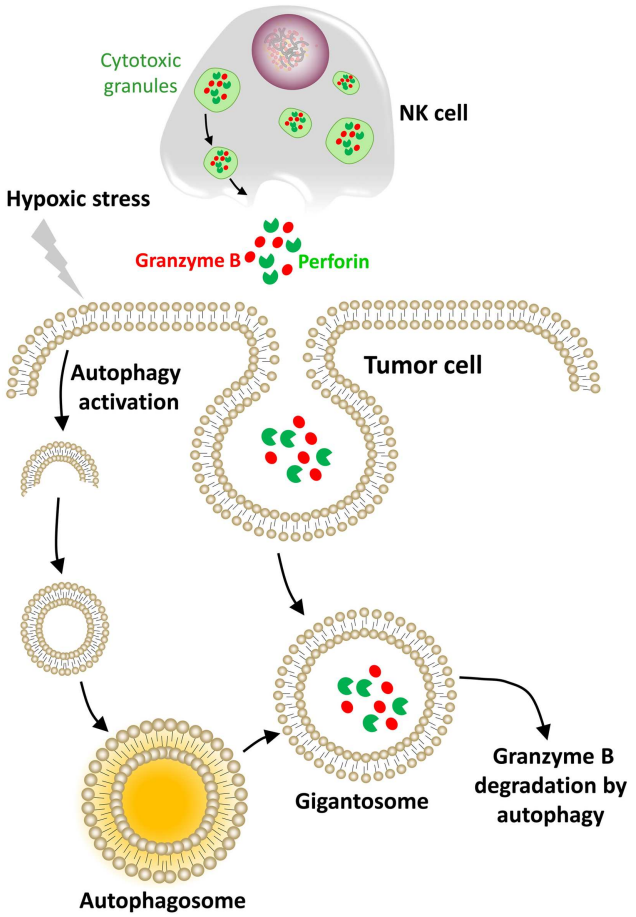

FIGURE 4 | Hypoxic stress activates autophagy in tumor cells as an intrinsic resistance mechanism to NK-mediated killing. In our model, the cytolytic effectors perforin and granzyme B enter the target cells by endocytosis and then are found in enlarged endosomes called "gigantosomes." In hypoxic cells, the activation of autophagy leads to the formation of autophagosomes that fuse with "gigantosomes" to form amphisomes. The fusion between amphisomes and lysosomes selectively degrades granzyme B in this compartment, making hypoxic tumor cells less sensitive to NK-mediated lysis.

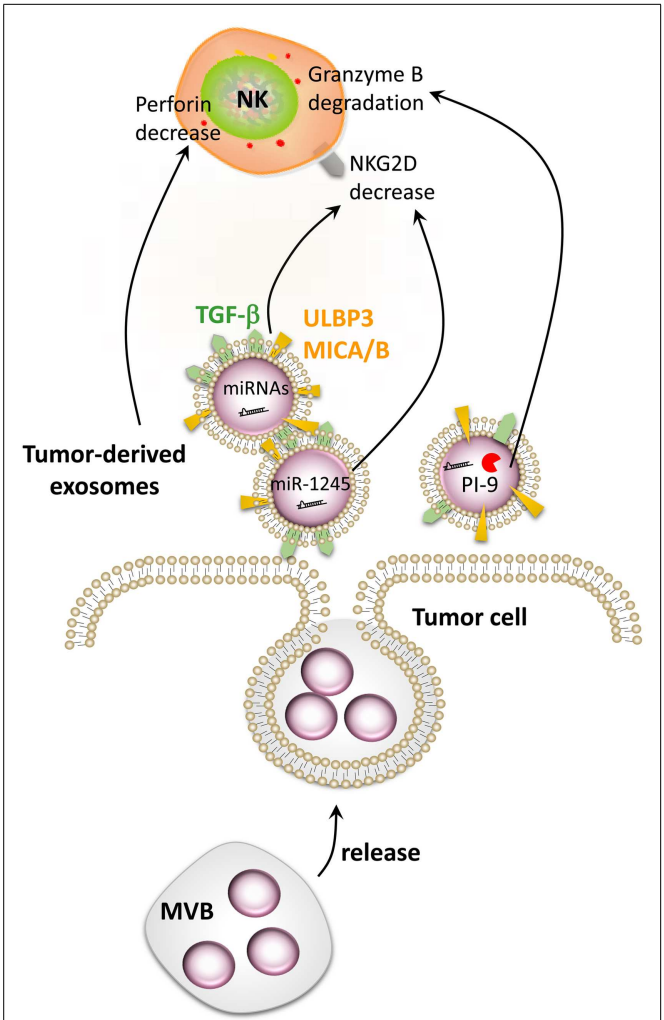

FIGURE 5 | Impairment of NK cell function by tumor-derived exosomes. Tumor cells secrete extracellular vesicles called exosomes. Tumor-derived exosomes contain numerous factors able to modulate the function of NK cells such as MICA/B, ULBP3, TGF-β, PI-9, and different microRNAs. Exosome-derived MICA/B, ULBP3, TGF-β, and miR-1245 can decrease NKG2D on the surface of NK cells, while PI-9 degrades granzyme B. Tumor-derived exosomes can also decrease the level of perforin in NK cells by a still-unknown mechanism.

remain stable for 48 h (104, 107). The transfer of TDE-bearing, membrane-anchored TGF-β, MICA, and MICB leads to the down-regulation of NKG2D expression at the surface of NK cells and impairs their cytotoxic functions (**Figure 5**) (99, 108). However, TDEs can only weakly impair the NK cell proliferation compared with their strong negative effect on the proliferation of CD8+ T cells (109). Nevertheless, numerous studies highlighted TGF-β as a major immunosuppressive molecule for NK cells (108, 110, 111). Indeed, an elevated plasma level of TGF-β was detected in lung or colorectal cancer patients compared with healthy volunteers. This increase inversely correlated with NKG2D surface expression on NK cells in these patients (110). Recently, TGF-β was shown to block NK cell activation by repressing gene expression and antagonizing IL-15-induced proliferation (111). A striking observation was also done by Clayton et al. who identified exosomal TGF-β1 as a more potent contributor to antiproliferative effects than the soluble form (109).

Several cancer models have generated evidence supporting the important roles of TDEs. Indeed, mammary carcinoma exosomes promote tumor growth by suppressing NK cell function in mice (104). A decrease in splenic NK cell cytotoxicity was observed after *in vivo* injection of TDEs. Moreover, a reduction in the number and the percentage of NK cells was observed in the lungs 3 days after exosome injection, without a reduction in the viability of the NK cells. Interestingly, TDEs also reduced the expression of the NK pore-forming and cytolytic protein perforin (**Figure 5**) (104, 111), whereas the level of granzyme B was unaffected (104). A decrease in NK cell proliferation in response to IL-2 was also

observed after treatment with exosomes derived from different tumor cell types (breast and melanoma) due to the inhibition of the JAK-STAT signaling. However, TDEs did not affect DC maturation but hampered their ability to stimulate the immune response (104). The granzyme B-inhibitory serpin proteinase inhibitor-9 (PI-9) has also been identified inside exosomes (112) and could also play an important role in the resistance of tumor cells to NK cells (**Figure 5**). Taken together, these data highlight the crucial role that TDEs may have on the tumor immunosurveillance by affecting the NK cell receptors, proliferation, and release of cytotoxic molecules, thus impairing an effective anti-cancer immune response.

Numerous studies have provided evidence that hypoxic stress may influence the composition of TDEs. Indeed, to substitute oxygen deprivation and a lack of nutrients, tumor cells induce the expression of angiogenic factors to overcome hypoxic stress through the formation of new blood vessels from existing vasculature. In addition to secreted VEGF, several chemokines (G-CSF, GM-CSF, CXCL16, and SDF-1) and exosomes were shown to be important mediators for tumor cells to overcome hypoxic stress (102). In this context, it has been reported that tumor cells under hypoxic stress secrete numerous proteins sequestered in exosomes involved in cell–cell communication, cell growth, and malignant transformation. Other studies have focused on how hypoxia-induced membrane vesicles stimulate angiogenesis in malignant and angiogenic brain tumor glioblastoma multiforme (GBM). Indeed, hypoxic cancer cells release exosomes containing tissue factor (TF) acting on surrounding endothelial cells in a paracrine manner, leading to the activation of a protease-activated receptor 2 (PAR2)-ERK signaling pathway (113). PAR2 has been recently identified as a regulator of the innate immune response and a mediator of cell proliferation and migration. Also called thromboplastin, TF forms a complex with the tissue protease factor VIIa and is necessary for the initiation of thrombin formation. Because hypoxic tumors are often characterized by endothelial cell hyperplasia and hypercoagulation, the combined presence of newly generated fibrin and activated platelets has been shown to protect the tumor from NK cells and immune surveillance (114). Further findings obtained with GBM cells indicates that hypoxic conditions stimulated tumor cells to generate exosomes containing proteins that reflect the hypoxic status of the tumor cells. These findings support the hypothesis that the microenvironment significantly impacts the TDE composition. The enrichment in exosomes of specific hypoxia-related RNAs and proteins (cytokines, growth factors, and MMP) could indeed be associated with a poor patient prognosis. In addition, hypoxic TDEs mediated a strong paracrine stimulation of angiogenesis and activation of cancer cells, leading to an acceleration of tumor growth in a mouse xenograft model (115). TDEs systematically contain several members of the ADAM family, mostly ADAM10 (107), which is able to shed NKG2D ligands from the cell membrane (116). Finally, besides stimulating the production of exosomes with a specific content, hypoxia has also been shown to enhance exosome release by cancer cells (92).

Besides solid tumors, circulating tumor cells, such as leukemic cells, escape NK surveillance at a systematic level in blood. It is important to note that leukemic cells are constantly recirculating in the bone marrow, where the environment is maintained in constant hypoxia (117). Recent studies have shed light on mechanisms of tumor cell escape from NK-mediated killing that could be used as new therapeutic approaches. These mechanisms include the shedding of soluble (BAG6, and MICA) or exosome-derived inhibitory molecules (TGF-β) in various malignancies such as acute myeloid leukemia (118), chronic lymphocytic leukemia (119), and Hodgkin's lymphoma (119).

SECRETED microRNAs

As described above, under hypoxic conditions, most cell types undergo important metabolic changes orchestrated by members of the HIF transcription factor family. It is well documented that HIF-1α is a potent inducer of miR-210 (120), which has been described to be released by tumor cells (121, 122). It has been shown that miR-210 released by leukemic and metastatic cancer cells may be transported by exosomes and enter endothelial cells (121, 122). In the recipient cells, miR-210 is able to induce angiogenesis and promote tumor growth. These data highlight the role of exosomal miR-210 in the shaping of the TME and the potential action on various cell types present at the tumor site. Although the data available are limited, we believe that exogenous miRNAs can impair the anti-tumor function of immune cells (**Figure 5**). In line with this concept, it has been shown that the TGF-β1-induced miR-1245 downregulated the NKG2D receptor on NK cells and impaired NKG2D-mediated functions (123). The influence of exogenous miRNAs on NK cells is currently unknown but understanding this new regulatory mechanism may help to improve the outcome of NK-based immunotherapy.

CONCLUSION

Recent developments in cancer immunotherapies have now begun to explore the use of NK cells (15, 124). Particularly, strategies designed to improve NK-mediated killing using tumor-specific mAbs have shown promising results in preclinical and some clinical settings (125). This review has summarized the different mechanisms involved in the impairment of NK-mediated tumor killing and highlighted that the majority of these mechanisms likely evolve within the TME. In this regard, it should be emphasized that the composition and characteristics of the TME are important in determining the anti-tumor immune response. For example, different subsets of the immune system, including NK cells, DCs, and effector T cells, are capable of driving potent anti-tumor responses. However, the ability of tumor cells to exploit other cells present in the TME is now widely regarded as a critical factor that switches the immune response from a tumor-destructive profile to a tumor-promoting profile. Such a microenvironment may also favor the development of immunosuppressive populations of immune cells, such as MDSCs, TAMs, and Treg cells.

Despite recent advances in cancer immunotherapy, the therapeutic outcome was often disappointing in many clinical protocols. Given the important immunomodulatory effects of the TME, it stands to reason that it may represent a therapeutic target that can be manipulated to improve the anti-tumor immune response. Thus, the first clinical interventions that aim to target the microenvironment to enhance tumor immunity are under active evaluation.

Overall, investigations oriented toward the identification of novel therapeutic strategies, aiming to improve the anti-tumor immunotherapy, should pay closer attention to the TME to awake or reawake immune cells and/or to redirect such a microenvironment from a pro-tumor to an anti-tumor state. Given its central role in tumor progression and resistance to therapy, the hypoxic TME should be considered as a new critical therapeutic target in oncology. We believe that a better characterization of the TME can provide important prognostic and predictive values independent of the tumor phenotype.

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Human immunodeficiency syndromes affecting human natural killer cell cytolytic activity

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Natural killer (NK) cells are lymphocytes of the innate immune system that secrete cytokines upon activation and mediate the killing of tumor cells and virus-infected cells, especially those that escape the adaptive T cell response caused by the down regulation of MHC-I. The induction of cytotoxicity requires that NK cells contact target cells through adhesion receptors, and initiate activation signaling leading to increased adhesion and accumulation of F-actin at the NK cell cytotoxic synapse. Concurrently, lytic granules undergo minus-end directed movement and accumulate at the microtubule-organizing center through the interaction with microtubule motor proteins, followed by polarization of the lethal cargo toward the target cell. Ultimately, myosin-dependent movement of the lytic granules toward the NK cell plasma membrane through F-actin channels, along with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor-dependent fusion, promotes the release of the lytic granule contents into the cleft between the NK cell and target cell resulting in target cell killing. Herein, we will discuss several disease-causing mutations in primary immunodeficiency syndromes and how they impact NK cell-mediated killing by disrupting distinct steps of this tightly regulated process.

Keywords: NK cells, primary immunodeficiency, NK cell cytotoxicity, cytotoxic lymphocytes, cytotoxic synapse, lytic granules

INTRODUCTION

Natural killer (NK) cells comprise 5–15% of human peripheral blood lymphocytes and play an important role in the clearance of virally infected cells, as well as the elimination of cancer cells (1, 2). The main population of NK cells differentiates from hematopoietic stem cells in the bone marrow, and fully matures as $CD56^{+}CD16^{+}$ NK cells in secondary lymphoid organs (3). While several recent studies have elegantly demonstrated the presence of a memory immune response in NK cells, previously only attributed to T and B cells of the adaptive immune system (4, 5), NK cells are generally recognized as innate immune cells by the fact that their receptors do not undergo DNA rearrangement processes. Instead NK cells express various germline-encoded activating and inhibitory NK receptors on their cell surface (6–8). Integrated signaling from both activating and inhibitory receptors is thought to enable NK cells to distinguish healthy "self" from infected or stressed unhealthy or "non-self" cells (9, 10). Therefore, activation of NK cells can be achieved via the ligation of a number of activating receptors with their corresponding ligand on the target cell (natural cytotoxicity). In addition, NK cells are also able to detect antibody-coated targets through the low affinity Fc receptor CD16 (FcγRIIIA) and thereby mediate antibody-dependent cellular cytotoxicity (ADCC) (11, 12). The main consequence of NK cell activation is the direct killing of bound unhealthy target cells through the release of perforin and granzymes from a preformed secretory lysosome known as the lytic granule (13, 14). In addition, NK cells can affect the overall immune response through the secretion of chemokines and cytokines (3, 15–18) as well as through direct interaction with other immune cells (2, 5).

Research conducted over the past quarter of a century has uncovered the process of NK cell development, identified and demonstrated the function of activating and inhibitory receptors, and provided a wealth of information regarding the signaling pathways that are linked to these receptors and the proteins that are critical to the delivery of the lethal cargo to the target cell. Significantly, the study of human primary immunodeficiency syndromes (PIDs) has provided invaluable knowledge and insight regarding the role of NK cells in the immune system, and in addition has also resulted in the identification of genes whose protein products regulate distinct steps that are critical to the development of cellular cytotoxicity by this population of innate immune cells. Among the PIDs, several NK cell deficiencies have been identified including rare isolated NK cell deficiencies, where there is total absence or very low number of NK cells in the peripheral blood, which is known as classical natural killer cell deficiency (CNKD) (19, 20). MCM4 and GATA2 are the only two genes identified thus far in which mutations are linked to CNKD, however there remain several cases of CNKD where the underlying genetic cause has not been identified (20). Clearly, sequencing of the genomes in patients with CNKD, which are not caused by mutation of either MCM4 or GATA2, will undoubtedly reveal other genes that play an important role in NK cell development or survival.

The term NK cell deficiency does not just mean an absence of NK cells in the immune system; it also includes the absence of specific NK cell effector functions, like cytotoxicity, despite the

presence of normal NK cell numbers. This has been termed functional NK cell deficiency (FNKD) (19, 20). FNKDs have provided us with very valuable insights into the roles of specific NK cell functions in immunity as well as essential proteins required for NK cell effector activities, when the responsible gene has been identified. So far, the only known human genetic mutation leading to FNKD is in the *FCGR3A* gene, which encodes CD16 (20, 21). Similar to patients of CNKD, patients carrying homozygous missense mutations in CD16 (L66H) showed susceptibility to human papilloma virus (HPV) or herpesviridae members. In contrast to CKND, these patients had normal numbers of NK cells, but unexpectedly showed normal ADCC, whereas natural cytotoxicity was defective (20). The fact that the altered amino acid found in these patients is located outside of the immunoglobulin domain (Ig domain) responsible for IgG binding (22) suggests why ADCC of NK cells from the patients is normal. In addition, novel co-stimulatory roles of CD16 mediated by the distal Ig domain of CD16 (23) provided important insights that might explain why the patients' NK cells showed defective natural cytotoxicity.

Lastly, there are several additional human PIDs that demonstrate defects in NK cell numbers and effector functions. Since many immune cells other than NK cells are also affected, there are additional complications and difficulties in understanding the complex immunological roles of NK cells in these diseases. However, the identification of specific gene mutations has illuminated molecular pathways that are important for NK cell development and effector functions, which are also shared in other immune cell types. In this review, we will specifically focus on PIDs where the mutated gene products impact the intracellular pathways that regulate the development of NK cell-mediated cytotoxicity (**Table 1**). For detailed discussions about human diseases involved in NK cell development and differentiation, NK cell signaling, or other NK cell effector functions, the reader is referred to other excellent reviews on these topics (19–21, 24).

BIOGENESIS OF LYTIC GRANULES AND THEIR MATURATION

Cell-mediated killing by NK cells and cytotoxic T lymphocytes (CTLs) is achieved by directed release of lytic granules toward bound target cells. Lytic granules are dual-function organelles that exhibit characteristics of a degradative lysosome, but can also secrete granule constituents for cell-mediated killing and are therefore often referred to as a secretory lysosome. This specialized organelle is mostly observed in hematopoietic lineage cells, but it is also found in melanocytes that produce pigment proteins called melanins (25, 26). Both secretory lysosomes and conventional lysosomes are acidic organelles (pH 5.1–5.4), morphologically similar, and contain proteins like acid hydrolases that are required for their degradative function as well as receptor proteins including members of the lysosomal-associated membrane protein (LAMP) family. On the other hand, the main distinction of secretory lysosomes from conventional lysosomes is that secretory lysosomes undergo a regulated secretion process and they contain additional contents that are cell-type-specific. In the case of NK cells and CTLs, these specific secreted contents include perforin and granzymes, which are essential for cytotoxic activity. On the other hand, the main secreted proteins of melanocytes are melanins, which are pigments responsible for skin color. Cells containing secretory lysosomes use common secretory machineries, but cell-type-specific secreted contents enable each cell type to perform different effector functions. This is why human genetic diseases such as Chediak–Higashi syndrome (CHS) and type 2 Hermansky–Pudlak syndrome (HPS), caused by impaired function of the secretory lysosome, are characterized not only by severe immune deficiencies including defective cytotoxicity by NK cells and CTLs but also by hypopigmentation due to defects in melanosome formation and excessive bleeding due to the absence of dense granules in platelets (26–28).

In contrast to CTLs, which need to be activated, the genes encoding perforin and granzymes are constitutively transcribed in NK cells thus allowing them to immediately kill an infected or stressed cell upon initial contact and without the need for additional gene expression (29, 30). Perforin is essential for NK cell-mediated cytotoxicity, since it is the only molecule that delivers apoptosis-inducing granzymes into the target cell. Perforin is initially synthesized as an inactive precursor in the ER and undergoes multiple posttranslational modifications including proteolysis and glycosylation during its transit to the Golgi and finally to the lytic granule (31). However, the exact pathway that sorts perforin from the *trans*-Golgi network (TGN) into lytic granules is unresolved. After arriving into lytic granules, perforin is further processed by cathepsin L to develop into a mature form. In the case of granzymes, five different granzymes, granzyme A, B, H, K, and M, are expressed in human NK cells and CTLs. They are synthesized as pro-enzymes and modified to have a mannose-6-phosphate moiety in the *cis*-Golgi, which facilitates their trafficking to the endosome by the mannose-6-phosphate receptor (M6PR) before finally arriving into the lytic granule (32, 33). In lytic granules, granzymes are further processed into an active form by various cathepsins (34–36). Granzymes are serine proteases and each granzyme has different substrate specificity and induces apoptosis in the target cell in both caspase-dependent and caspase-independent manners (37, 38).

An important question to be asked is: How do cytotoxic lymphocytes protect themselves from these dangerous molecules? Significantly, the activity of perforin is achieved by the membrane attack complex/perforin (MACPF) domain, which has cytolytic activity, and a C2 domain that enables binding to membrane in a calcium-dependent manner (37, 39, 40). Importantly, the low level of Ca^{2+} in lytic granules maintains perforin in an inactive conformation. In addition, calreticulin, another component of the lytic granule, inhibits perforin activity (41), and the acidic environment of lytic granules provides protection to NK cells since the activity of both perforin and granzymes are inhibited at this low pH. Furthermore, the low pH environment favors association of serglycin, a proteoglycan matrix found within lytic granules, with both perforin and granzymes. This interaction keeps both perforin and granzymes in an inactive state until they are secreted into a neutral pH environment (42). Interestingly, the mechanism by which perforin facilitates the transport of granzymes into the target cell is still unclear (37, 40). According to the prevailing model, perforin is released into the cytotoxic synaptic cleft where it binds calcium, oligomerizes on the target membrane, and forms pores. The high concentration of calcium as well as the neutral pH at the interface between NK cells and target cells relieves perforin from its

Table 1 | Human primary immunodeficiency syndromes with defective NK cell cytotoxicity.

NK cytotoxicity process	Disease	Gene mutated	Protein affected	NK cell defects in cytotoxicity	Rescued by IL-2
Lytic granule biogenesis	Familial hemophagocytic lymphohistiocytosis type 2 (FHL2)	*PFR1*	Perforin	Exocytosis of lytic granules is normal, but no cytotoxicity is achieved due to absence of pore-forming molecule	No
	Papillon–Lefèvre syndrome (PLS)	*CTSC*	Cathepsin C	Granzyme B in lytic granules is not fully processed, causing defective cytotoxicity	Yes
	Hermansky–Pudlak syndrome type 2 (HPS2)	*AP3B1*	β3A-subunit of adaptor protein 3	Enlarged lytic granules impaired movement along microtubules? Less perforin in lytic granules?	?
	Chediak–Higashi syndrome (CHS)	*CHS1/LYST*	CHS1/LYST	Enlarged lytic granules. Impaired exocytosis of lytic granules (unknown cause)	?
Adhesion to target cells	Leukocyte adhesion deficiencies type 1 (LAD-I)	*ITGB2*	β2-subunit of integrin (CD18)	Impaired adhesion to target cells. Impaired polarization	Yes
	Leukocyte adhesion deficiencies type 3 (LAD-III)	*FERMT3*	Kindlin-3	Impaired adhesion. Impaired specific activating receptor-mediated cytotoxicity, but normal natural cytotoxicity	?
F-actin rearrangement	Wiskott–Aldrich syndrome (WAS)	*WASP*	WASP	Impaired adhesion to target cells. Impaired reorganization of F-actin and integrins. Impaired polarization of lytic granules.	Yes
	WASP-interacting protein (WIP) deficiency	*WIPF1*	WIP	Reduced surface expression of some NK activating receptors. No detectable WASP.	No
	Dedicator of cytokinesis 8 (DOCK8) deficiency	*DOCK8*	DOCK8	Impaired adhesion to target cells. Impaired reorganization of F-actin and integrins. Impaired polarization of lytic granules.	No
Polarization of lytic granules toward CS	MYH9-related diseases (MYH9-RD)	*MYH9*	Myosin IIA	Impaired exocytosis of lytic granules	?
Fusion of lytic granules into PM	Griscelli syndrome type 2 (GS2)	*RAB27A*	RAB27A	Impaired exocytosis of lytic granules	Yes
	Familial hemophagocytic lymphohistiocytosis type 3 (FHL3)	*UNC13D*	Munc13-4	Impaired exocytosis of lytic granules	No
	Familial hemophagocytic lymphohistiocytosis type 4 (FHL4)	*STX11*	Syntaxin-11	Impaired exocytosis of lytic granules	Yes
	Familial hemophagocytic lymphohistiocytosis type 5 (FHL5)	*STXBP2*	Munc18-2	Impaired exocytosis of lytic granules	Yes

? = Unknown or equivocal.

inhibited conformation and triggers its activity (40). It is assumed that the established pore is large enough for granzymes to get into target cells by simple diffusion (21, 37, 40). Last but not least, how do NK cells prevent themselves from potential self-destruction from released cytotoxic components? Cell surface expression of cathepsin B has been suggested to provide protection of cytotoxic lymphocytes by cleaving perforin (43). In addition, it was recently shown that the surface expression of LAMP-1 (CD107a) also protects NK cells from degranulation-associated damage by inhibiting binding of perforin to NK cells (44). Therefore, it seems there exist multiple layers of protection at the final degranulation step that ensure unidirectional cytotoxicity. In the following paragraphs, we will focus our discussion on PIDs with impaired NK cell cytotoxicity caused by: (1) defects in the contents of lytic granules and (2) impaired biogenesis and maturation of lytic granules.

PIDs AFFECTING CONTENTS OF LYTIC GRANULES

Familial hemophagocytic lymphohistiocytosis type 2

Familial hemophagocytic lymphohistiocytosis type 2 (FHL2) is an autosomal recessive disorder comprising 13–58% of all FHL cases and is caused by mutation of the *PFR1* gene, which encodes perforin (45). Most of the mutations identified in FHL2 patients occur within regions critical for perforin maturation, or impair proper folding, oligomerization, or Ca^{2+}-mediated membrane binding (31, 46). Interestingly, each mutation can dramatically impact the level of mature perforin, ranging from absent to normal. Additionally, the intrinsic activities of the mutated perforin correlate with the age of FHL onset and the severity of the disease (47–52). Significantly, the inability of the mutated perforin to form pores on target cell membranes results in the absence of cytotoxic function of NK cells from FHL2 patients. Perforin loss did not

affect the level of other lytic granule components (granzymes and cathepsins) or the steps leading to lytic granule polarization and membrane fusion (45, 53). Therefore, the normal degranulation (examined by surface expression of CD107) observed in NK cells from FHL2 patients provides us an important criterion to distinguish FHL2 patients from FHL patients caused by mutation of other genes (53). In many cases, FHL2 patients usually further develop other diseases including leukemia, juvenile rheumatoid arthritis, and macrophage activation syndrome (48, 54–61), suggesting an important role for perforin and cytotoxic activity mediated by NK cells and $CD8^+$ T cells in limiting or preventing these diseases. In addition, the non-redundant role of perforin activity in cellular cytotoxicity suggests the intervention of perforin activity as a potential therapeutic target in human diseases caused by abnormal cytotoxicity of cytotoxic lymphocytes (52).

Papillon–Lefèvre syndrome

Papillon–Lefèvre syndrome (PLS) is a rare autosomal recessive disease caused by mutation of the gene encoding cathepsin C, *CTSC* (62–64). This disease is clinically characterized by palmoplantar keratosis, early onset of severe periodontitis, and susceptibility to viral infections. Cathepsin C is a lysosomal cysteine protease, which is responsible for the processing of granzyme A and B (36, 65). Consequently, NK cells from PLS patients primarily contain immature granzyme B, and hence, their NK cells show impaired cytotoxic activity (34). Interestingly, the impaired processing of granzyme B as well as the defective cytotoxicity could be restored by treatment of interleukin-2 (IL-2), suggesting that an IL-2 signaling pathway is able to process granzyme B in a cathepsin C-independent manner (34, 66).

PIDs AFFECTING BIOGENESIS AND MATURATION OF LYTIC GRANULES

Hermansky–Pudlak syndrome type 2

Hermansky–Pudlak syndrome is an autosomal recessive disease clinically characterized by oculocutaneous albinism and excessive bleeding (67, 68). Among the currently identified nine different types of HPS, Hermansky–Pudlak syndrome type 2 (HPS2) is the only type known to cause immunodeficiency in addition to other clinical symptoms (67, 69, 70). HPS2 is caused by mutation of the *AP3B1* gene that encodes the β3A-subunit of adaptor protein 3 (AP3), which is part of a heterotetrameric protein complex (67, 71). Mutations leading to the loss of the β3A-subunit affect the stability of other subunits and thereby induce loss of the entire complex (67). The AP3 complex mediates sorting of integral membrane proteins from the endosome and TGN to the lysosome and secretory lysosome (72, 73). Considering the ubiquitous expression of AP3, it is very interesting to note that its absence causes severe functional defects only in cells with secretory lysosomes, indicating that AP3 is required for the biogenesis of secretory lysosomes and/or in sorting of secretory lysosome-specific proteins.

In the case of melanocytes, the AP3 complex was shown to be responsible for sorting of tyrosinase, a protein required for melanin synthesis, into the melanosome (74). Additionally, AP3-deficiency was shown to cause defective cytotoxicity of both NK cells and CTLs (17, 75–77). While perforin levels in CTLs have been reported to be within the normal range in some patients with HPS2 (76), NK cells from two siblings with HPS2 showed less intracellular perforin levels compared to NK cells from healthy controls (75), suggesting that the defective NK cell-mediated cytotoxicity observed in these patients is at least partially a result of diminished perforin. Considering the fact that these patients had similar mRNA expression of perforin, correct sorting of existing perforin into lysosomes and the existence of mature perforin in patient NK cells, perforin biosynthesis seems to be independent of AP3 complex (75). Yet it remains possible that the AP3 complex is involved in sorting of proteins critical for perforin stability once perforin has made it into the lytic granule. This discrepancy between CTLs and NK cells from these patients might be due to further cell-type-specific roles of AP3 between NK cells and CTLs since lytic granule biogenesis is known to be different between these two cell types; lytic granules are preformed in resting NK cells, whereas lytic granules are generated after activation in CTLs (78–80). Clearly more patients need to be accumulated to better understand these different observations. It is also interesting to note that enlarged lytic granules were observed in CTLs of HPS2 patients (77). Further elucidation of the AP3 complex target proteins required for lytic granule sorting as well as other potential roles of the AP3 complex in the biogenesis of lytic granules will be able to answer these questions.

Lastly, Clark et al. suggested that the AP3 complex might also play a more direct role(s) in the cytotoxic process of CTLs. They observed that lytic granules from AP3-deficient CTLs fail to cluster around the microtubule-organizing center (MTOC) when conjugated with appropriate target cells (77). This seems to be due to a failure of lytic granules to move along the microtubules or an inability to attach to microtubules. In addition, it is not clear whether this observed defect is a result of a direct role for AP3 in mediating lytic granule clustering at the MTOC, or due to a sorting failure of a protein(s) critical for attachment and/or movement of lytic granules along microtubules. Definitely, future studies elucidating the mechanism resulting in the phenotypes of AP3-deficiency in CTLs and NK cells will be very exciting.

Chediak–Higashi syndrome

Chediak–Higashi syndrome is another disease caused by defective lytic granule generation in NK cells and CTLs. The disease is inherited in an autosomal recessive manner and the main clinical symptoms of CHS are partial albinism and recurrent infections in the lung and skin. Mild coagulation defects and varying degrees of neurologic dysfunctions are also commonly observed in CHS patients. Although patients are often able to clear bacterial infections with the help of antibiotics, they instead develop an accelerated phase of hemophagocytic lymphohistiocytosis; seen by infiltration of macrophages and lymphocytes into the major organs of the body (81). Clinically, CHS and Griscelli syndromes (GSs; discussed in later section) present with identical features. However, at the cellular level, CHS can be distinguished from GS by the fact that CHS cells contain abnormally enlarged lysosomes and secretory lysosomes. Therefore, this cellular feature is used as the key diagnostic criterion of CHS (82–84). In 1996, the responsible gene for CHS was mapped to *CHS1/LYST* gene (*beige* gene in the mouse model of CHS) (85, 86). The *CHS1/LYST* gene is highly conserved in all species, and a unique region at the C-terminal end

of the protein called the BEACH (Beige and Chediak) motif defines a family of proteins homologous to the CHS1/LYST protein. Our current understanding suggests that all BEACH family proteins participate in vesicle trafficking (83, 87).

CHS1/LYST proteins are expressed at very low levels in all cell types, and are involved in maintaining the morphology of lysosomes and secretory lysosomes (83). However, similar to HPS2, only cells with secretory lysosome function are impaired (83, 88). In fact, although few in number, giant lytic granules are observed in NK cells and CTLs from CHS patients, and defects in cytotoxicity of both cell types have been reported from many studies (19, 21). In the case of NK cells from CHS patients, their numbers are within normal range, but the absence of CHS1/LYST causes defects in natural cytotoxicity as well as ADCC (89–95). NK cells from CHS patients are able to efficiently bind target cells suggesting that the defect in NK cell-mediated cytotoxicity is due to problems in the actual cytotoxicity process. However, detailed molecular roles of CHS1/LYST in NK cell-mediated cytotoxicity have not been reported to date. Interestingly, treatment with interferons (IFNs) increase the cytotoxic capacity of NK cells from CHS patients (92, 93, 96), suggesting that IFN signaling is able to rescue cytotoxicity defects of CHS NK cells, at least partially.

Most of our knowledge regarding the cellular functions of CHS1/LYST protein were obtained from studies of CTLs from CHS patients. Unexpectedly, while giant lysosomal organelles were observed in the patients' cells, the biogenesis, processing, and expression level of lytic proteins such as perforin and granzymes were normal, as were their sorting into lytic granules after activation. However, as time went on, the lytic granules began to fuse together becoming giant organelles (97, 98). Based on these observations, it is likely that the CHS/LYST protein either prevents abnormal fusion between lysosomal organelles or separates lysosomal membranes after normal fusion events, thus regulating lysosomal size. Significantly, CTLs from CHS patients were unable to secrete their granules in response to TCR ligation (97), suggesting that the cytotoxicity defects observed in NK cells and CTLs from CHS patients result from an exocytosis defect. Further studies elucidating the roles of CHS1/LYST in lysosomal fusion and exocytosis as well as the roles of other BEACH family proteins will provide clearer insight into the NK and CTL phenotypes observed in patients as well as the biogenesis of lytic granules and melanosomes.

ADHESION OF NK CELLS TO TARGET CELLS

Natural killer cells rapidly accumulate at the site of inflammation through the stimulation of inflammatory chemokine receptors and adhesion molecules expressed on their cell surface (1). Although the roles of weak adhesion molecules like selectin family members have not been directly examined in NK cell – target cell binding, it is generally appreciated that the first step toward the development of NK cell cytotoxicity is weak integrin-mediated adhesion to target cells (99). Signals from this weak adhesion, along with the initial signaling resulting from the engagement of activating/inhibitory receptors on the NK cell with corresponding ligands on the target cell, determines whether the NK cell should detach (strong inhibitory signal) or establish a firm adhesion (strong activation signals) leading to the formation of the cytotoxic synapse (CS). Along with high affinity interactions toward their ligands, integrins were also observed to cluster at the CS of NK cells, strengthening adhesion (avidity) (100–102) and promoting the scanning of target cells (**Figure 1**) (11, 12, 99). In addition to being an important mediator of cell–cell adhesion, integrins can serve as co-stimulatory molecules enhancing cytotoxicity signals from other activating NK receptors (103–105), as well as promoting lytic granule polarization following ligand engagement (104, 106–108).

LEUKOCYTE ADHESION DEFICIENCY TYPE I

Leukocyte adhesion deficiencies (LAD) are autosomal recessive diseases caused by mutations in the genes encoding adhesion molecules or molecules critical for processing/activation of adhesion molecules. Leukocyte adhesion deficiency type I (LAD-I) is the most common type of LAD, and is caused by mutation in the gene encoding the β2-subunit of the integrin (CD18), *ITGB2* (**Figure 1**) (109). A variety of mutations have been found in *ITGB2*, which can impact the expression of CD18 as well as its ability to bind a ligand (110). The main clinical symptoms of these patients are recurrent bacterial and fungal infections in the skin and mucosa, absence of pus formation at the infection sites, and impaired healing after infection. An abnormally high number of leukocytes in the blood are also a common feature of this disease, indicative of inefficient recruitment to sites of inflammation (19, 110). Supporting the essential role of integrins in mediating cytotoxicity signaling, NK cells from LAD-I patients showed defective natural cytotoxicity as well as ADCC compared to control NK cells (111–113). Although the defective cytotoxicity of patients' NK cells seems to be mainly due to impaired adhesion to target cells, it was recently suggested that LFA-1 (CD11a/CD18) might be required for clustering of lytic granules to the MTOC after target cell binding (114). However, further studies are required to clearly conclude that impaired lytic granule convergence observed in NK cells of LAD-1 patients is due to the absence of LFA-1. It remains possible that the defect observed in LAD-I NK cells might result from impaired/delayed signaling from other NK receptors caused by defective adhesion to target cells. One study suggested that the cytotoxicity defects in LAD-I NK cells could be rescued at least partly via activation with IL-2 (115). In this regard, it is very interesting to note that activation of NK cells with only IL-2 treatment promoted clustering of lytic granules (114). How IL-2 signaling is able to rescue cytotoxicity of NK cells (adhesion to target cells or clustering of lytic granules) is unclear and will require further investigation.

LEUKOCYTE ADHESION DEFICIENCY TYPE III

Leukocyte adhesion deficiency type III (LAD-III) is caused by mutation of the *FERMT3* gene, which encodes Kindlin-3 (110). Kindlin-3 is expressed only in cells of the hematopoietic lineage (116), and like talin, regulates integrin-mediated adhesion by binding directly to the integrin β-chain (**Figure 1**) (117, 118). Patients with LAD-III have severe recurrent infections, leukocytosis, and persistent bleeding (110). As expected from the main role of Kindlin-3 in integrin-mediated adhesion, defective integrin activation was observed in platelets, lymphocytes, and polymorphonuclear leukocytes (PMNs) derived from LAD-III patients (119–125). Recently, functions of NK cells from a single

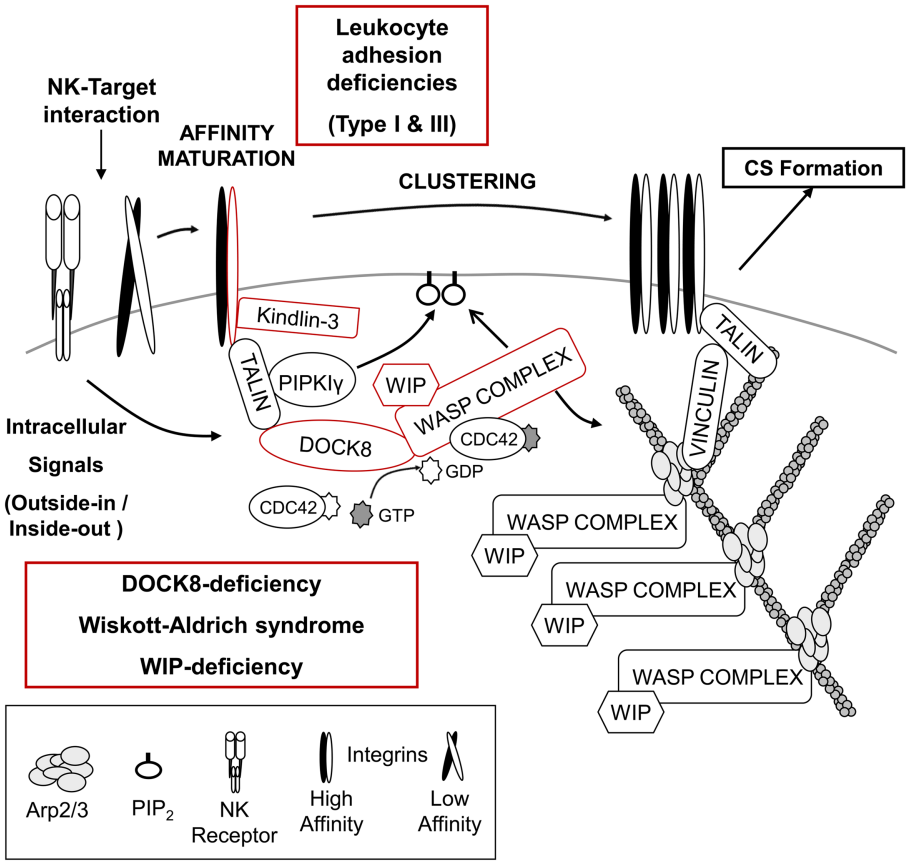

FIGURE 1 | Regulation of NK – target cell adhesion and generation of F-actin at the cytotoxic synapse. Initial signals from the NK – target interaction recruit multiple proteins responsible for integrin-mediated adhesion and high affinity maturation as well as the accumulation of F-actin at the cytotoxic synapse (CS). Absence of either the integrin β2-subunit (CD18) or its regulator, Kindlin-3, results in leukocyte adhesion deficiency type I or type III, respectively. NK cells from both diseases present defective cytotoxicity due to failure of efficient target binding and/or defective NK cell activation. The DOCK8–WASP complex is also recruited to the NK-target interface, and a "DOCK8–CDC42–WASP" pathway is likely responsible for F-actin reorganization, which might facilitate integrin-mediated adhesion and provide the F-actin meshwork critical for organization of the CS. Absence of either DOCK8 or WASP results in primary immunodeficiency disorders and NK cells from these patients show impaired NK cytotoxic activity (see text for details).

female infant patient were examined (126). Interestingly, specific activating receptor-mediated cytotoxicity was defective, whereas natural cytotoxicity mediated by multiple NK receptors was normal. It was suggested that Kindlin-3 might function by lowering the signaling threshold required for NK cell activation. Clearly, more patients will be needed to further define its role in NK cell-mediated cytotoxicity. In addition, further insights into the cellular roles of Kindlin-3 in inside-out/outside-in signaling by integrins as well as elucidation of the relationship of Kindlin-3 and talin in integrin regulation will be very helpful in understanding LAD-III.

ACTIN REORGANIZATION AT THE CYTOTOXIC SYNAPSE

Essential signaling molecules like NK activating/inhibitory receptors are accumulated at the center of the interface known as the central supramolecular activation cluster (cSMAC), whereas F-actin and integrins are localized at the periphery of the interface forming a ring-shaped peripheral supramolecular activation cluster (pSMAC). Among these distinct molecular patterns at the CS, F-actin accumulation at the pSMAC is thought to be one of the early and critical events in NK cell-medicated cytotoxicity, since clustering of NK receptors as well as adhesion molecules are observed to be dependent on F-actin polymerization (13, 99, 101). Two primary immunodeficiencies causing defective F-actin accumulation at the CS of NK cells have provided some clues on how F-actin accumulation might be regulated at the CS.

WISKOTT–ALDRICH SYNDROME

Wiskott–Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disorder characterized by recurrent infections, prolonged bleeding, eczema, thrombocytopenia, and impaired cellular and humoral immunity (127, 128). The disease is caused by mutations of the *WAS* gene, which encodes the hematopoietically expressed WAS protein (WASP) (129, 130). Depending on the disease severity and type of mutation, WAS is further subcategorized as X-linked thrombocytopenia (XLT) or X-linked neutropenia (XLN) (127, 128). WASP is the founding member of the WASP superfamily of F-actin nucleation promoting factors (NPFs), which stimulate the formation of branched F-actin

via the ubiquitously expressed Arp2/3 complex (**Figure 1**) (131). All members of the WASP superfamily contain a C-terminal verprolin-connecting-acidic (VCA) domain that interacts with profilin-G-actin and the Arp2/3 complex (127, 128, 132). WASP is thought to play diverse roles in immune cells including formation of the immunological synapse (IS), migration, and phagocytosis (127, 128, 132). WASP-interacting protein (WIP; discussed in the next section) constitutively stabilizes WASP in an autoinhibited conformation (133) in which the VCA domain is stabilized through an interaction with the basic region (BR) and CDC42/RAC GTPase binding domain (GBD). Cell stimulation results in the cooperative interaction of the BR with phosphatidylinositol (4,5)-biphosphate (PIP_2) and active CDC42 (CDC42–GTP) with the GBD, thereby leading to the release of the bound VCA domain so that it can bind the Arp2/3 complex and promote branched F-actin generation (134–138).

Wiskott–Aldrich syndrome protein was observed to localize at the pSMAC of the CS along with F-actin in target-bound NK cells, and the roles of WASP in NK cell-mediated cytotoxicity have been examined using NK cells from WAS patients (101, 139, 140). Although the proportion of NK cells in the blood was within the normal range or higher in WAS patients, WAS NK cells showed defective natural cytotoxicity as well as ADCC (139, 140). Impaired cytotoxicity of WAS NK cells was due to inefficient conjugate formation with target cells, a failure in F-actin accumulation at the cell–cell contact site, clustering of integrins and CD2, and polarization of lytic granules at the CS (**Figure 1**) (101, 139, 140). Interestingly, *ex vivo* as well as *in vivo* treatment with IL-2 was able to rescue impaired cytotoxicity of WAS NK cells (139, 141). IL-2 treatment of WAS NK cells restored target binding efficiency, F-actin accumulation, and polarization of lytic granules to the CS. The IL-2-mediated rescue of the defect in WAS NK cells was found to be independent of WASP function, but dependent on another WASP family member, WAVE2 (141). These findings suggest that there exists two distinct pathways for Arp2/3-generated F-actin reorganization at the CS in NK cells, and future studies should enable the use of IL-2 as a therapy to improve clinical symptoms of WAS including severe herpes simplex virus (HSV) infection caused by defective NK cell-mediated cytotoxicity.

Activation of NK cells by target cells or stimulation of CD16 was shown to activate CDC42 in NK cells (139), and accumulating observations suggest that the spatiotemporal activation of CDC42 in NK cells is important in CS formation and cytotoxic activity (142, 143). Therefore, based on current knowledge, NK cell activation results in the generation of active CDC42 at the CS, and this leads to the activation of WASP. Activated WASP will then contribute to F-actin reorganization at the CS, which will mediate the clustering of CD2, integrins, and NK receptors. However, there are still many important questions to be answered regarding the role and regulation of WASP in NK cell-mediated cytotoxicity. For example: (1) What are the upstream signaling pathways that lead to the activation of CDC42 and the generation of PIP_2 that are required for WASP activation at the CS? (2) How is WASP accumulated at the CS after the stimulation of NK cells? and (3) How does WASP mediate the polarization of lytic granules toward the CS? Future studies aimed at addressing these and other important questions will provide insight into how WASP is regulated and mechanistic insight into its role in the development of NK cell-mediated killing.

WASP-INTERACTING PROTEIN DEFICIENCY

Deficiency of WASP-interacting protein was recently identified in a female infant patient (144). The patient had a homozygous mutation in the *WIPF1* gene and both of her parents were heterozygous for the same mutation, suggesting that the disease is inherited in an autosomal recessive manner. WIP is ubiquitously expressed in humans, although higher levels are observed in hematopoietic cells (145). The clinical features were very similar to those observed in WAS patients, including recurrent infections, thrombocytopenia, eczema, and immunodeficiency. This is not surprising as WIP is thought to maintain the stability of WASP and regulate WASP activity (**Figure 1**) (145, 146). Consistent with this, WASP protein was not detected in the patient's T cells, despite normal *WAS* mRNA expression. However, several WASP-independent functions of WIP have been observed in NK cells. First of all, WIP was shown to be essential for formation of a multiprotein complex composed of WIP, WASP, actin, and myosin IIA after NK cell activation (147). WIP was also found to associate with the lytic granules in NK cells and WIP-depleted NK cells failed to polarize their lytic granules toward the CS (148). In addition, the expression level of major NK activating receptors (NKG2D, NKp30, and NKp46) was lower on WIP-deficient NK cells compared to control NK cells. These findings also suggest that patients with WAS symptoms cannot be simply diagnosed based on WASP expression at the protein level, and will require sequence analysis of the *WAS* and *WIPF1* genes to distinguish between WAS and WIP deficiency.

DEDICATOR OF CYTOKINESIS 8 DEFICIENCY

Another PID that causes defective F-actin accumulation at the CS of NK cells is the loss of dedicator of cytokinesis 8 (DOCK8). DOCK8 is a member of the DOCK180 family of guanine nucleotide exchange factors (GEFs) that mediate activation of Rho family proteins and contribute to multiple cellular processes including cell migration and phagocytosis (149–151). DOCK8 deficiency is a relatively recently identified primary combined immunodeficiency that shows an autosomal recessive pattern of inheritance (152, 153). The main clinical symptoms of these patients are recurrent infections in the lung and skin, high IgE levels in the serum, and severe allergies (152–154). The roles of DOCK8 in human NK cells have been examined recently using NK cells from DOCK8-deficient patients as well as primary human NK cells and human NK cell-lines depleted of DOCK8 by RNAi (155, 156). DOCK8-deficient/depleted NK cells showed defective natural cytotoxicity and specific activating receptor-mediated NK cytotoxicity, suggesting that functional defects of NK cells in DOCK8-deficient patients could be part of the cause for the frequent sinopulmonary infections observed in these patients (152–154, 157). Defective accumulation of F-actin and clustering of integrins at the IS were also observed in DOCK8-deficient/depleted NK cells as well as B cells and $CD8^+$ T cells from DOCK8-mutant mice (155–158). Polarization of lytic granules toward the CS was also defective in DOCK8-deficient/depleted NK cells (155, 156). Mechanistically, DOCK8 was found to interact with WASP and talin, and to mediate their localization to the CS of NK

cells (**Figure 1**) (155). Significantly, DOCK8 was found to have GEF-specificity toward CDC42 (155, 159). These findings provide an important link between the signaling pathways that might activate WASP and may help explain the remarkable similarities between WAS and DOCK8 deficiency. In fact both diseases share many clinical symptoms [recurrent infections by certain types of viruses, and high IgE levels] (127, 128, 132, 152–154, 157), as well as phenotypes observed in NK cells [defective cytotoxic activity, integrin-mediated adhesion, F-actin accumulation, and polarization of lytic granules (101, 139, 140, 155, 156)].

Surprisingly, the addition of IL-2 was unable to rescue the cytotoxic defects seen in DOCK8-deficient NK cell suggesting that DOCK8 is likely involved in the regulation of other signaling pathways in addition to its role in localizing and potentially activating WASP. However, based on the current findings, it is very tempting to hypothesize that DOCK8 mediates the recruitment of WASP to the CS of NK cells after activation where it can generate active CDC42 thus leading to the activation of WASP (**Figure 1**). Another DOCK8-interacting partner, talin, associates with phosphatidylinositol phosphate kinase type Iγ (PIPKIγ), which produces PtdIns (4,5)P2 (PIP_2), a molecule required for optimal activation of WASP (134–138, 160–162). The activated WASP is then able to interact with Arp2/3 inducing the generation of a branched F-actin network, and this enhances the clustering of integrins and NK receptors. Future studies testing the hypothesis of the "DOCK8–CDC42–WASP" pathway should provide a clearer picture of the formation of the CS. Lastly, one remaining question regarding the role of DOCK8 and WASP is how they mediate the polarization of lytic granules toward the CS. Based on current knowledge, it is most likely that polarization of lytic granules toward the CS occurs later than F-actin accumulation (101) since cytochalasin D (Cyt D)-treated NK cells also showed defective polarization of lytic granules (140). However, considering the fact that CDC42 is best known for regulating cellular polarity and is responsible for MTOC polarization in T cells, macrophages, and dendritic cells (DCs) (163–167), DOCK8 might regulate MTOC polarization through the activation of other CDC42 effector molecules in addition to regulating the CDC42–WASP pathway leading to F-actin accumulation.

POLARIZATION OF LYTIC GRANULES TOWARD CYTOTOXIC SYNAPSE

Another important step required for NK cell-mediated cytotoxicity is the polarization of lytic granules toward the CS. This event occurs after the rearrangement of F-actin at the CS and requires both F-actin polymerization and microtubule function, since lytic granules fail to polarize toward the CS when either F-actin accumulation or the microtubule network are disrupted (101). As NK cells start to form conjugates with target cells, lytic granules are rapidly clustered around the MTOC (**Figure 2**) (107). This retrograde, minus-end-directed transport of lytic granules on microtubules is Src kinase-dependent, and mediated by the dynein/dynactin motor complex that constitutively associates with lytic granules and tethers them to the microtubules (107, 114, 168). In conjunction with the clustering of the lytic granules, the MTOC polarizes toward the CS, thereby delivering the lethal cargo to the site of target cell contact (101, 107, 168). Importantly, this process occurs along with maturation of the CS, as it requires F-actin accumulation, rearrangement of NK receptors, and clustering of adhesion molecules.

Current knowledge suggests that activation of extracellular-signal-regulated kinase (Erk), Vav1, and protein tyrosine kinase 2 (PYK2) are required for MTOC polarization in NK cells (169–171). In addition to these molecules, several other proteins have been identified that impact the polarization of lytic granules to the CS (**Figure 2**). For example, interrupting the cellular function of the CDC42-interacting protein-4 (CIP4) was shown to impair MTOC polarization as well as NK cell-mediated cytotoxicity (172). CIP4 was suggested to link the microtubule and F-actin network at the CS through its ability to interact with microtubules and WASP. In this regard, the scaffold protein IQGAP1 also has the potential to interact with F-actin and microtubules, and silencing of IQGAP1 was also shown to impair NK cell cytotoxicity due to defective MTOC polarization toward the CS (173). However, MTOC polarization and cell-mediated killing were found to be normal in mouse CTLs lacking IQGAP1 (174), highlighting again that distinct mechanisms regulating cell-mediated killing are engaged in these two cell types. The Ral GTPase, RalA, also regulates MTOC polarization toward the CS (175). Very recently, the small GTP-binding protein, Arl8b, was found to interact with KIF5B, the heavy chain of the microtubule plus-end-directed motor kinesin-1, and mediate MTOC polarization in NK cells (176). How and where the kinesin-1 motor is recruited to mediate movement of both the MTOC and lytic granules toward the CS remains unclear. In CTLs, a Rab27a/Slp3/kinesin-1 complex was reported to be critical for final transport of polarized lytic granules along with microtubules toward the CS (**Figure 2**). Interestingly, polarization of the MTOC toward the CS was normal when kinesin-1 was interrupted in CTLs (177). It will be interesting to analyze these seemingly different roles of kinesin-1 in both NK cells and CTLs. Lastly, although not examined in NK cells, activity of CDC42 might also be essential for MTOC polarization considering similar roles in other immune and non-immune cells (163–167).

Following the clustering of the lytic granules at the MTOC, the MTOC docks near the plasma membrane (PM) facilitating fusion of lytic granules into the secretory cleft formed between the NK cell and target cell (168, 178). Previous studies using conventional confocal microscopy suggested that the lytic granules pass through an actin-cleared region at the center of the CS (101, 102); however, recent approaches using super-resolution microscopy suggest that the secretory region at the center of the CS is not totally absent of F-actin. Instead, a hypodense branched F-actin meshwork exists in the central region of the CS that is rearranged to produce small pores through which the lytic granules travel (179, 180). The previously suggested role of myosin IIA, an actin motor protein, in the final transit of lytic granules through the actin-rich region of the CS are consistent with this new finding and will be discussed in the next section (**Figure 2**). It still remains to be determined how the F-actin network at the secretory region is regulated to allow lytic granule movement by the myosin motor protein.

MYH9-RELATED DISEASE

Myosin IIA is an actin-based molecular motor that promotes movement along F-actin and is known to be involved in numerous

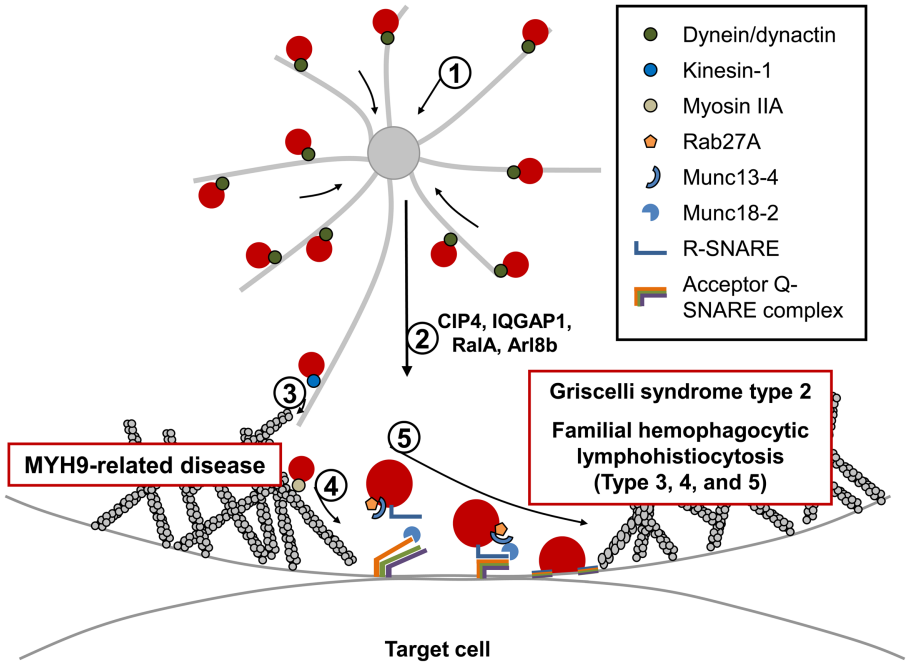

FIGURE 2 | Fusion and exocytosis of lytic granules during NK cell-mediated cytotoxicity. Directed release of lytic granules toward bound target cells is a multi-step process: (1) lytic granules are rapidly clustered around the MTOC. This retrograde (minus-end-directed) movement along the microtubules occurs rapidly and is mediated by the dynein/dynactin motor complex. (2) The MTOC, along with accumulated lytic granules, are polarized toward the CS, and several proteins have been identified to be critical for this step. (3) Polarized lytic granules are further delivered toward the CS along the microtubule (anterograde transport) in a kinesin-1-dependent manner. (4) Myosin IIA facilitates the final transit of the lytic granules through the hypodense F-actin meshwork at the CS. NK cells from patients with MYH9-related diseases fail to degranulate. (5) The final fusion of lytic granules with the plasma membrane occurs in an orchestrated manner and requires multiple molecules including Rab27a, its effector protein Munc13-4, SNARE proteins, and their accessory proteins. Mutations in proteins involved in this final fusion step have been found to cause immunodeficiency disorders including Griscelli syndrome type 2, familial hemophagocytic lymphohistiocytosis types 3, 4, and 5, all of which have similar clinical symptoms, and defective lytic granule exocytosis.

cellular processes (181). Myosin IIA is a hexameric protein composed of two heavy chains, two regulatory light chains, and two essential light chains. The heavy chain of myosin IIA contains a motor domain at the N-terminal head that binds to F-actin to perform its motor function through ATP hydrolysis, whereas the C-terminal region performs filament formation, cargo binding, and regulatory functions (182–184). In humans, mutations in *MYH9* gene, which encodes the heavy chain, lead to autosomal dominant diseases collectively known as MYH9-related diseases (MYH9-RDs), which include May–Hegglin Anomaly, Sebastian Syndrome, Fechtner Syndrome, and Epstein Syndromes (185, 186). The main clinical features of MYH-RD are macrothrombocytopenia and granulocyte inclusions.

Previous studies using the myosin light chain kinase inhibitor ML-9 (1-[5-chloronaphthalene-1-sulfonyl]-1*H*-hexahydro-1,4-diazepine) inhibited NK cell-mediated cytotoxicity (187), suggesting that myosin IIA would be critical for NK cell effector activity. Consistent with this notion, NK cells from MYH9-RD patients as well as human NK cells that were either inhibited pharmacologically or depleted of myosin IIA function had impaired NK cell-mediated cytotoxicity (188–190). Molecularly, it was found that inhibition or depletion of myosin IIA did not affect the early steps of NK cytotoxicity including target binding, F-actin accumulation, or polarization of lytic granules, but caused impaired degranulation (**Figure 2**). It was further found that a pool of myosin IIA is associated constitutively with lytic granules, and facilitates the final transit of the lytic granules through the F-actin meshwork at the secretory region of the CS (190). It is important to note that there might be other important roles of myosin IIA in NK cell-mediated cytotoxicity. For example, as mentioned in the previous section, it was shown that myosin IIA and actin are recruited to WIP during NK cell activation (147). Lastly, a large fraction of myosin IIA is found associated with the F-actin at the cell cortex, suggesting that there are likely other functions of myosin IIA in addition to its role in regulating lytic granule trafficking at the CS (147, 188, 190). Future studies regarding other cellular roles of myosin IIA in the NK cytotoxicity and their connection with roles in lytic granule transport will be interesting.

FUSION OF LYTIC GRANULES WITH THE PLASMA MEMBRANE

Exocytosis of lytic granules requires membrane fusion between lytic granules and the PM. An increasing number of studies suggest that small GTPases, including Rab–GTPases, known to regulate membrane trafficking and fusion are important regulators of lytic granule exocytosis in NK cells (191, 192). Following

GDP to GTP exchange, these small GTPases are able to interact with their effector proteins involved in protein sorting, motor activity, and tethering, thereby impacting membrane trafficking. Therefore, they are suggested to play important roles, at least in determining specificity of the initial tethering step (**Figure 2**) (193, 194). In fact, mutation in Rab27a is known to be responsible for a PID known as Griscelli syndrome type 2 (GS2), which will be discussed in detail below (195, 196).

In addition to Rab proteins, the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which are known to mediate most membrane fusion events in eukaryotic cells, also participate in exocytosis (197–199). There are approximately 40 SNARE family proteins in humans with different combinations of SNARE proteins expressed in different cell types. In addition, each SNARE protein exhibits a distinct localization pattern suggesting that selective pairings of SNARE members in specific organelles (like lytic granules) constrain their target binding (197–199). All SNARE proteins contain a coiled-coil SNARE motif at the center, and are structurally categorized as either an R-SNARE or Q-SNARE (further subclassified as Qa-, Qb-, Qc-, or Qb,c-SNARE). Membrane fusion is induced when an R-SNARE protein, such as those found on the lytic granule membrane binds to its cognate Q-SNARE proteins expressed on the PM (**Figure 2**). Bundling of four SNARE motifs (one from the R-SNARE, and three motifs from two or three Q-SNAREs) makes a stable SNARE complex, and the exerted mechanical force pulls the two membranes closer to promote membrane fusion. Interestingly, familial hemophagocytic lymphohistiocytosis type 4 (FHL4) is the result of a mutation in the gene encoding a Qa-SNARE protein called syntaxin-11 (STX11) (200, 201). In addition to the selectivity of SNARE proteins, accessory proteins including SM (Sec1/Munc18-like), Munc13-like, and synaptotagmin associate with SNARE proteins to regulate membrane fusion both temporally and spatially (197–199, 202). Mutations in Munc13-4 and Munc18-2/syntaxin-binding protein 2 (STXBP2) are responsible for familial hemophagocytic lymphohistiocytosis type 3 (FHL3) and familial hemophagocytic lymphohistiocytosis type 5 (FHL5), respectively (**Figure 2**) (203–205). Lastly, the entry of extracellular Ca^{2+} is essential for lytic granule exocytosis (206). Although PIDs regarding this category are not discussed in this review (please refer to reviews (21, 207) for more details), NK cells from patients deficient in either the ER calcium sensor STIM1 or the PM calcium channel ORAI1 showed defective cytotoxicity due to impaired exocytosis of lytic granules, although polarization of lytic granules toward the CS was normal (206). Significantly, Munc13 proteins and synaptotagmin proteins contain two calcium-dependent phospholipid-binding C2 domains and are known to regulate activity of the SNARE complex in a Ca^{2+}-regulated manner (197, 199, 202). Likewise, members of the synaptotagmin-like protein (Slp) family also share C-terminal tandem C2 domains. Slps were also shown to interact with activated Rab27 (Rab27–GTP) via the Slp homology domain (SHD) and participate in docking granules to the membrane (208). Unfortunately, we still do not have a clear picture as to which of these proteins are regulating the membrane fusion steps in NK cell-mediated cytotoxicity. However, as discussed below, clues to understanding this seemingly complicated and tightly regulated mechanism of lytic granule docking and fusion have been uncovered by functionally antagonizing these molecular pathways and examining NK cells from PID patients deficient in these key molecules.

GRISCELLI SYNDROME TYPE 2

Griscelli syndrome type 2 is an autosomal recessive human disease caused by mutations in the *RAB27A* gene that encodes the small GTPase Rab27a (195, 196). GS2 is clinically characterized by severe immunodeficiency, an accelerated phase of HLH, partial albinism, and distinctive silvery-grayish hair (209, 210). GS2 is distinguished from other subtypes of GS (GS1: mutation in *MYO5A* encoding myosin Va; GS3: mutation in *MLPH* encoding melanophilin) from the fact that only GS2 is associated with the development of hemophagocytosis (211). Since all three responsible proteins for GS are required for distribution of melanins in melanocytes (212), hypopigmentation due to defective melanosome function is a common feature of all subtypes of GS. On the other hand, the fact that cytotoxic lymphocytes only express Rab27a among the three proteins explains why abnormal immune function is only observed in GS2 (213). NK cells from GS2 patients showed defective cytotoxicity which could be at least partially restored by treatment of IL-2 (192, 214–217). In CTLs of GS2 patients as well as Rab27a mutant *Ashen* mice, docking of lytic granules at the PM and the subsequent degranulation process were impaired (195, 204, 218, 219). Supporting the role of Rab27a observed in CTLs, degranulation of activated NK cells from GS2 patients was defective (192), and depletion of Rab27a in the human NK cell-line, NKL, showed a decreased number of lytic granules at the PM (**Figure 2**) (191). As discussed above, synaptotagmin-like proteins 1–3 (Slp1–3), which are effectors of Rab27a, are expressed in CTLs and involved in lytic granule exocytosis (177, 220, 221). Interestingly, it was recently found that Slp3 interacts with the kinesin-1 motor protein. This is significant, as the motor activity of kinesin-1 was suggested to deliver the slp3/Rab27a complex toward the CS of CTLs (177). Lastly, while Rab27a is in a distinct compartment from lytic granules in both resting NK cells and CTLs (192, 222), when they are activated, Rab27a is recruited to lytic granules. Whether Rab27a binding to Slp3 or some other effector molecule mediates this recruitment remains to be determined.

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 3

Familial hemophagocytic lymphohistiocytosis type 3 is caused by mutation in *UNC13D*, which encodes Munc13-4 (204). Like other types of FHL, FHL3 patients present with hyperactive CTLs and macrophages in the peripheral blood. Patients usually have fever, hepatosplenomegaly, defective coagulation, and features of hemophagocytosis (204, 223, 224). Initial studies of Munc13-4 function reported that Munc13-4 is highly expressed in hematopoietic cells and involved in cytotoxicity of CTLs by mediating the exocytosis of lytic granules (204). In line with this finding in CTLs, cytotoxic activities (both natural cytotoxicity and ADCC) were defective in NK cells from FHL3 patients, whereas levels of cytokines produced by activated NK cells were normal (48, 53, 225). Consistent with the defect being at the level of lytic granule fusion, the polarization of lytic granules toward the CS was normal, but

the patient NK cells failed to degranulate (**Figure 2**) (53, 54). As indicated above, Munc13-4 contains one diacylglycerol (DAG) binding C1 domain and two Ca^{2+}-binding C2 domains (204), suggesting it regulates SNARE conformation in Ca^{2+}-dependent manner like other Munc13 members in a process called "priming," which guides incomplete SNARE components for full assembly. However, it is still unclear which target SNARE proteins are regulated by Munc13-4 and how it acts mechanically during NK cell degranulation. However, one of the interesting findings to note is that Munc13-4 was found to be an effector of activated Rab27a (Rab27a–GTP) (226, 227). When NK cells become activated, both proteins were found to associate with lytic granules, and the recruitment of each protein was dependent on the other (192). It is also very intriguing to note that the recruitment of these proteins to lytic granules also requires myosin IIA, since inhibition of myosin activity blocked their recruitment (192). Clearly much more work is needed to delineate the mechanisms by which Rab27a and Munc13-4 regulate lytic granule docking and fusion.

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 4

Familial hemophagocytic lymphohistiocytosis type 4 is caused by mutations in the gene that encodes the Qa-SNARE protein STX11 (200, 201). STX11 is considered an atypical member of the syntaxin family, since it lacks the hydrophobic transmembrane domain found in other syntaxin family members that is involved in membrane binding (228). Instead, STX11 is able to associate with membranes via a cysteine-rich region located at its C-terminal end (229). NK cells from FHL4 patients or human NK cells depleted of STX11 showed defects in both natural cytotoxicity and specific activating receptor-mediated cytotoxicity (54, 230). Polarization of lytic granules toward the CS was normal in these NK cells, but they failed to degranulate suggesting the roles of STX11 in the late step of lytic granule release (**Figure 2**). Recently established STX11-deficient mice also support findings from the patients (231, 232). Interestingly, the NK cell defects from FHL4 patients were partly rescued with IL-2 treatment (54). It is of interest that STX11 localizes to the late endosome and the TGN (233, 234). Over-expressed STX11 also showed a distinct localization pattern that was different from both Rab27a and perforin in resting NKLs, but upon activation, they colocalized with each other at the CS. It is unclear why these proteins (Rab27a, Munc13-4, and STX11) exist in distinct subcellular compartments prior to activation, but one possible explanation would be the separation of exocytic machineries and lytic granules in the resting state to prevent premature granule exocytosis. Another important issue remaining to be resolved is the identification of the other SNARE complex members that contribute to membrane fusion. Two R-SNARE proteins, VAMP4 and VAMP7, were recently shown to colocalize with perforin in activated NK cells, and mediate lytic granule exocytosis (235, 236). The Qb,c-SNARE protein, SNAP23, is known to interact with STX11 in B cells; therefore, it is intriguing to hypothesize that it might also mediate degranulation of lytic granules in NK cells in cooperation with STX11. STX11 was also identified to interact with Munc18-2/STXBP2, which might play important roles in priming the SNARE complex containing STX11 (203, 205).

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 5

Familial hemophagocytic lymphohistiocytosis type 5 is a recently identified type of FHL caused by mutation in *STXBP2*, which encodes Munc18-2 (also called STXBP2) (203, 205). FHL5 patients have either a very low level of Munc18-2 or no expression due to homozygous or compound heterozygous mutations in the *STXBP2* gene. In addition to common clinical features of FHL, some FHL5 patients additionally presented with colitis, bleeding disorders, and hypogammaglobulinemia (210, 224). Munc18-2 is a member of the SM (Sec1/Munc18-like) protein family, and likely guides appropriate SNAREs for productive complex formation (197–199, 202). Interestingly, Munc18-2 was found to colocalize and interact with STX11 and the protein expression level of STX11 seems to correlate with that of Munc18-2. However, the absence of STX11 did not affect Munc18-2 protein levels suggesting that Munc18-2 might be the main SM protein regulating the STX11 SNARE complex (203). Importantly, NK cells from these patients presented with impaired cytotoxic activity due to defective degranulation (**Figure 2**) (203, 205, 224, 237). Interestingly, similar to what has been observed in NK cells from FHL4 patients, IL-2 stimulation of NK cells from FHL5 patients partially rescued the cytotoxicity defects, suggesting that the IL-2 pathway is able to bypass the defective Munc18-2/STX11 pathway in both FHL4 and FHL5.

CONCLUSION

Advances in clinical diagnostics has substantially increased the identification of patients with PIDs that affect NK cell numbers or effector functions. In addition to furthering our understanding of NK cells in the immune system (CKND and FKND), this group of diseases has substantiated the important roles that NK cells play in immune surveillance. Furthermore, the quantitative and/or qualitative defects in NK cells derived from these patients have highlighted essential molecular machineries shared among immune cells and their importance in NK cell-mediated cytotoxicity. In fact, these loss-of-function mutations have provided invaluable insight into the molecular processes regulating the development of cell-mediated killing by NK cells. However, there are many issues that still remain unanswered. First, how can IL-2-mediated signaling pathways restore NK cell functions in multiple PIDs? Secondly, what are the NK cell-specific mechanisms that differentiate the cytotoxic processes engaged in NK cells from those of CTLs (from biogenesis to exocytosis of lytic granules)? Lastly, how do the signaling molecules that regulate the detailed secretory pathway of lytic granules in NK cells cooperate with the multiple tethering and fusion molecules to promote lytic granule exocytosis? It is clear that PIDs have substantially impacted our understanding of NK cell biology and the cellular mechanisms that control the tightly regulated process of lytic granule release. Further mechanistic insight into the process of NK cell-mediated killing will hopefully reveal novel therapeutic approaches to treat PID patients.

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Perforinopathy: a spectrum of human immune disease caused by defective perforin delivery or function

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Congenital perforin deficiency is considered a rare cause of human immunopathology and immune dysregulation, and classically presents as a fatal illness early in infancy. However, we propose that a group of related disorders in which killer lymphocytes deliver only partially active perforin or a reduced quantum of wild-type perforin to the immune synapse should be considered part of an extended syndrome with overlapping but more variable clinical features. Apart from the many rare mutations scattered over the coding sequences, up to 10% of Caucasians carry the severely hypomorphic *PRF1* allele C272 > T (leading to A91V mutation) and the overall prevalence of the homozygous state for A91V is around 1 in 600 individuals. We therefore postulate that the partial loss of perforin function and its clinical consequences may be more common then currently suspected. An acute clinical presentation is infrequent in A91V heterozygous individuals, but we postulate that the partial loss of perforin function may potentially be manifested in childhood or early adulthood as "idiopathic" inflammatory disease, or through increased cancer susceptibility – either hematological malignancy or multiple, independent primary cancers. We suggest the new term "perforinopathy" to signify the common functional endpoints of all the known consequences of perforin deficiency and failure to deliver fully functional perforin.

Keywords: perforin, perforinopathy, granzyme, NK cell, protein misfolding, FHL, immune deficiency

INTRODUCTION

Perforin (PRF, encoded by the *PRF1* gene) is a pore-forming toxin (1, 2) stored in the secretory granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (collectively known as cytotoxic lymphocytes, CLs) (3). During an immune response, CLs form an immune synapse (IS) with various types of antigen-presenting cell, and release PRF and granzyme serine proteases into the synaptic cleft (4). The extracellular milieu of the IS (high calcium and neutral pH) promotes perforin membrane binding and insertion culminating in pore formation (5, 6), an indispensable requirement for granzymes to enter the target cell cytoplasm and trigger a multitude of cell death signaling pathways (7–12).

A SPECTRUM OF CLINICAL PRESENTATIONS REFLECTING FUNCTIONAL PRF DEFICIENCY

It was first appreciated around the turn of this century that the complete loss of perforin activity results in a fatal, autosomal recessive immunoregulatory disorder of infancy (median age of onset <12 months), familial hemophagocytic lymphohistiocytosis (FHL) (13), which can be cured only by heterologous bone marrow transplantation (14). The inability to clear antigen-presenting cells by impaired CLs causes an uncontrolled activation and expansion of CD4+ and CD8+ T cells that secrete high levels of interferon-γ. This cytokine is central to disease pathogenesis, as it leads to macrophage activation and secondarily to the overproduction of pro-inflammatory cytokines by these cells. This in turn is manifested clinically as intractable fever, liver and spleen enlargement, and hemophagocytosis in the bone marrow and lymphoid organs, leading to severe anemia and leukopenia (15). It is generally thought that macrophage activation and infiltration in bone marrow leads to hemophagocytosis in FHL patients. Recently, it was shown that interferon-γ specifically potentiated phagocytosis of erythrocytes by macrophages, thus suggesting a key mechanism for anemia in these patients (16). Variations in clinical presentation stem in part from the fact that there are up to five independent genetic causes of primary FHL and related syndromes (4). Linkage analyses have identified four genetic causes that account for 80–90% of FHL, while a further locus mapping to Chr 9 is yet to be defined (17). Inactivating perforin mutations are responsible for approximately 50% of all cases (type 2 FHL, FHL2), while mutations in three other genes, *UNC13D*, *STX11*, and *STXBP2* (FHL3, 4, and 5 respectively) impair or ablate the delivery of perforin to the IS (18–21). Thus, all the causes of FHL are causally linked by the failure to deliver sufficient active perforin to the IS.

Natural killer cells (innate immunity) and CTLs (adaptive immunity) were first recognized for their key role/s in the defense against viruses; more recently these cells have also been appreciated as being critical for immune surveillance against a variety of malignancies, particularly those of hematopoietic origin (22, 23) or of multiple cancers in the same individuals (24). Both these

roles reflect the importance of perforin in initiating the apoptosis of dangerous cells, either those harboring an intracellular pathogen, or possessing the potential for uninhibited growth and spread, to the detriment of the host. Given this, it is perhaps surprising that (infantile) FHL rarely presents with overwhelming sepsis syndrome caused by viral infection (with the rare exceptions of persistent or fulminant Epstein–Barr virus infection) and almost never with malignancy. Thus, most cases of FHL are not manifested directly through the consequences of failed "target cell death" but rather, indirectly, through an apparent skewing of the immune response toward exaggerated cytokine secretion, the second major type of effector function of CTL/NK cells.

Recent work on CTL/NK cells has highlighted their regulation of various inflammatory pathways through their cross-talk with other components of the immune system (25–27); the consequent aberrations in inflammatory responses leading from failed perforin production can occur in response to known human pathogens, although a specific pathogen that triggers the onset of FHL is rarely identified. These phenomena strongly indicate that perforin and the pathways that synthesize and deliver it to the IS play a more fundamental role in immune homeostasis, centered more particularly on adaption of the neonate to the myriad "non-pathogenic" antigenic stimuli he/she will encounter after leaving the womb. In this context, we postulate it is the failure to clear "constitutive" antigen-presenting cells through perforin-dependent (largely granzyme-mediated) cell death that induces the increased secretion of interferon-γ from the killer lymphocyte, in turn provoking severe dysregulation of pro-inflammatory and chemokine cascades in by-stander cells. The molecular and cellular mechanisms causally linking failed target cell apoptosis and the hypersecretion of interferon-γ are of fundamental importance to the pathophysiology of perforinopathies, and ought to be the focus of intensive research. The consequential effects on macrophages, including secondary pro-inflammatory cytokine secretion and grossly increased macrophage phagocytic activity are the major manifestations of disease and are most marked when perforin activity is completely abolished, but less so if some residual perforin activity persists, for example due to inheritance of perforin missense mutation/s that are not completely inactivating. This theme is taken up again below, as we believe that the variable clinical presentations probably flow on from this fact. While this paper focuses on perforin, it has also been noted for some time that granzymes (for example, granzymes A and M) can influence inflammatory pathways (25, 27), sometimes in a perforin-dependent manner, and at other times without the need for perforin to be present. While the significance of granzyme-mediated inflammatory pathways for human health are yet to be defined and hyper- (or hypo-) inflammatory syndromes are not yet described, future research should keep this possibility in mind. This is particularly so because granzyme genes can show considerable polymorphism, both in mice (28) and humans (29).

On the basis that congenital defects in CTL/NK that influence the secretion of active perforin may become clinically evident at various stages of life, we would like to propose that this group of disorders be considered as perforinopathies under three subheadings: acute, subacute, and chronic, depending on the stage of disease onset (**Table 1**). As alluded to above, earlier presentations tend to reflect cytokine-mediated immunopathology; later in life, a patient may present more cryptically with relatively weaker inflammatory manifestations or none at all (**Figure 1**). We think it is quite likely that clinicians managing these less urgent clinical presentations may not have previously considered perforinopathy in the differential diagnosis, particularly in children presenting with a variety of inflammatory disorders beyond infancy.

ACUTE PRESENTATIONS OF PERFORINOPATHY – FHL

For the purpose of this discussion, we define acute perforinopathy as that resulting in a clinical presentation prior to the age of 24 months, with median of approximately 9 months (this may vary depending on the genetic cause of FHL). As discussed above, the pathogenesis involves a cascade of downstream events following on from the inability of NK cells and CTL to present functional perforin and, therefore, kill a cognate target cell. Affected infants typically present with "classic" FHL and meet all or most of the criteria described in HLH-2004 (15). The diagnosis is confirmed by the loss of NK cytotoxicity (which is convenient to test, as the NK cells of healthy individuals display constitutive, pathogen-independent cytotoxicity) and identification of mutations in candidate genes (*PRF1*, *UNC13D*, *STX11*, *STXBP2*). Affected infants are typically very unwell, and may require admission to a high-dependency or intensive care unit; given the autosomal recessive inheritance of most of these disorders, a sibling may have previously been similarly affected, while parents are almost always unaffected carriers. Patients first have their condition stabilized, and are then prepared for heterologous bone marrow transplantation, the only potentially curative therapy (14, 15).

Acute perforinopathy is caused by detrimental mutations in *PRF1* or in proteins responsible for its delivery from the lumen of cytotoxic granules to the IS, *UNC13D*, *STX11*, or *STXBP2* (4). The molecular basis of many disease-causing *PRF1* mutations has been investigated directly using recombinant expression systems (23, 30–33), and is supported by the X-ray crystal structure of perforin (1). In contrast, the biochemical bases of pathological mutations in the other three proteins, Munc13-4, Syntaxin11, and Munc18-2, remain largely unexplored due to the lack of sufficiently informative experimental systems. As a result, the loss of molecular function is predicted (34, 35), either on the basis of undetectable NK cell activity and/or severely reduced protein expression due to degradation. In the context of an early onset FHL (acute perforinopathy), the known structural and cellular defects correlate well with disease severity. Indeed, in the case of *PRF1*, genuinely "null" mutations are most commonly caused by nonsense or frame-shift mutations and in-frame deletions that completely abrogate function. By contrast, a careful genotype-phenotype analysis has revealed that only about 50% of missense mutations ever reported resulted in a complete loss of perforin function, either due to direct interference with a critical functional domain or unscheduled post-translational modifications such as glycosylation (30, 36). The remaining *PRF1* missense mutations were more commonly associated with atypical presentations of FHL or with seemingly unrelated pathologies (immunoregulatory or otherwise) in older children, adolescents, and even adults, who did not necessarily presented with FHL at all (23, 37). If

Table 1 | Possible clinical manifestations of perforinopathy.

	Acute perforinopathy	Sub-acute perforinopathy	Chronic perforinopathy
Age at onset	0–2 years	>2 years	Adolescents – adults
Cause	Bi-allelic mutations in *PRF1*, *UNC13D*, *STX11*, *STXBP2*, leading to complete loss-of-function	Bi-allelic mutations in *PRF1* and, putatively, in *UNC13D*, *STX11*, *STXBP2*, leading to partial loss-of-function	Monoallelic mutations in *PRF1* [e.g., polymorphism 272C > T (Ala91Val)], and possibly, other FHL-causing genes, are contributing not causative factors
Onset	"Classical" FHL – meets all or most of the criteria described in HLH-2004	Difficult to diagnose as does not meet minimal essential criteria of HLH-2004: e.g., inflammatory disease that responds to corticosteroid therapy and has remitting/relapsing clinical course; hematological malignancy	May include a range of conditions, including hematological malignancy, macrophage activation syndrome, lymphoproliferative disease, but not FHL
Diagnostic features	Intractable fever and hepatosplenomegaly are early and prominent Investigations (including NK function assays) described in HLH-2004 will strongly suggest FHL, and disease is confirmed by DNA sequencing	Once FHL is suspected, conduct tests as described in HLH-2004. Severe reduction of NK cell function that prompts genetic analysis may indicate FHL. Test siblings for bi-allelic mutations in FHL-related genes	Mild reduction of NK function. No symptoms described in HLH-2004 are expected
Therapy	Use protocols in HLH-2004; heterologous stem cell transplantation is the only curative therapy	Initially, corticosteroid therapy When genetic cause is identified, HLH-2004 and heterologous stem cell transplantation, as the only cure. If asymptomatic siblings are carriers of bi-allelic mutations in FHL-related genes, preventative stem cell transplantation may be considered	Disease-specific therapy, ranging from corticosteroids to stem cell transplantation

residual perforin activity delays the clinical onset beyond the age of 12 months, we term the presentation "subacute."

SUB-ACUTE PRESENTATIONS OF PERFORINOPATHY

Sub-acute perforinopathies have a wide spectrum of manifestations, all of which are all caused by a partial ("sub-total") loss of CL cytotoxicity due to bi-allelic mutations in one of the four genes described above. Unlike the acute form of the disease, sub-acute perforinopathies may be difficult to diagnose due to their generally milder and more "patchy" clinical presentations, an intermittent clinical course, a range of ages of onset and their frequent response to non-specific immune-suppressive or immune-ablative therapies (38, 39). These patients are more likely to present with a precipitating infection or with an isolated inflammatory manifestation such as pneumonitis that can lead the treating clinician away from the underlying diagnosis. While the only curative therapy for such patients is still ultimately bone marrow transplantation, combination drug therapy may induce remissions of variable duration. These non-curative interventions may be the only available therapies that influence disease outcome, but they have frequent unwanted effects such as opportunistic infection, growth retardation, and bone marrow suppression. Alleviating symptoms that might have categorized them as FHL patients might also delay diagnosis in both the patient and their younger

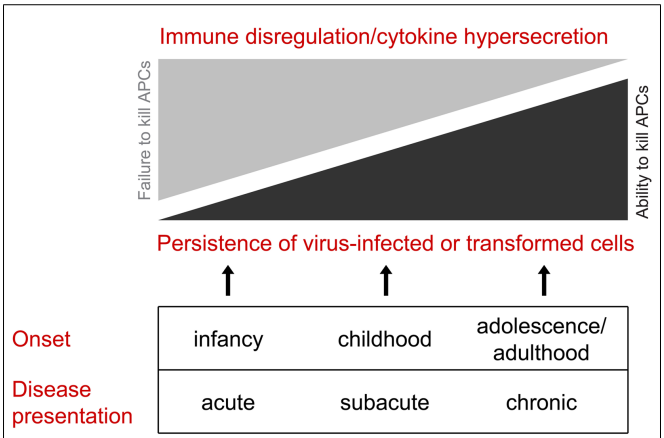

FIGURE 1 | All of the clinical presentations of perforin deficiency (including the failure to deliver active perforin into the immune synapse) result from failed killing of an antigen-presenting cell. Early in life, the clinical manifestations are more likely to reflect cytokine hypersecretion due to chronic stimulation of CD4 and CD8 CTLs and NK cells, resulting in disordered immune hemostasis affected both the lymphoid and myeloid compartments. Later in life, hyper-inflammatory presentations are less likely, and the clinical scenario is more likely to reflect failure to clear dangerous target cells, particularly virus-infected cells and neoplastic cells.

siblings (38). Considering the fact that "classic" FHL is relatively rare and commonly described in infants or very young children, we believe sub-acute perforinopathies may be under-diagnosed, and thus may delay consideration of curative stem cell transfer as a definitive treatment option.

How common are sub-acute perforinopathies? The inevitable answer is "We do not know." Due to the lack of direct functional data on Munc13-4, syntaxin 11, and Munc18-2 mutations, we will discuss perforin deficiency as a paradigm for partial loss of CL function. The first indication that bi-allelic perforin mutations may lead to atypical FHL came from the observation that individuals who inherit two mutant alleles of *PRF1*, one of which is the common polymorphism C272 > T, encoding the ostensibly conservative Ala91Val (A91V) substitution, presented at an older age than "classic" FHL, and with variable symptoms (13, 38, 39). The initial suggestion that the "polymorphism" may be pathogenic was initially met with skepticism, due largely to the very high frequency of the allele among Caucasians (8–17% are heterozygotes) and the rareness of early onset FHL, which was considered at that time to be the only authentic manifestation of the disease (40). However, over the next several years, additional clinical research and biochemical studies have confirmed the original prediction that the A91V substitution is a genuine mutation that impacts severely on perforin structure and function (31, 41). While a prospective cohort study has never been reported, it is nonetheless clear from many reported case studies that inheritance of A91V in the homozygous state or its co-inheritance with a genuinely null allele is very strongly associated with atypical FHL or other immunopathologies that are delayed compared with "classic" disease (sub-acute perforinopathies). In the largest epidemiological studies reported to date [involving over 2,600 healthy individuals – (42–44)], healthy A91V homozygotes have been reported only in one study (42); every other reported case of A91V homozygocity was invariably associated with a pathology. However, the statistical power of these studies linking the genotype with disease has been insufficient to achieve significance. Based on the Mendelian inheritance of perforin mutations, it is predicted that at least 1 in 600 Caucasian individuals should be homozygous for A91V. Assuming strong penetrance of an associated immunopathology in these individuals, such a perforinopathy would be almost 100 times as common as classic ("acute") FHL. Remarkably, at this frequency, A91V-related immune deficiency would rank among the most common congenital disorders; it would be approximately four times as frequent as cystic fibrosis and at a par with Down syndrome.

Is partial perforin deficiency a common phenomenon, and is it caused only by A91V? A retrospective analysis of all reported cases of FHL associated with bi-allelic *PRF1* mutations uncovered a previously unappreciated dichotomy: half of the patients who carried at least one missense mutation in *PRF1* presented "acutely," with a FHL-like syndrome before the age of 12 months, while the remaining 50% had significantly delayed FHL, or presented with other, seemingly unrelated pathologies (23, 37, 45). Most of these patients either developed hematological malignancies (usually beyond the age of 10 years, which we consider to be a "chronic" presentation) or presented with unusual or protracted viral infections (23, 46, 47). Critically, biochemical analysis of multiple missense mutations associated with atypical FHL has led to the realization that partial perforin deficiency results from perforin misfolding: all reported cases of atypical, delayed FHL had some recoverable perforin-mediated cytotoxicity *in vitro* (23). Structural studies recently supported these findings, as wild-type perforin was shown to be a thermo-labile protein, with a melting temperature (the temperature at which the protein denatures and starts to lose function) only slightly above 40°C (5). Even though this analysis was conducted *in vitro*, this finding has raised the intriguing possibility that the protracted, cytokine-induced fever of FHL might further aggravate the disease by accentuating the protein misfolding defect. Given the diversity of clinical presentation that encompasses cancer, atypical onset of viral infections, and attenuated FHL-like syndromes, it is likely that a significant proportion of sub-acute perforinopathies go undiagnosed.

Identification of missense mutations that confer partial activity for the proteins encoded by the *UNC13D*, *STX11*, and *STXBP2* genes may broaden the spectrum of recognized sub-acute perforinopathies. A number of non-synonymous polymorphisms in these genes have already been cataloged in single nucleotide polymorphism (SNP) databases, and future studies will determine whether any of these variations can affect the rate and/or efficiency of secretory granule exocytosis and membrane fusion, steps that ultimately determine the amount of perforin secreted into the IS and its rate of delivery.

CHRONIC PRESENTATIONS OF PERFORINOPATHY

We consider chronic perforinopathies to be disease states that represent as a spectrum of immune-mediated diseases associated with monoallelic mutations in FHL-related genes. The presentations typically do not have a strong resemblance to classic FHL and may include blood cancers and macrophage activation syndrome in patients with juvenile rheumatoid arthritis. Typically, age of onset will be beyond 5 years of age. In addition, some studies have reported an association of *PRF1* polymorphisms and the outcome of allogeneic bone marrow transplantation. All of these associations are contentious, as studies that find no link outnumber those supporting such an association.

Once again, the most reliable and "testable" information comes from the analysis of *PRF1* polymorphisms, as the functional consequence of mutations is well understood. Considering the fact that hundreds of FHL patients with known genetic defects have been diagnosed around the world since 1999, the number of their family members who are known carriers of monoallelic mutations, should exceed 1,000. This is a significant cohort of unrelated individuals, who may be potentially investigated in prospective longitudinal studies on immune surveillance of infections and cancer and for dysregulated immune homeostasis.

An association between presumed partial CL deficiency due to monoallelic A91V perforin polymorphism, and acute lymphoblastic leukemia or macrophage activation syndrome, has been demonstrated in several studies that have necessarily involved a limited number of individuals (48, 49), but no statistical difference was observed in follow up studies when larger cohorts of patients were examined (24, 43). The likely reason for this apparent discrepancy may be the relatively low statistical power of smaller studies.

Another possibility is that a partial deficiency of CL function may predispose more strongly to a specific subtype of malignancy or other pathology. For example, while the largest analysis of patents with acute lymphoblastic leukemia did not reveal an enrichment of A91V carriers compared to healthy controls, a subset of the ALL patients who also had BCR-ABL translocations were more likely to carry the A91V allele than the control group (43). Similarly, in a study that retrospectively examined patients who had been diagnosed with more than one primary malignancy during their lifetime, a significant proportion of patients diagnosed with both melanoma and B cell lymphoma were carriers of A91V or another perforin mutation that is partly inactivating (R28C), than those who had been diagnosed with either disease alone (24).

We have tried to explore the molecular basis for how a monoallelic mutation might adversely affect overall cytotoxic function in an inherited condition classically considered to be recessive. Molecular analysis has revealed that A91V has a partial dominant-negative effect on the function of wild-type perforin; partly misfolded perforin might interfere with the expression or trafficking of normal perforin through the endoplasmic reticulum/Golgi to the secretory granules (31, 50). Once wild-type and misfolded perforin molecules are "mixed" and stored together, it is easy to imagine that perforin pores with a heterogeneous composition of monomers might have disproportionately poor function. We also speculate that in heterozygous individuals, the wild-type allele may be under-expressed relative to the mutated allele, thus further diminishing overall CL function. This possibility needs to be formally excluded, but has been described for many other polymorphic genes [e.g., Ref. (51)].

Further analysis of CL function in patients with immune-mediated disease is warranted, particularly employing recent advances in single-cell microscopy technology, which may reveal CL deficiencies that would otherwise remain undetected in cell population-based experiments (6). Such technologies may validate the more common statistical approaches, where for example, the frequency of a *PRF1* mutation in cancer patients is compared to that in healthy controls. In acute perforinopathy, there is a clear causal relationship between the disease phenotype and the patient's genotype, but this is not the case in cancer (even assuming a clinician considered testing for perforin genotype, which would be unusual). Rather, most chronic perforinopathies are likely to present "indirectly." In cancer, the *PRF1* gene [and, most likely, *UNC13D*, *STX11*, and *STXBP2* (52)] would be considered to be a tumor suppressor gene, but is not the cause of malignancy *per se* and is certainly not the only causal factor. Rather, impaired perforin function reduces the surveillance of cancerous cells, which were generated through an unrelated genetic event. This notion raises a question of what constitutes an appropriate control in these studies. Since the hypothesis is that carriers of monoallelic mutation(s) in *PRF1* or related genes predispose to immune-mediated diseases with uncertain age of onset, taking a "snapshot" of a healthy population may underestimate the impact of mutations. In contrast, it was recently shown that individual differences between the cytotoxic activity of NK cells (and cytotoxic T cells) are much smaller than previously thought (6). This opens an avenue for "personalized" analysis of CL function that may shed light on predisposition of an individual to an immune pathology, prospectively and retrospectively.

SUMMARY

In this discourse, we have proposed the term "perforinopathy" to denote the wide and diverse spectrum of manifestations of perforin deficiency, both temporal and clinical. Early (acute) clinical presentations are often fatal and usually represent dysregulated immune homeostasis and the resultant hypercytokinemia ("cytokine storm") and hemophagocytosis consequent on macrophage activation. Sub-acute presentations still occur in infancy/early childhood, may typically have a more benign course that is still principally inflammatory and may accompany other pathologies such as juvenile idiopathic arthritis or virus infection. Chronic manifestations of perforin deficiency may appear as late as adulthood, and the principal cause of symptoms is the failure to clear specific dangerous cells, particularly pre-malignant cells. Hematopoietic malignancies seem to be a common outcome, and some patients may present well into adulthood with more than one primary cancer: early onset malignant melanoma and lymphoma is the commonest combination we have observed. Although most of the data supporting our proposed "classification" is centered on our knowledge of *PRF1* gene mutations, we hope that this paper will stimulate further study on polymorphisms and mutations of other genes that play a role in delivering functional perforin to the target cell.

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Familial hemophagocytic lymphohistiocytosis: when rare diseases shed light on immune system functioning

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The human immune system depends on the activity of cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and NKT cells in order to fight off a viral infection. Understanding the molecular mechanisms during this process and the role of individual proteins was greatly improved by the study of familial hemophagocytic lymphohistiocytosis (FHL). Since 1999, genetic sequencing is the gold standard to classify patients into different subgroups of FHL. The diagnosis, once based on a clinical constellation of abnormalities, is now strongly supported by the results of a functional flow-cytometry screening, which directs the genetic study. A few additional congenital immune deficiencies can also cause a resembling or even identical clinical picture to FHL. As in many other rare human disorders, the collection and analysis of a relatively large number of cases in registries is crucial to draw a complete picture of the disease. The conduction of prospective therapeutic trials allows investigators to increase the awareness of the disease and to speed up the diagnostic process, but also provides important functional and genetic confirmations. Children with confirmed diagnosis may undergo hematopoietic stem cell transplantation, which is the only cure known to date. Moreover, detailed characterization of these rare patients helped to understand the function of individual proteins within the exocytic machinery of CTL, NK, and NKT cells. Moreover, identification of these genotypes also provides valuable information on variant phenotypes, other than FHL, associated with biallelic and monoallelic mutations in the FHL-related genes. In this review, we describe how detailed characterization of patients with genetic hemophagocytic lymphohistiocytosis has resulted in improvement in knowledge regarding contribution of individual proteins to the functional machinery of cytotoxic T- and NK-cells. The review also details how identification of these genotypes has provided valuable information on variant phenotypes.

Keywords: cellular cytotoxicity, natural killer, hemophagocytosis, mutation analysis

INTRODUCTION

Defense of an organism against pathogens and cancer is accomplished by the cooperation between different cell types of the human immune system, in particular cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and NKT cells (1). Although these cells use different receptors to identify a target cell, once activated they all employ a similar, highly coordinated machinery that delivers the lethal hit by the secretion of secretory lysosomes (2). Secretory lysosomes in CTL, NK, and NKT cells are lysosome-related organelles that contain perforin, a tetrameric protein, which is able to form pores across lipid bilayers and serine proteases, such as granzymes. Upon release of perforin into the gap formed between the effector and target cell, perforin pores allow granzymes to enter the target cell and to trigger apoptosis by initiation of the caspase cascade (3). Although in this review we will address more specifically some features of the NK cells, because they are suitable for rapid diagnostic assays, it is important to recognize that T-cells share most of these features and have great relevance in most of the human disease related to impairment of this machinery.

Natural killer cells serve as pivotal sentinels within the immune system as they respond quickly to a pathogenic infiltration and alert the host about infections. They are able to recognize and lyse tumor cells and virus-infected cells, even without any previous sensitization. NK cells originate in the bone marrow and are released into the bloodstream upon maturation, thus being able to respond to stimuli such as pathogen molecules, cytokines, or by the interaction with any target cell that expresses ligands for activating NK cell receptors. Since inhibitory receptors recognize the ubiquitously expressed MHC class I molecules, NK cells do not attack healthy cells. On the contrary, NK cell activation occurs when a potential target cell lacks surface MHC class I, as observed in cancer or infection. This concept has been defined as the "*missing self-hypothesis*." As a result, NK cells behave as fine-tunable effector cells: their regulation may occur at different levels, since the number of receptors which are expressed on their

surface may be modified according to the level and type of circulating cytokines. The balance of signals received from activating and inhibitory receptors determines the outcome of NK cell function. Furthermore, an increasing interest is directed to the interaction (*cross-talk*), which occurs between NK cells and other cells engaged in the early phase of inflammatory response (4), which have major implications for the ability of the organism to react to infection and cancer.

Activation and priming of cytotoxic T- and NK-cells induces the formation of secretory lysosomes, which contain lytic molecules. The complex machinery of handling and re-locating these granules became more readable by investigation of human experimental models represented by a congenital immune deficiency in which defective granules activity has a pivotal pathogenic role.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome observed more often, but not exclusively, in children. Accumulation of reported cases and investigation of consanguineous families allowed to define that the familial form of HLH is indeed a congenital immune deficiency; to date, four subtypes are defined by mutations in the following genes: *PRF1* in familial hemophagocytic lymphohistiocytosis type 2 (FHL2), *UNC13D* in familial hemophagocytic lymphohistiocytosis type 3 (FHL3), *STX11* in familial hemophagocytic lymphohistiocytosis type 4 (FHL4), and *STXBP2* in familial hemophagocytic lymphohistiocytosis type 5 (FHL5). These subtypes will be described below in more details. Furthermore, some patients with other congenital immune deficiencies [X-linked lymphoproliferative disorder, Griscelli syndrome, Chédiak–Higashi syndrome (CHS), and Hermansky–Pudlak syndrome (HPS)] may develop HLH. A fully overlapping clinical picture may be observed, most often following viral infection, in patients with no evidence of genetic defect; these cases have been usually defined as "secondary" or HLH. Patients with HLH are often older than those with underlying genetic defect, but the clinical course may be equally life-threatening. The approach to patients with HLH/FHL is aimed at achieving clinical remission while defining the diagnosis. Definition of FHL has immediate therapeutic implications inasmuch hematopoietic stem cell transplantation (HSCT) is mandatory for FHL, but not for HLH, with the only exception of those cases in which the disease recurs and proves to be familial (and potentially linked to other or unknown genes) or treatment-dependent.

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Familial hemophagocytic lymphohistiocytosis is a genetically heterogeneous disorder caused by mutations in genes involved in the secretory lysosome-dependent exocytosis pathway. More than 60 years after the first FHL case was reported, five independent, FHL-causing loci have been identified and the underlying genetic defect has been described for four of them (**Table 1**). Moreover, we have progressively learned that other immune deficiencies have a clinical picture that may initially lead to a diagnosis of FHL. The genes involved are numerous and some of them are difficult to analyze due to the size of the gene and heterogeneity of mutations. Yet, around 70% of cases are explained by mutations in only two genes: *PRF1* and *UNC13D*, which cause the FHL2 and FHL3 subgroups, respectively (5).

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 2 (OMIM 603553)

Nearly 15 years after its genetic definition, it is now clear that FHL2 accounts for 20–50% of all FHL cases, depending on the cohort studied (7–9). Mutations in the perforin gene (*PRF1*, OMIM 170280) are responsible for this phenotype (8–10). *PRF1* has three exons: exon 2 and 3 code a 555 amino-acid polypeptide. Over 120 different mutations have been identified to date: 101 missense/non-sense mutations and 21 deletion/insertion mutations (8, 10–17). Since the pathogenic role of some mutations, especially some single amino-acid substitutions, is difficult to assess, their functional characterization is still required. In a recent comprehensive *in silico* analysis of 76 missense mutations in *PRF1*, An et al. used a structural approach to explain the effect of a mutation on the ability of perforin to oligomerize, thereby offering an explanation for the observed defect in cytotoxicity in FHL2 patients (18).

Accumulation of a sufficient number of cases helped to establish connections between specific mutations and particular ethnic groups: c.1122G > A (p.W374X) was found to have a high

Table 1 | Overview of some characteristics of genetic disorders associated with occurrence of hemophagocytic lymphohistiocytosis.

Subtype	OMIM number	Mutated gene	Locus	Affected protein	Animal model
FHL1	603552		9q21.3–22	Unknown	None
FHL2	267700	*PRF1*	9q21.3–q22	Perforin	$Pfn1^{-/-}$
FHL3	608898	*UNC13D*	17q25.1	Munc13-4	Jinx
FHL4	603552	*STX11*	6q24	Syntaxin 11	None
FHL5	613101	*STXBP2*	19p13	Munc18-2	None
GS2 (Griscelli)	607624	*RAB27A*	15q21	RAB27a	Ashen
CHS (Chédiak–Higashi)	214500	*LYST*	1q42.1–q42.2	LYST	Beige
HPS2 (Hermansky–Pudlak)	608233	*ADTB3A*	5q14.1	AP-3	Pearl
XLP-1	308240	*SH2D1A*	Xp25	SAP	$Sh2d1a^{-/-}$
XLP-2	300635	*XIAP*	Xp25	XIAP	$XIAP\text{-}ps1^{-/-*}$

**The $XIAP\text{-}ps1^{-/-}$ knockout mouse described in 2009 by Kotevski et al. did not phenocopy the XLP-2 human disease [Ref. (6)].*

incidence in Turkish patients (9); mutation c.50delT (p.L17FsX) is very frequent in patients of African-American origin (19); while the c.1090-1091delCT (p.L364fsX) mutation has only been identified in Japanese patients (10).

Mutation c.272C > T (p.A91V) has a particularly high frequency in Southern European population, ranging from 2.5 up to 10% (20, 21). It seems to be present at a very low frequency in African-American subjects and Sub-Saharan Africans, with no reported cases of the polymorphism in Japan, supporting the concept of a Mediterranean origin of the mutation. Although its impact on the protein structure and function has been documented (22, 23), its pathogenic role in FHL has been considered controversial. It is now clear that patients with FHL2 frequently show A91V mutation in combination with another pathogenic mutation; association of A91V with another missense/hypomorphic mutation frequently results in a late-onset of FHL2 (24). Interestingly, in addition to the 106 mutations described in FHL2 patients, other mutations have been associated with different phenotypes (see below).

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 3 (OMIM 608898)

Familial hemophagocytic lymphohistiocytosis type 3 is caused by mutations in the *UNC13D* gene (OMIM 608897), which encodes the 1,090 amino-acids-long protein Munc13-4. Munc13-4 is involved in the priming of secretory granules and their fusion with the plasma membrane, and its loss-of-function consequently impairs the release of perforin and granzyme into the synaptic cleft (25).

Familial hemophagocytic lymphohistiocytosis type 3 covers between 30 and 40% of FHL patients, based on different geographic areas and ethnic groups. The clinical picture of FHL3 patients is undistinguishable from that of FHL2 patients. However, evaluation of patient CTL and NK cells by flow-cytometry showed a clear difference: while FHL2 patients have no perforin but show no degranulation defect, cells from FHL3 patients have normal perforin expression but reduced to absent degranulation (26) (**Table 2**).

UNC13D consists of 32 coding exons. To date at least 112 different mutations in *UNC13D* have been reported as a cause of FHL3: 60 missense/non-sense, 25 splicing/regulatory, 25 deletion/insertion mutations, as well as 2 complex gene rearrangements (25–28). After some initial difficulties in recognizing biallelic mutations in patients with FHL3, it became evident that not only exonic mutations, but also variations outside exons and splice sites are a common cause of FHL3 (29). Since then, additional contribution confirmed this issue. In 2011, Meeths et al. described two novel mutations frequently occurring in Northern European populations: the deep intronic mutation c.118-308G > A selectively impairs *UNC13D* transcription in lymphocytes, thus abolishing Munc13-4 expression while the 253-kb inversion affects the 3′-end of the transcript, also abolishing Munc13-4 expression (30). Another deep intronic mutation, c.118-307G > A, was recently reported in a Chinese patient and documented to impair *UNC13D* transcription, possibly by disrupting a transcription factor binding-site or enhancer element (31). Two mutations have been described in specific populations: the c.1596 + 1G > C mutation is described as the most common *UNC13D* mutation in Japan (29) while mutation c.754-1G > C is predominantly found in Korean patients (32). Altogether, the mutation analysis of patients with FHL3 turned out to be far more engaging than that of patients with FHL2.

Recently in a genotype–phenotype study of 84 patients with FHL3 from Italy, Germany, and Sweden, Sieni et al. described that central nervous system (CNS) involvement is more common in patients with FHL3 than with FHL2. Moreover, the combination of fever, splenomegaly, thrombocytopenia, and hyperferritinemia appears to be the most easily and frequently recognized clinical pattern in FHL3, and in association with a defective granule release assay may lead to clinical suspicion of FHL3 (28).

While FHL2 and FHL3 subgroups account for the majority of patients with FHL, additional genetic subgroups have been progressively identified.

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 4 (OMIM 603552)

Familial hemophagocytic lymphohistiocytosis type 4 is caused by mutations in the *STX11* gene (OMIM 605014) (33). This gene consists of two exons and encodes the 287 amino-acid-long SNARE protein Syntaxin 11 (Stx11).

Table 2 | Overview of the functional characteristics of genetic disorders associated with occurrence of hemophagocytic lymphohistiocytosis.

Subtype	Protein expression	NK cell function	Degranulation
FHL1	Unknown	Unknown	Unknown
FHL2	Perforin reduced to absent, detectable by flow-cytometry	Defective killing	Normal
FHL3	Munc13-4 defect, detectable by western blot	Variably impaired	Reduced
FHL4	Syntaxin 11 defect, detectable by western blot	Variably impaired	Reduced
FHL5	Munc18-2 defect, detectable by western blot	Variably impaired	Reduced
GS2 (Griscelli)	Rab27a defect, detectable by western blot	Variably impaired	Reduced
CHS (Chédiak–Higashi)	Lyst defect, detectable by western blot	Variably impaired	Reduced
HPS2 (Hermansky–Pudlak)	AP-3 defect, detectable by western blot	Variably impaired	Reduced
XLP-1	SAP defect, detectable by flow-cytometry	Inhibitory 2B4, defective vs. B-EBV	Normal
XLP-2	XIAP defect, detectable by flow-cytometry (with limitations) and western blot	Enhanced AICD	Normal

Although original reports of FHL4 were restricted to families of Turkish/Kurdish origin, more recently patients of different origins have been identified with a defect in *STX11*. Despite the initial clustering of cases, at least 12 different *STX11* mutations have been described to date: 5 missense/non-sense mutations, 5 small deletions, 1 small deletion, and 1 gross deletion (34–36). Patients with FHL4 seem to have a later onset and a less severe clinical presentation of the disease compared to FHL2 and FHL3 (37). Mutations in *STX11* have never been associated with variant phenotypes, different from FHL4.

Recently, Sepulveda et al. tried to elucidate the role of *STX11* mutations in the pathogenesis of FHL. They generated a Stx11-deficient ($Stx11^{-/-}$) murine model that faithfully reproduced the manifestations of HLH and represented a suitable model for studying FHL4 *in vivo* and the role of Stx11 *in vitro*. By comparing the severity of HLH in $Stx11^{-/-}$ mice with that observed in $Rab27a^{-/-}$ and $Prf1^{-/-}$ mice, they established a correlation between the murine mutants and the age at HLH onset in their human counterparts (38). Furthermore, in a recent report the *STX11* L58P mutation revealed that both the N-terminus and Habc domain of Stx11 are required for binding to Munc18-2, implying similarity to the dynamic binary binding of neuronal syntaxin 1 to Munc18-1 (39).

FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 5 (OMIM 613101)

Familial hemophagocytic lymphohistiocytosis type 5 is due to mutations in *STXBP2* (also named *MUNC18-2*; OMIM 601717) (40, 41) and has been reported to account for up to 20% of cases with FHL in the German series (42). Since 2010, 40 different mutations of *STXBP2* have been described: 15 missense/non-sense, 10 splicing/regulatory, and 15 deletion/insertion mutations (41, 43–46). FHL5 does not appear to be restricted to a specific geographic region.

In contrast to what was observed for the comparison between FHL2 and FHL3, some clinical presentations of FHL5 seem to be different from other classical manifestations of FHL. Gastrointestinal symptoms, such as chronic diarrhea, gastro-esophageal reflux, and abdominal pain are present in a significant number of patients. Renal tubular dysfunction was also observed in one patient. This could be explained by an impaired expression and function of Munc18-2/STXBP2 protein in cells other than cytotoxic lymphocytes, including intestinal and renal epithelium (47). The defect caused by insufficient function of STXBP2 protein in the neutrophils is associated with defective mobilization of the granules. As a result, the cell is unable to kill bacteria; insufficient clearance of *E. coli* might be one or the main reason for the frequency of gastrointestinal symptoms in patients with FHL5 (42, 46–48).

Since platelets contain syntaxin-binding proteins with non-redundant functional roles, platelets from FHL5 patients have defective secretion, with decreased Munc18-2 and Stx11 levels. These data demonstrated a key role for Munc18-2, perhaps as a limiting factor, in platelet exocytosis, suggesting that it regulates Stx11 (49). These data together with those of Pagel et al. (41) suggest that, although bleeding histories may be too variable to be a sufficient diagnostic criteria, platelet function assays may be worth investigating in patients with FHL.

ADDITIONAL GENETIC IMMUNE DEFICIENCIES ASSOCIATED WITH HLH

In addition to the described four subgroups of FHL, in which HLH is usually the primary manifestation, a few additional genetic conditions may cause a clinical syndrome largely overlapping that of FHL but in which additional, distinctive clinical features occur.

X-LINKED LYMPHOPROLIFERATIVE DISEASE 1 (DUNCAN DISEASE, OMIM 308240)

X-linked lymphoproliferative disease 1 (XLP-1) is a rare congenital immunodeficiency caused by mutations in *SH2D1A* (Xq25), the gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) (50, 51).

In immune-competent individuals Epstein–Barr virus (EBV) causes infectious mononucleosis, a common, usually self-limited disease. In XLP-1, the lack (or dysfunction) of SAP causes the selective inability to control infection by EBV, a γ-herpes virus that infects B-cells (52–55). Several immunological defects have been identified, including defective NK and $CD8^+$ T-cell-mediated cytolytic responses against EBV-infected cells, which lead to B-cell accumulation and persistence of reactive inflammatory responses (56). In the absence of SAP, 2B4 receptor (member of SLAM family), when engaged by its ligand CD48, delivers inhibitory instead of activating signals (53). It has been recently demonstrated that in XLP-1 NK cells the co-engagement of 2B4 with different activating receptors inhibits NCR, CD16, and activating KIRs, characterized by ITAM-dependent signaling pathways. In contrast, the 2B4 dysfunction does not affect the activity of DNAM-1 and NKG2D triggering receptors. Thus, while $CD48^+$ B-EBV and lymphoma B-cells devoid of NKG2D and DNAM-1 ligands were resistant to lysis, the preferential usage of these receptors allowed XLP-1 NK cells to kill lymphomas that expressed sufficient amounts of the specific ligands (57). Better knowledge of the underlying dysfunction could be turned into a diagnostic tool. Patients with XLP-1 may present with different phenotypes: fulminant mononucleosis, B-cell lymphoma, lymphoproliferation, and dysgammaglobulinemia (56, 58, 59), but also with HLH (60). Thus, differential diagnosis is relevant. To this issue, immunological screening for intracytoplasmic SAP expression and rapid assays to examine 2B4 receptor function, which is inhibitory instead of activating in *SH2D1A* mutated patients (53, 57), may be applied (Manuscript in preparation).

In our review of the literature, 100 *SH2D1A* mutations were found: 46 missense/non-sense, 14 splicing, 2 regulatory mutations, 9 small deletions, 6 small insertions, and 23 gross deletions (50–58, 61). Interestingly, this gene is characterized by a high number of deletions including the entire gene. Intronic mutations have also been described affecting *SH2D1A* transcription but not mRNA splicing, and leading to markedly reduced level of SAP protein (61). Thus, the strategy of mutation analysis of this gene must be designed to include these possible variants.

X-LINKED LYMPHOPROLIFERATIVE SYNDROME TYPE 2 (OMIM 300635)

A subset of patients with an XLP-like phenotype was recently found to have mutations in *BIRC4*, the gene encoding the X-linked inhibitor of apoptosis protein (XIAP) and has been linked to

another subgroup, named X-linked lymphoproliferative syndrome type 2 (XLP-2) (62).

X-linked inhibitor of apoptosis protein is an essential ubiquitin ligase for pro-inflammatory signaling downstream of the nucleotide-binding oligomerization domain containing (NOD)-1 and -2 pattern recognition receptors. Recently, the XIAP baculovirus IAP repeat (BIR2) domain was recognized as a hotspot for missense mutations in XLP-2. XLP-2-BIR2 mutations severely impair NOD-1/2-dependent immune signaling in primary cells from XLP-2 patients and in reconstituted XIAP deficient cell lines. XLP-2-BIR2 mutations abolish the XIAP–RIPK2 interaction resulting in impaired ubiquitylation of RIPK2 and recruitment of linear ubiquitin chain assembly complex (LUBAC) to the NOD-2-complex. These new findings document that impaired immune signaling in response to NOD-1/2 stimulation is a general defect in XLP-2 and demonstrate that the XIAP BIR2–RIPK2 interaction might be even targeted pharmacologically to modulate inflammatory signaling (63).

In a comparison of the clinical phenotypes associated with XLP-1 and XLP-2, EBV infection was the common trigger of HLH in 92% of XLP-1 and 83% of XLP-2. HLH (XLP-1, 55%; XLP-2, 76%) and hypogammaglobulinemia (XLP-1, 67%; XLP-2, 33%) occurred in both groups, although with different proportions. Survival rates and mean ages at the first HLH episode did not differ for both groups, but HLH was more severe with lethal outcome in XLP-1. Only XLP-1 patients developed lymphomas while XLP-2 patients preferentially displayed chronic hemorrhagic colitis, recurrent splenomegaly often associated with cytopenia and fever (64).

In a similar study, Marsh et al. reported an early disease onset during infancy for XLP-2-linked HLH and a high relapse rate; however, this seemed to occur even in the absence of an EBV infection. Some XLP-2 patients develop hypo/dysgammaglobulinemia resulting from humoral immune system derangement. Intriguingly, and in contrast to XLP-1, XLP-2 was never associated with common variable immunodeficiency (64).

In a third consortium review of 25 patients, the majority initially presented with manifestations other than HLH, such as Crohn-like bowel disease ($n = 6$), severe infectious mononucleosis ($n = 4$), isolated splenomegaly ($n = 3$), uveitis ($n = 1$), periodic fever ($n = 1$), fistulating skin abscesses ($n = 1$), and severe Giardia enteritis ($n = 1$). Subsequent manifestations included celiac-like disease, antibody deficiency, splenomegaly, and partial HLH. Screening by flow-cytometry identified 14 of 17 patients in this cohort (65). Given these clinical differences, XIAP deficiency must be considered in a wide range of clinical presentations. It has recently been suggested that XIAP deficiency would be better classified if defined as an X-linked subtype of FHL, rather than as a second type of XLP (64).

To date, 41 mutations are known in *BIRC4*: 20 missense/non-sense, 2 splicing mutations, 2 regulatory mutations, 16 deletions/insertions, and 1 complex rearrangement (62, 64, 66, 67). The phenotypic differences may be the result of differences in the molecular basis of each disease. However, neither genotype, nor protein expression, nor results from cell death studies were clearly associated with the clinical phenotype.

Although HSCT remains the milestone for cure of FHL, some discrepancy recently emerged in the outcome of patients with XLP-2. In an international survey of 19 patients, 7 received myeloablative (MAC) regimens, 1 received an intermediate-intensity regimen, and 11 received reduced intensity conditioning (RIC) regimens predominantly consisting of alemtuzumab, fludarabine, and melphalan. The probability of survival was very low in the MAC group, with all but one patient dying from transplantation-related toxicities (especially veno-occlusive disease and pulmonary hemorrhage); otherwise, 55% of those who received RIC survived at a median of 570 days after HSCT. The probability of surviving in the RIC was enhanced by disease inactivity at the time of HSCT. Based on these findings, MAC regimens should not be used for patients with XIAP deficiency. The reason may be connected with the loss of XIAP anti-apoptotic functions in XLP-2 patients (68).

IMMUNODEFICIENCIES ASSOCIATED WITH HLH AND PARTIAL ALBINISM

At present three syndromes are known to cause HLH and manifest with partial albinism.

CHÉDIAK–HIGASHI SYNDROME (OMIM 214500)

Around 85% of CHS patients develop HLH in the first decade of life. They show markedly defective cytotoxicity of both NK cells and CTL. The genetic defect is caused by mutations in the *LYST* gene (69), which encodes a 3,801 amino-acid protein. Each clinical manifestation of CHS (albinism, bleeding tendency, recurrent bacterial infections, neurologic dysfunction, and HLH) (70, 71) is associated with a defect of a specific cell type and the formation of enlarged lysosomes in these cells.

The presence of giant inclusion bodies of lysosomal origin in a variety of granule-containing cells, including hematopoietic cells and melanocytes, has thus become the hallmark of the disease (72). This feature together with HLH and oculo-cutaneous albinism can address clinical suspicion toward CHS and direct molecular analysis to *LYST* sequencing. In our revision of the available literature, a total of 56 mutations were found to be reported at the time of writing: 23 missense/non-sense, 4 splicing, 20 small deletions, 8 small insertions, and 1 gross deletion. As expected, disruptive mutations correlated with the severe form of the disease (73). The wider diffusion of mutation analysis, despite the big size of the gene, provided an increased number of reports from different geographic areas during the last few years, confirming that CHS has no ethnic or geographic boundaries.

A rare neurologic disorder, named hereditary spastic paraplegia (HSP) and characterized by leg spasticity, weakness, hyperreflexia, and additional neurological symptoms, was recently reported in two adult siblings with HSP and homozygous *LYST* pathogenic mutation. Large peroxidase-positive granules were observed in both patients' granulocytes, while pigment deficiency, immune deficiency, and bleeding tendency were not observed. This example illustrates nicely how the clinical spectrum of CHS may be much broader than recognized at present (74).

GRISCELLI SYNDROME TYPE 2 (OMIM 607624)

Griscelli syndrome is a rare autosomal-recessive disorder characterized by partial oculo-cutaneous albinism and HLH (75). Among

GS subtypes, only patients with type 2 develop HLH. Mutations causing Griscelli syndrome type 2 (GS2) were mapped to the *RAB27A* gene, which is composed of 5 coding exons and encodes for a 221 amino-acid protein that belongs to the superfamily of small Rab GTPase. CTLs and NK cell activity defect results from the inability of cytotoxic granules to dock to the plasma membrane whereas hypopigmentation is accounted for by a defective release of melanosomes from melanocyte dendrites. Change in phagosomal function and antigen cross-presentation of Rab27a-deficient dendritic cells has been reported *in vitro*. In the mouse model (ashen mice), the dendritic cells are unable to perform a sufficient antigen cross-presentation (76).

To date 34 *RAB27A* mutations are known: 15 missense/non-sense, 4 splicing, and 15 deletions/insertion. In 2010, Meeths et al. sequenced *RAB27A* in patients diagnosed as HLH and found one mutated family (77). Since the clinical picture of the two syndromes is indistinguishable, they concluded that the diagnosis of GS2 may be overlooked, particularly in fair-haired patients with hemophagocytic syndromes.

HERMANSKY–PUDLAK TYPE 2 (OMIM 608233)

The term HPS encompasses nine different human autosomal-recessive genetic disorders, sharing partial oculo-cutaneous albinism and bleeding disorders (78, 79). Furthermore, patients with Hermansky–Pudlak type 2 (HPS2) also show an increased susceptibility to infections, resulting from both congenital neutropenia and impaired cytotoxic activity. Mutations of the gene encoding the β-3A subunit of *adaptor protein-3* (AP-3) complex are the cause of HPS2 (80). To date, 20 mutations are known in this gene and are associated with HPS2: 7 missense/non-sense, 1 splicing, 11 deletions/insertion, 1 complex rearrangement, and a chromosome 5 inversion that disrupts the gene sequence (81). To date, only one HPS2 patient has been reported who developed HLH. However, since this patient also carried a potentially contributing heterozygous RAB27A mutation, the risk to develop HLH in HPS2 remains unclear (82). The pearl mouse model of HPS2, upon infection with lymphocytic choriomeningitis virus, developed all the key features of the disease, which yet was only transient (82). In a cohort of 22 HPS2 patients, only one additional patient with HLH was identified; two developed incomplete, transient HLH-like episodes, although the cytotoxicity or degranulation capacity was impaired in all 16 patients tested (83). Although future reports might clarify the genotype–phenotype correlations, the risk for HLH in HPS2 appears lower than in Griscelli or CHS (79, 82).

CLINICAL PICTURE OF FHL

Most patients with FHL are brought to the attention of the pediatrician because of long-lasting fever, which does not respond to antibiotic therapy. Physical examination usually shows hepatosplenomegaly (84); up to 30% of cases also show neurological abnormalities, from irritability to cranial nerve palsy or seizures. Cerebrospinal fluid analysis shows alterations in more than half of patients, with pleocytosis, increased protein, or both. Several studies have defined the pattern of alterations evident at neurologic imaging: parenchymal atrophy, diffuse abnormal signal intensity in the white matter on T2-weighted images, focal hyperintense lesions, delayed myelination, or parenchymal calcification (85, 86).

Characteristic bio-markers are elevated ferritin, triglycerides, α-chain of the soluble interleukin-2 receptor (sCD25), and low fibrinogen. Hemophagocytosis by activated macrophages (which became popular by being included in most of the different names used for this disease since its original report in 1952) may be lacking at initial bone marrow examination. This negative finding does not preclude the diagnosis of HLH, as well as the presence of hemophagocytosis alone does not make the diagnosis of HLH, and should be considered supportive evidence only. Thus, the relevance of hemophagocytosis in the set of diagnostic criteria might even be questionable. Additional findings in a minority of patients are: lymphadenopathy, icterus, rash, edema, high levels or transaminases, bilirubin, and lactate dehydrogenase. Accumulation of cases allowed to characterize unusual presentations: acute liver failure or isolated CNS involvement may be observed. This led to the practice to consider HLH in the differential diagnosis of patients scrutinized for possible liver transplant (87). Identification of high levels of selected cytokines provided a likely explanation for some clinical features: fever is induced by IL-1 and IL-6; pancytopenia results from high levels of IFN-γ and TNF-α and also from hemophagocytosis; hypertriglyceridemia results from the inhibition of lipoprotein lipase by TNF-α; ferritin is secreted by activated macrophages, also responsible for the high levels of plasminogen activator, which cause high plasmin levels and hyperfibrinolysis. The picture of the contribution of the individual cytokines and chemokines is entirely under thorough evaluation by several investigators.

DIAGNOSTIC STRATEGY

To facilitate an initial approach to the disease, in 1994 the Histiocyte Society defined a set of diagnostic criteria; they were subsequently revised in 2004 (**Table 3**) (88). Nevertheless, diagnosing

Table 3 | Revised diagnostic guidelines for hemophagocytic lymphohistiocytosis (HLH).

The diagnosis of HLH can be established if either 1 or 2 below are fulfilled
1. A molecular diagnosis consistent with HLH
2. Clinical and laboratory criteria for HLH fulfilled (5/8 criteria below)
Fever
Splenomegaly
Cytopenia (affecting ≥2 of 3 lineages in peripheral blood)
Hemoglobin <9 g/dl (in infants <4 weeks: Hb <10 g/dl)
Platelets $<100 \times 10^9$/l
Neutrophils $<1.0 \times 10^9$/l
Hypertriglyceridemia and/or hypofibrinogenemia
Fasting triglycerides ≥3.0 mmol/l
Fibrinogen ≤1.5 g/l
Hemophagocytosis in bone marrow or spleen or lymph nodes
Low or absent NK cell activity
Ferritin ≥500 μg/l
Soluble CD25 (i.e., soluble IL-2 receptor) ≥2,400 U/ml

Supportive evidences are cerebral symptoms with moderate pleocytosis and/or elevated protein, elevated transaminases and bilirubin, LDH.

FHL still represents a challenge for the pediatrician. Although most of the cases develop the disease when they are very young, about 20% of cases only present once the subject is older than 2 years (76); later onset, including the young adult age, is increasingly reported (89–92). The information of parental consanguinity may be useful but is expected in no more than 25% of cases. The family history may include the early death of a sibling with undefined cause, or diagnosed as "lymphoma or infection." Evidence of partial albinism, or "light hairs" is not frequent but, when present, turns to be very informative.

The set of alterations described above, however are not specific. Thus, in many cases leukemia is suspected at first but quickly ruled out by bone marrow examination, which shows hemophagocytosis in about one-half of all cases. Similarly, none of the biochemical abnormalities described earlier are exclusive to HLH. Investigators wonder if these criteria might be simplified. The evidence of defective NK cell activity, although very suggestive for the diagnosis, is laborious and restricted to a limited number of laboratories also due to the usual need for radionuclide reagents. The real role of hemophagocytosis is repeatedly questioned. To this issue, we observed that the combination of fever, splenomegaly, and thrombocytopenia, in the absence of leukemia, represents the starting point to suspect FHL (28). When this is associated with hyperferritinemia, the clinician may consider it as a sufficient basis to suspect HLH and thus address the diagnostic work-up already within a few hours from admission (**Figure 1**). The level of D-dimers is usually abnormal even when INR/PTT is normal. The possible contribution of additional parameters, such as plasma levels sCD25, sCD163, neopterin, and IFN-γ appears increasingly convincing (86).

As in many other immune deficiencies, common pathogens, especially viruses, may represent an excessive challenge for the child with FHL and thus trigger the onset of the disease. It is very important to remember that patients with visceral leishmaniasis

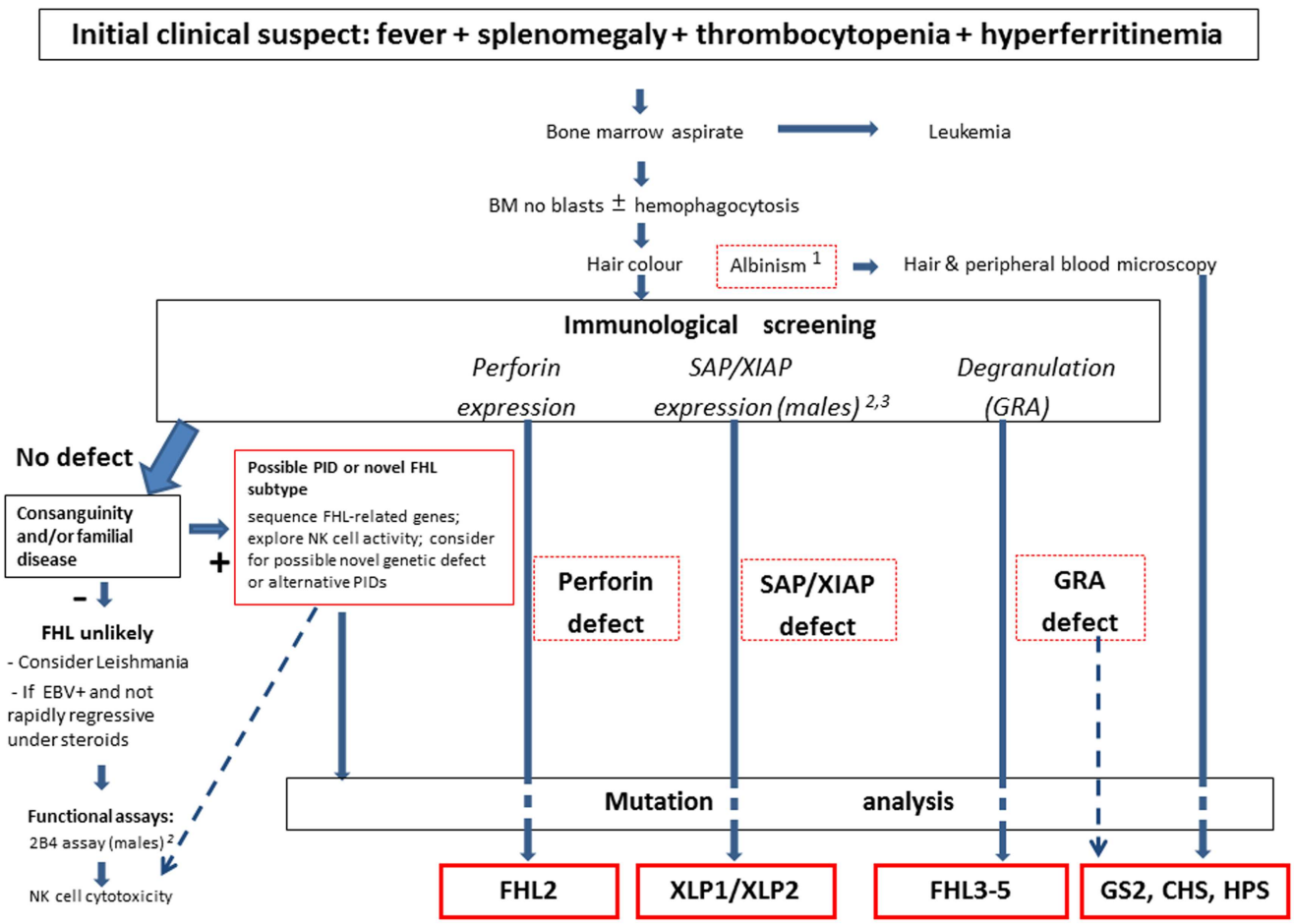

FIGURE 1 | Overview of the current diagnostic approach to FHL. The clinical suspect is raised by a few elements, which may trigger the use of the functional laboratory screening at flow-cytometry. Mutation analysis remains the gold standard for confirmation of the diagnosis, with therapeutic implications. Notes: 1. Some patients with pigment dilution syndromes (GS, CHS, or HPS) may have gray or very blond hairs, not necessary albino discoloration. 2. Although lack of SAP expression at flow-cytometry is invariably associated with XLP-1, in rare cases patients with XLP-1 may have residual, reduced, or even normal SAP expression; thus the 2B4 assay may be used to discriminate between normal (activating 2B4) or defective (inhibitory 2B4) SAP function. 3. The use in flow-cytometry of currently available anti-XIAP mAb does not provide fully satisfactory results, while it is more suited for western blot analysis. Thus mutation analysis may be necessary to rule out the diagnosis in the presence of suggestive clinical picture.

may present with a very similar clinical picture (93), but especially in non-endemic areas this may not be readily included in the differential diagnosis. Unfortunately, patients with undiagnosed leishmaniasis have been diagnosed and then treated as FHL, with major consequences. Thus, the use of polymerase chain reaction (PCR) for investigation of selected infectious agents, including EBV, cytomegalovirus (CMV), and Leishmania appears to be recommended.

If a patient shows the set of initial parameters that match the diagnostic hypothesis, such as fever, splenomegaly, thrombocytopenia, and elevated ferritin, the attending physician should approach an immunology laboratory, which is able to perform a functional screening. Impaired NK cell cytotoxicity, measured as lysis of K652 cells by peripheral blood lymphocytes in a standard chromium release assay, became the hallmark of FHL. However, the variability of NK cell percentages in PBL among different individuals and the use of radioactivity present major limitations to get precise and standardized results in different laboratories. The use of the NK cytotoxicity assay is useful when it shows reduced or absent activity, but normal NK function assay should not definitely exclude the diagnosis of FHL.

Thus, a set of tools for the screening of FHL and other genetic immune deficiencies associated with HLH has been developed. A deficient intra-cytoplasmic expression of perforin by NK cells can identify patients with FHL2 (14). Furthermore, defective intracellular expression of SAP and XIAP are associated with XLP-1 and XLP-2, respectively. For patients with normal findings at the above described assays, Marcenaro et al. have originally demonstrated that surface CD107a expression represents a rapid tool for identification of patients with degranulation defect (25). CD107a (LAMP1) lines the lysosome containing perforin and granzymes to prevent the NK and CTL itself from damage. After activation, the granules move to the synapse with a fine-tuned process of polarization and docking to the cell membrane. After the degranulation into the synapse, CD107a can be identified on the surface of NK and CTLs. Mutations of genes that produce proteins necessary for this secretory mechanism result in the absence of CD107a measured by flow-cytometry. In FHL2, perforin is low or absent but degranulation still takes place. Thus it is important to measure perforin expression before the degranulation assay is done. This is true as well for XLP-1 and XLP-2 which are not associated with failure of degranulation, and these proteins should be measured at least in male patients with suspected genetic HLH. It is important to note that although a minority of patients with a clinical diagnosis of secondary HLH may have an abnormal resting NK cell degranulation, none shows abnormal degranulation using interleukin-2 (IL-2)-activated NK cells (94).

A degranulation defect, which is present in the majority of FHL cases, was first documented in patients with FHL3 (95), and rapidly became the standard for their identification. Thereafter, additional reports confirmed this finding also in patients with FHL4, FHL5, GS2, CHS, and HPS2 (94). In patients with a high probability for genetic defect, in whom flow-cytometry screening of relevant molecules was normal, assessment of cytotoxic activity appears mandatory. Repeated evidence for complete or partially defective killing and/or degranulation should be taken as strong support for the diagnosis of primary HLH (94). In addition, the clear evidence of a functional defect can guide therapy even before a mutation is identified.

Figure 1 describes an overview of our current diagnostic strategy for FHL. In summary, these assays provide an initial confirmation of the clinical diagnosis, and direct the mutation analysis, which yet remains the gold standard for the diagnosis. In 80% of familial HLH cases the genetic error can be identified by sequencing. Knowledge of the genetic background has a great impact on the treatment of the patient: it supports indication towards HSCT, helps with the selection of a matched donor, and represents a useful tool to counsel the family, and to offer prenatal diagnosis if requested.

TREATMENT OF FHL

Historical reports showed that patients with HLH have an exceedingly high risk of mortality within weeks (84), unless an appropriate treatment is started promptly. According to the patient condition, the treatment may be started even before the results from some diagnostic studies become available. The immediate aim of therapy is to suppress the hyper-inflammatory state and to kill not only the exuberant lymphocytes, but also the pathogen-infected antigen-presenting cells. This removes the stimulus, thus breaking the vicious loop of continuous but ineffective activation of cytotoxic cells. The first international cooperative study HLH94 set the combination of dexamethasone and etoposide as the standard of care (88, 96). This strategy brings most patients into a state of disease control within 4–8 weeks. This may buy enough time for further diagnostic tests. For those patients with evidence of a genetic defect, HSCT is strongly recommended, it being the only treatment that can cure FHL to date. Yet, this is not an easy and universal solution. Although recent advances in transplantation procedures and supportive therapy minimize the transplant-related mortality, this is particularly true for 20% of the patients who have a matched familial donor, the procedure remains risky and by no means a guarantee for survival. For the remaining cases, a matched unrelated, or partially matched familial donors, or cord blood units, are the possible alternative sources for HSCT. Since treatment related mortality in this setting remains a big threat, it is extremely important that a recommendation for HSCT is correctly defined. The use of RIC for HSCT provided a significant improvement especially by reducing the unacceptably high level of toxicity connected with the use of ablative regimens (and in particular the veno-occlusive disease resulting from exposure to busulphan) (97, 98). Although the use of RIC may be associated with incomplete donor chimerism, the use of anti-thymoglobulin (ATG) or alemtuzumab and fine tuning of its timing has considerably improved the treatment results (99). Moreover, as in other constitutional or acquired immune disorders, mixed chimerism may turn out to be sufficient to control the disease by replacing the defective function (100, 101).

For patients with normal function at initial screening, the current treatment strategy also suggests to allow a chance for treatment withdrawal after disease resolution in order to avoid, potentially unnecessary, HSCT. For patients with refractory disease or with disease reactivation, which appear unable to remain disease-free in the absence of chemo-immunotherapy,

transplantation will be considered even in the absence of a documented genetic defect.

One major problem in the cure of patients with FHL remains the persistent, unacceptably high rate of mortality in the early, pre/transplant phase. In 1993, Stephane et al. (102) reported an ATG-based regimen associated with a more rapid response rate, explained by its direct attack of CTL, which are thought to drive the disease process. These data were updated and expanded by the same group in 2007 (103). Unfortunately, this regimen may lead to shorter remissions and higher reactivation rates, when compared to the standard etoposide-based regimen. Because these two regimens have complementary strengths (and weaknesses), a pilot study has been designed to adopt a hybrid regimen, which combines initial ATG with subsequent weekly doses of etoposide. This approach has been adopted in a pilot study run in parallel in Europe and in the USA, under the name EURO-HIT-HLH (EudraCT Number 2011-002052-14) and HIT-HLH (ClinicalTrials.gov Identifier: NCT01104025), respectively.

Furthermore, data derived from the animal model suggest that blocking the IFN-γ activity may induce disease control without cytoreduction. Thus, the feasibility and the therapeutic potential of a novel human anti-IFN-γ agent denominated NI-05-01 is currently explored in a phase II study run on an international setting (EudraCT Number 2012-003632-23).

Finally, the hypothesis to cure patients with FHL using gene transfer is supported by its monogenic origin. To this issue, the recent report that gene transfer corrects the cellular and humoral defects in SAP($^{-/-}$) mice provides proof of concept for gene therapy in XLP-1 (104).

MACROPHAGE ACTIVATION SYNDROME

Children and adults with autoimmune diseases, especially systemic onset juvenile idiopathic arthritis (s-JIA), may develop a clinical syndrome closely resembling, or even overlapping, HLH. This condition has been repeatedly defined as "macrophage activation syndrome" (MAS). Specific diagnostic criteria for MAS complicating JIA have been developed. In particular, persistent continuous fever ≥38°C, falling leukocyte count, falling platelet count, increased liver enzymes, hyperferritinemia, falling erythrocyte sedimentation rate, hypofibrinogenemia, hypertriglyceridemia, and evidence of hemophagocytosis in the bone marrow characterize severe MAS (105). About 7% of patients with JIA develop life-threatening MAS (105–107). Although treatment with immunoglobulin and cyclosporine A has been defined as the current standard for MAS, in some patients a more aggressive therapy, similar to that of HLH, may be required. Several cases of s-JIA-associated MAS dramatically benefiting from the IL-1 receptor antagonist, anakinra, after inadequate response to corticosteroids and cyclosporine A have now been reported (108). It is noteworthy that, although most patients with MAS have normal or reduced NK cell function, they show reduced expression of perforin or SAP, and heterozygous mutations in one FHL-related genes (109–111), thus launching a bridge between FHL and the pathogenic mechanisms of MAS. Attempts to harmonize the nomenclature between HLH and MAS are ongoing in cooperation by pediatric hematologists and rheumatologists.

VARIANT PHENOTYPES RECOGNIZED IN ASSOCIATION WITH PARTIAL CYTOTOXIC DEFECTS

Whereas the complete cytotoxic defect, due to biallelic disruptive mutations in one of the FHL-related genes, leads to full-blown FHL, with the typical and rapidly fatal course, the clinical impact of a less complete or partial defect in this pathway remains yet to be clarified.

However, over the last years, reports of later onset of FHL up to adult age and associations between monoallelic mutations of the FHL-related gene and conditions other than FHL suggest that, although far from causing the full-blown picture of FHL, partial insufficiency of the cytotoxic machinery could pave the way to alternative phenotypes.

LATER ONSET OF FHL

The age at diagnosis of FHL is usually very young, with a peak incidence between 1 and 6 months of age (84). Nevertheless, over the years sporadic cases of FHL in older patients have been reported, pointing to unexpected later onset. In 2001, Allen et al. reported four familial cases of HLH diagnosed on a clinical basis, at an age comprised between 9 and 17 years (89). Soon after, Clementi et al. described the first adult cases in two siblings developing FHL2 at 22 and 21 years of age (91). This was followed by other reports of FHL2 in older children or adolescents of different ethnic origins: one 7-years-old patient from Russia, two North American patients of 8 and 10 years (17), one 10-year-old Turkish patient (9), three Japanese patients of 7–12 years (112), and more recently, three Columbian patients aged 5–12 years (113). The oldest patients reported to date are a Spanish man of 49 years, homozygous for *PRF1* A91V (114), and a 62-year-old Japanese man, compound heterozygous for a *PRF1* missense and a non-sense mutation (115). In 2011, Zhang et al. described 10 adult patients with FHL due to biallelic mutations in *PRF1* ($n = 7$), *MUNC13-4* ($n = 2$), and *STXBP2* ($n = 1$) (24), showing that not only FHL2 could present later in the life.

Despite these sporadic reports, adult patients are still at most considered as affected by the "secondary" non-genetic form of HLH, which has several implications in treatment and outcome. Thus, with the aim to raise the attention of adult specialists to this rare disease we recently described our experience in diagnosing FHL in subjects older than 18 years in Italy. Out of the 197 patients referred to the Italian Registry of HLH in which a genetic defect in FHL-related genes had been identified, 11 (6%) were older than 18 years with a median age of 23 years (range, 18–43 years). FHL2 was the most frequent subtype ($n = 6$) with A91V the most frequent single mutation. The other genetic diagnoses were: FHL3 ($n = 2$), FHL5 ($n = 1$), XLP-1 ($n = 2$). Only one-half of these patients presented with the full-blown picture of HLH, while the other half had atypical manifestations at the onset, which brought them often to the attention of non-hematological specialists. This led to frequent delay in diagnosis and treatment. The clinical course was aggressive and led to early death in 8 of 11 (72%) patients supporting the indication to treat these patients until HSCT (92).

To our knowledge, all the patients with later onset of FHL reported to date have at least one missense mutation, while none had biallelic disruptive mutations. This is in keeping with the

results of the genotype–phenotype studies of FHL, which established a close correlation between biallelic disruptive mutations and early age at the onset of FHL (10, 28). Thus, we can speculate that in the presence of hypomorphic genetic defects, residual protein allows some level of NK- and T-cell function, sufficient to cope with common infectious agents, the usual triggers of FHL, at least for several, or even many years. This is also described in a gene-expression profiling study showing that patterns of up- and down-regulated genes separated patients with "late-onset" and "relapsing" forms of FHL from patients with an "early onset and rapidly evolving" form of the disease (116).

ASSOCIATION BETWEEN MONOALLELIC MUTATIONS OF FHL-RELATED GENES AND CONDITIONS OTHER THAN FHL

Cellular cytotoxicity by NK cells and CTLs plays a central role in immune surveillance and tolerance through granule-dependent exocytosis pathway or death-receptor pathway. This gave rise to association studies aimed at investigating if genes involved in programed cell death of lymphocytes could contribute to cancer and autoimmune susceptibility (117).

Lymphoma

The description of the association between different kind of lymphoma and HLH (118, 119) has been followed by studies reporting the link between monoallelic *PRF1* mutations and lymphomas (120–124). Lymphoma or HLH was reported in two siblings with *PRF1* mutations (91), and then autoimmune lymphoproliferative syndrome and lymphoma in a patient with heterozygous *Fas* and *PRF1* mutations (122). Later on, Clementi et al. described four patients with Hodgkin or non-Hodgkin lymphoma who had biallelic perforin mutations and four additional patients with monoallelic *PRF1* mutations (123). A possible predisposing role of *PRF1* variants for ALCL was later established, with the finding of mutations in 27% of children with this subtype of lymphoma (124). We recently decided to extend this study to a larger, unselected population of children with ALCL, and to investigate them for even other FHL-related genes. In line with the previous data, 23 of the 84 (27%) children with ALCL were found to have monoallelic mutations in one of three genes: 21 patients (25%) carried a total of 10 different mutations of *PRF1*, 2 additional patients had missense mutations of the *UNC13D* gene, but no mutations were found in the gene *SH2D1A*. The observation that *PRF1* is involved in a quarter of patients with ALCL suggests a correlation between insufficient cellular cytotoxicity and development of ALCL. Less frequent involvement of *UNC13D* may suggest that subjects heterozygous for mutations in this gene may have others escape mechanisms to prevent lymphoma. *SH2D1A*, the XLP-1 gene, is not related to childhood ALCL (125). Yet, the observation that 5 of 158 males presenting with B-cell NHL (3.2%) had *SH2D1A* mutations raises the issue of prospective screening for XLP-1 in males with B-cell lymphoma (126).

As an extension of their previous association study on lymphoma, Santoro et al. in 2005 sequenced *PRF1* in 100 children with acute lymphoblastic leukemia detecting the most frequent *PRF1* mutation (A91V) in 12/100 patients and 5/127 controls ($p = 0.014$) (19). A similar study of the Cincinnati group, on a much larger cohort, confirmed this association only for *BCR-ABL* positive acute lymphoblastic leukemia (127).

The role of *PRF1* mutations in the predisposition to cancer has been addressed repeatedly especially by the Australian group. In a first study they analyzed 23 individuals with biallelic *PRF1* mutations whose onset of FHL was delayed or abolished and found that 11/23 presented as the primary clinical illness with B- or T-cell lymphoma or acute or chronic leukemia (128). In the following study of 81 European or Maghrebian families in which hematological malignancies had been diagnosed, they found that 3.7% had a *PRF1* variant. The A91V mutation and the N252S polymorphism were already known, while the novel A211V missense substitution was observed in two related Tunisian patients. However, they also report that the lytic function of perforin was not affected by over-expression of mutated *PRF1* in rat basophilic leukemia (119). Subsequently they examined the effect of perforin deficiency in four models of mouse B-cell lymphomagenesis. Perforin was shown to act as a suppressor of B-cell malignancies characteristically driven by v-Ablor bcl-2, whereas Mlh loss cooperated in accelerating spontaneous B-cell lymphomas characteristic of pfp loss. No protective role for perforin was observed in the more aggressive E-myc model of B-cell lymphoma. These transgenic models have allowed to pinpoint the role of perforin in surveillance of B-cell lymphomagenesis (129).

Autoimmune lymphoproliferative syndromes

Following the previous identification of a heterozygous mutation of *PRF1* in an autoimmune lymphoproliferative syndromes (ALPS) patient, in 2006 Clementi et al. analyzed 14 ALPS and 28 Dianzani autoimmune lymphoproliferative disease (DALD) cases and found a different amino-acid substitution in 2 of 14 ALPS and 6 of 28 DALD. This suggested that variations in genes involved in the cytotoxicity of CTL and NK cells may also influence ALPS and DALD presentation (130). To assess the potential role of *UNC13D* gene in the susceptibility to ALPS and DALD, Aricò et al. recently sequenced *UNC13D* in 21 ALPS and 20 DALD patients detecting 4 rare missense variations in 3 heterozygous ALPS patients. Transfection of the mutant cDNAs into HMC-1 cells showed that they decreased granule exocytosis, compared to the wild-type construct. These data suggested that rare loss-of-function variations of *UNCD13D* are risk factors for ALPS development (131).

Boggio et al. analyzed *SH2D1A* in ALPS and DALD patients based on the observation of Komori et al. (132) that suggested an opposite epistatic relationship between the Fas and SAP defects in mice. They found that ALPS and DALD patients displayed an increased frequency of the 346T single nucleotide polymorphism (SNP) in *SH2D1A* causing a loss of the −346°C methylation site and correlating with increased SAP expression and decreased IFN-γ production. Based on this finding they suggested high SAP expression promotes development of ALPS and DALD in human (133).

Multiple sclerosis

The screening of *PRF1* mutations in 1,156 patients with multiple sclerosis showed a higher frequency of monoallelic or biallelic A91V mutations compared to the control group (1,788 subjects) (134).

Arthritis and other rheumatologic disorders

Several groups have reported decreased levels of perforin in CTLs and in particular NK cells, along with decreased NK cell function in patients with s-JIA (109, 110). The first case of association of s-JIA and FHL-related genes was reported by Hazen et al. in 2008, who described an 8-year-old girl with s-JIA and hemophagocytosis, without complete criteria of MAS, who was found to have compound heterozygous mutations of *UNC13D* and reduced NK cell cytotoxic function (135). Soon after, variations in *UNC13D* were identified in 3/18 patients with s-JIA/MAS of which two had biallelic mutations (136). Starting from this, Vastert et al. sequenced *PRF1* in 54 s-JIA patients: 11 of 56 (20%) patients were heterozygous for missense mutations in *PRF1*, and s-JIA patients with history of MAS presented an increased prevalence of A91V mutation (20%) compared with s-JIA patients without history of MAS (9.8%) (137). Recurrent MAS was recently associated with monoallelic W374X mutation in *PRF1* in a child with s-JIA (111).

Other associations

Mutations in the *PRF1* gene were also detected in one 4-year-old girl with primary necrotizing lymphocytic CNS vasculitis (138) and in an 11-year-old girl with panniculitis. They were biallelic in the first case, monoallelic in the second one (139). Pasqualini et al. recently reported an 11-year-old boy with a history of secondary HLH who developed cytophagic histiocytic panniculitis, in whom mutation analysis showed monoallelic missense mutation of the *STX11* gene (140).

The investigation of *PRF1* in type I diabetes showed that allelic frequency of N252S was significantly higher in patients than in controls (141).

X-linked inhibitor of apoptosis protein mutations have been proposed to be involved in other phenotypes. Weiss et al. proposed *XIAP* as a putative modifier gene in Wilson disease (142). Ferretti et al. suggested a role of *XIAP* variants as a predisposing factor for idiopathic periodic fever (IPF) development, possibly through its influence on monocyte function. They evidenced a polymorphism, P423Q, at higher frequency in a large cohort of IPF patients. They also demonstrated that 423Q allele, as compared with 423P, was associated with higher Xiap protein and messenger RNA expression and lower caspase 9 activation (143). Recently, Ou et al. genotyped the *XIAP* Q423P polymorphism in 100 pediatric patients diagnosed with HLH and found its frequency to be comparable in patients and healthy controls (144).

Although association studies should always be taken with caution, unless investigation documents its pathogenic value in this specific condition, taken together these data indicate that sometimes different phenotypes can be associated to monoallelic or presumably "hypomorphic" mutations of FHL-related genes. In particular, a partial defect of granule-dependent cytotoxicity appears to be involved in the predisposition to cancer and autoimmunity, suggesting a link between these different pathogenic pathways and, likely, the role of other yet unknown genes.

CONTRIBUTION OF FHL PATIENTS TO THE CLARIFICATION OF THE CYTOTOXIC MACHINERY

In the granule-dependent exocytosis pathway, target cell recognition by CTL and NK cells is followed by the polarized release of preformed cytolytic granules into the synaptic cleft formed between the effector and the target. Exocytosis of mature cytolytic granules from CTL and NK cells is a complex phenomenon and key for the clearance of virally infected or tumorigenic cells. The process that leads to fusion of cytolytic granules at the immunological synapse can be divided into four distinct steps: polarization, docking, priming, and fusion of cytolytic granules. The analysis of patient CTL and NK cells, the morphological comparison to cells from healthy individuals, assessing their ability to form cytotoxic granules and to polarize and secrete these has been critical to gain an insight into the molecular machinery that drives CTL and NK cell cytotoxicity. Only by careful analysis of patient CTL and NK cells we can now draw a molecular picture of how mutations in proteins hamper normal CTL and NK cell function (**Figure 2**).

Mutations in the small cytoplasmic signaling adaptor protein SAP (XLP-1) affect activation of NK cells. SAP associates with members of the SLAM family of trans-membrane receptors, including SLAM (CD150), LY9 (CD229), CRACC (CD319), CD84, NTB-A (CD352), and 2B4 (CD244) (145). 2B4 is a co-receptor expressed in NK and T lymphocytes and specifically recognizes CD48, which is present solely on hematopoietic cells. 2B4 engagement causes tyrosine phosphorylation of immune receptor tyrosine-based switch motifs (ITSM), recruitment of SAP and thus transduction of activating signals via Fyn-dependent processes (145, 146). In the absence of functional SAP, 2B4 associates with protein tyrosine phosphatases and delivers inhibitory signals (53, 55, 147). Consequently, lack of NK cell-mediated cytotoxicity in XLP-1 mainly stems from the inhibitory effect of 2B4, which, upon engagement by CD48 (up-regulated on B-EBV cells), impairs the function of activating receptors (52). Beyond their common chromosomal localization and their requirement for normal immune responses to certain viral infections, SAP and XIAP showed no structural or functional similarity, are not co-regulated, and do not appear to directly interact (65). SAP functions as an intracellular adaptor molecule involved in SLAM family signaling (51). XIAP is an inhibitor of apoptosis family member, known for its caspase-inhibitory and anti-apoptotic properties. In patients with SAP deficiency, HLH may develop for several reasons including absence of iNKT cells and defective T-cell reactivation-induced cell death (148–150). The mechanism involved in XIAP deficient patients is still unclear.

Activation and priming of NK and CTL leads to the formation of secretory lysosomes, which contain lytic molecules including perforin, granzymes (a family of serine proteases), granulysin, and other lysosomal enzymes, but also a proteoglycan matrix (serglycin) that maintains proteases in an inactive stage, perforin inhibitor (calreticulin), and Fas ligand. CTL from CHS patients, however, show enlarged lysosomal compartments with ER-specific membrane proteins and autophagic inclusions, indicating a function of Lyst during lysosomal fission (151, 152). Why these enlarged lytic granules fail to fuse is still not fully understood. It could be that the structures are simply too big to polarize and fuse.

Similar to the function of Lyst, the AP-3 complex is also required for proper lytic granule maturation in CTL. Defects in the β-3A subunit disrupt the entire AP-3 complex, lead to lysosomal protein mis-sorting in some cell types including

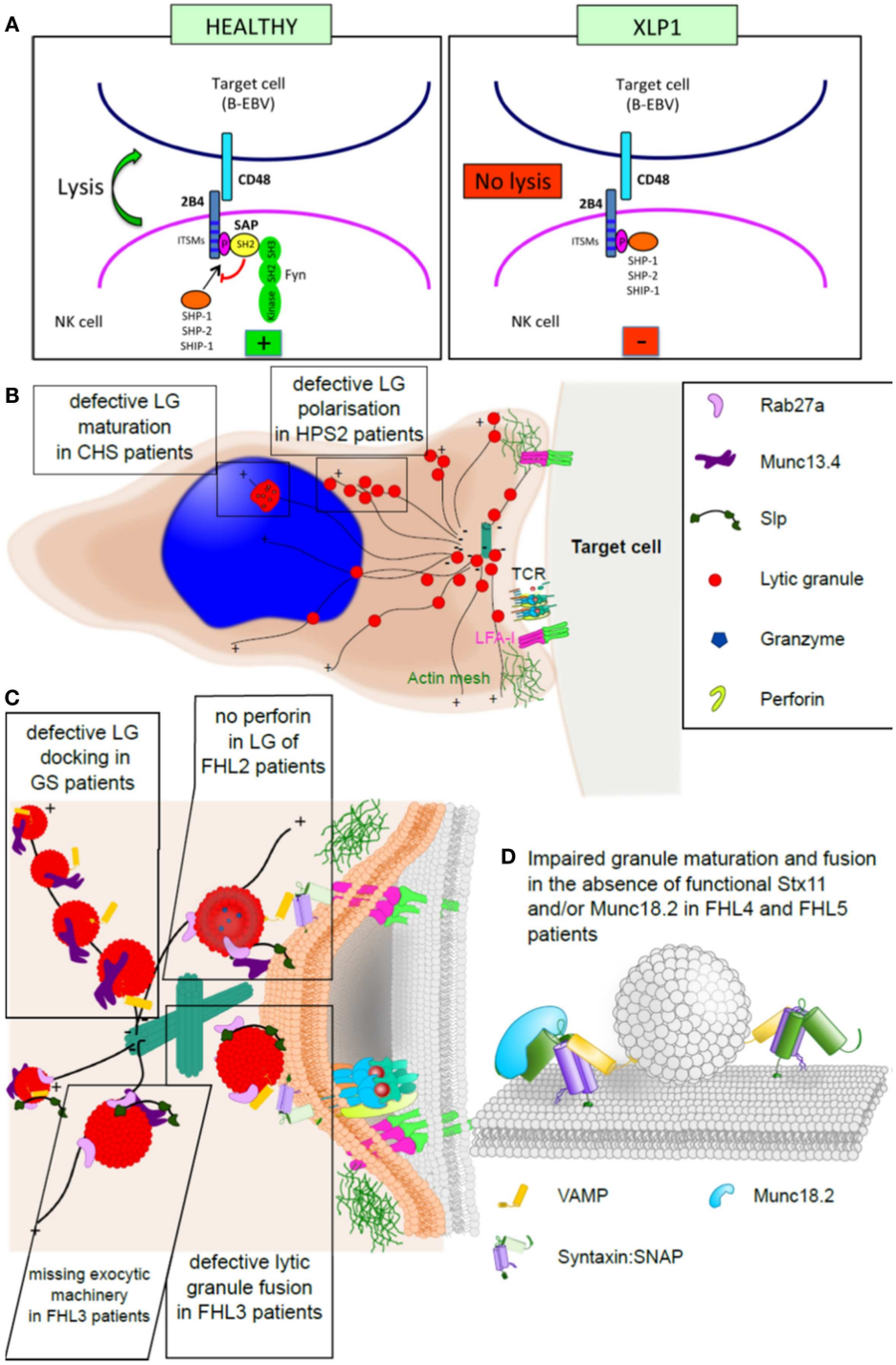

FIGURE 2 | Familial hemophagocytic lymphohistiocytosis and the immunological synapse. **(A)** Simplified model of an NK cell synapse formed by a healthy (left) or SAP-deficient (right) cell. In a healthy NK cell, SAP binds to the cross-linked and phosphorylated 2B4 receptor, which leads to the recruitment of Fyn and consequently lysis of the target cell. In the absence of SAP, 2B4 associates with protein tyrosine phosphatases (SHP-1, SHP-2, and SHIP) delivering inhibitory signals. **(B)** Cartoon of a CTL synapse to illustrate the granule maturation and polarization defect observed in CHS and HPS2 patients, respectively. **(C)** Magnification of the CTL: target synapse area from **(B)**, depicting the docking defect observed in GS2 patients, the molecular function of Munc13.4 in granule maturation and priming, which is impaired in FHL3 patients, and lack of perforin in the granules of FHL2 patients. **(D)** Model illustrating the fusion of membranes by the activity of SNARE proteins (such as syntaxin 11) and Munc18-2, which is lost in FHL4 and FHL5 patients.

melanosomes and platelets, as well as CTLs and NK cells (153, 154). In melanocytes AP-3 sorts tyrosinase and melanin-processing enzymes into melanosomes (155). However, the exact molecular function of AP-3 during secretory lysosome maturation in CTL and NK cells is not yet fully understood. Phenotypically, HPS2 patient cells show an increase in tubular–vesicular endosomes and secretory lysosomes that fail to polarize to the IS (156, 157). These observations suggest a role of AP-3 in the trafficking of a microtubule motor protein or its adaptor to secretory lysosomes (80). Interestingly, most HPS2 patients only show a mild HLH phenotype posing the question whether residual amounts of functional AP-3 are sufficient to sustain CTL and NK cell function, or whether loss of AP-3 may be compensated for by a different adaptor protein (157).

Once polarized, secretory lysosomes in healthy CTL dissociate from the microtubules and dock at the immunological synapse. In Rab27a-deficient GS2 patient CTL secretory lysosomes polarize normally at the immunological synapse but then remain attached to the microtubules, resembling beads on a string (158), providing evidence that Rab27a facilitates granule detachment and docking. Interestingly, loss of Munc13-4 from CTL impairs secretion at a similar stage; however, in contrast to GS2, secretory lysosomes in Munc13-4-deficient CTL detach from the microtubules and dock, but fail to fuse with the plasma membrane (95, 158). Taken together, these morphological phenotypes suggest that Rab27a and Munc13-4 function in consecutive steps during cytotoxicity, by mediating lytic granule docking and priming, respectively; a hypothesis strengthened by the finding that Rab27a and Munc13-4 interact directly (159–162).

As a member of the Rab family, the small guanine nucleotide-binding protein Rab27a operates as a molecular switch, and by that can controls a myriad of cellular processes. Besides the interaction with Munc13-4, active Rab27a also binds a class of proteins that is closely related to neuronal synaptotagmin I: the synaptotagmin-like proteins (Slps) (163). In melanocytes, Rab27a binds the synaptotagmin-like protein melanophilin and the plus-end directed actin motor myosin Va (164–166). This complex facilitates the polarization and docking of melanosomes at the cell's periphery and explains why loss of functional Rab27a causes albinism. In CTL, however, active Rab27a appears to interact with various, functionally redundant Slps (namely Slp1, Slp2a, and Slp3) since only over-expression of a dominant-negative construct against the highly conserved SHD domain of these Slps resulted in a striking cytotoxicity phenotype (165, 167–169). It is conceivable that Slp1, Slp2a, and Slp3 act as vesicle tethers at the plasma membrane or form the linker between Rab27a and a motor protein.

Munc13-4 is one of four mammalian homologs of *C. elegans* Unc13 (170). Munc13 proteins vary significantly in size and in the number of functional domains; however, the C-termini of all Munc13 proteins are highly homologous, encoding the recently defined MUN domain (171). One role fulfilled by Munc13-4 is that of a vesicle tether, arresting the movement of Rab27a-positive vesicles at the site of secretion and enabling vesicle priming (161, 172). This function is most likely exerted by the central MUN domain, due to its similarity to vesicle tethering proteins Sec6p and Vps53 (173–175). Moreover, Munc13-4 was recently shown to interact with the SNARE domain of Stx11 and t-SNARE complexes, thus offering a mechanism by which Munc13-4 could mediate vesicle priming and initiate fusion (176).

Besides its role as tether/priming component, which depends on the interaction with Rab27a, Munc13-4 has also been connected to lytic granule maturation in a Rab27a-independent mechanism by facilitating fusion between two distinct endosomal compartments (162). This model suggests that cytolytic granules in Munc13-4-deficient CTL lack the necessary fusion machinery, explaining why vesicle release is arrested after docking.

The final step during cytotoxicity is the release of cytotoxic mediators into the cleft between CTL and the target cell; a process that is facilitated by SNARE proteins, as well as SNARE accessory proteins (177). Stx11 is an unusual member of the syntaxin family of SNARE proteins as it associates with membranes through a cysteine-rich region at its C terminus, instead of the C-terminal trans-membrane domain (178). It still remains elusive whether lipid anchored SNARE proteins can facilitate membrane fusion, with reports arguing both ways (178–181). It is conceivable that Stx11 may form inhibitory, fusion incapable SNARE complex, potentially with Vti1b, and thus regulate membrane fusion indirectly. However, the observation that Stx11 can also form a SNARE complex with SNAP23 and VAMP8, which itself is crucial for CTL cytotoxicity (34, 182), supports an active role of Stx11 during membrane fusion.

Much of the molecular function of Stx11 depends on Munc18-2, another critical component of the CTL fusion machinery. One of the functions of Munc18-2 is to chaperone Stx11 as shown by reduced protein levels of Stx11 in Munc18-2-deficient CTL (40, 41). This role depends on the direct interaction between Munc18-2 and Stx11 and is most likely initiated and selected for by the N peptide of Stx11. Strikingly, the killing defect observed in CTL and NK cells from FHL4 and FHL5 patient can often be compensated for by *in vitro* activation with IL-2 (40, 41, 43, 46). This may be partially due to the up-regulation of surrogate proteins, namely Stx3 and Munc18-1. Interestingly, in the absence of Stx11 Stx3 is trafficked to the plasma membrane in a Munc18-2-dependent manner, adding evidence to the model that Stx11 is indeed involved in the final fusion event (183). Besides the chaperone function, detailed analysis of reported mutations additionally concluded that Munc18-2 will most likely function during SNARE complex formation, binding and fusion itself, similar to the role of its homolog Munc18-1 in neurons (184–186).

CONCLUSION

Familial hemophagocytic lymphohistiocytosis is a rare, life-threatening disease with a non-specific clinical presentation, which needs accurate clinical, immunological, and genetic diagnostic work-up. Current standard of therapy based on chemo-immunotherapy (dexamethasone and etoposide) allows rapid disease control in most but not all patients. Due to the remaining risk of early mortality, novel therapeutic approaches based on the use of anti-thymocyte globulin (ATG or anti-CD52/Campath), or an anti-IFN-γ human monoclonal antibody, are currently explored in international cooperative settings. Currently identified genetic defects allow the assignment of a genetic marker to over 80%

of the families, with proportions varying according to the geographic areas. In the remaining patients, some cases show either familial recurrence and/or the disease is refractory or recurs after initial treatment; these items strongly suggest an additional genetic defect beyond the ones we currently investigate. All such cases should be addressed to reference laboratories with research capabilities, where the combined use of confocal microscopy, cellular cytotoxicity assays, and protein expression studies may help to better characterize putative novel defects. In turn, those studies will broaden our current knowledge of the cellular cytotoxic machinery in humans. In the meanwhile, observations of mono or even biallelic mutations in FHL-related genes in patients with phenotypes different from FHL, may provide additional insights into our understanding of the role of this part of innate and adaptive immunity.

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Graded defects in cytotoxicity determine severity of hemophagocytic lymphohistiocytosis in humans and mice

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Primary hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disease of hyperinflammation resulting from immune dysregulation due to inherited defects in the cytolytic machinery of natural killer and T cells. In humans, mutations in seven genes encoding proteins involved in cytolytic effector functions have so far been identified that predispose to HLH. However, although most affected patients develop HLH eventually, disease onset and severity are highly variable. Due to the genetic heterogeneity and variable time and nature of disease triggers, the immunological basis of these variations in HLH progression is incompletely understood. Several murine models of primary HLH have been established allowing to study HLH pathogenesis under more defined conditions. Here we directly compare the clinical HLH phenotype in six HLH-prone mouse strains with defects in the granule-dependent cytotoxic pathway. A severity gradient of HLH manifestations could be identified that is defined by the genetically determined residual lytic activity of cytotoxic T lymphocytes (CTL) and their ability to control lymphocytic choriomeningitis virus, which was used as a trigger for disease induction. Importantly, analysis of cohorts of HLH patients with severe bi-allelic mutations in the corresponding genes yielded a similar severity gradient in human HLH as reflected by the age at disease onset. Our findings define HLH as a threshold disease determined by subtle differences in the residual lytic activity of CTL.

Keywords: cytotoxicity, hemophagocytic lymphohistiocytosis, inflammation, CTL, virus persistence, antigen persistence

INTRODUCTION

Primary hemophagocytic lymphohistiocytosis (HLH) is a rare life-threatening syndrome of hyperinflammation due to genetic defects in the perforin-dependent granule exocytosis pathway of natural killer (NK) and T cells (1–4). The HLH syndrome is characterized by uncontrolled inflammatory and immunopathological processes in various tissues as a result of infiltrating, excessively activated T cells, NK cells, and macrophages, accompanied by a massive cytokine production (IFN-γ, TNF, IL-6, IL-18) (5–7). Due to this loss in immune homeostasis, HLH patients present with prolonged fever, hepatosplenomegaly, severe cytopenia, and frequently with neurologic manifestations. In addition, elevated serum levels of ferritin, triglycerides, soluble CD25 (IL-2 receptor α chain), and liver enzymes, as well as hemophagocytosis in various tissues and reduced cytolytic activity of lymphocytes are characteristic criteria for HLH (**Table 1**) (8). Typically, patients with primary HLH develop disease in early childhood with a poor prognosis in the absence of therapeutic intervention (9–11).

Traditionally, "familial HLH" (FHL) has been defined as a genetic disease, in which the predisposition to HLH is the dominant feature (*PERFORIN* deficiency, *MUNC13-4* deficiency, *SYNTAXIN-11* deficiency, and *MUNC18-2* deficiency) (12–17), while "immunodeficiencies with albinism" (Chediak–Higashi syndrome (CHS) or *LYST* deficiency, Griscelli syndrome type 2 (GS2) or *RAB27A* deficiency, and Hermansky–Pudlak syndrome type 2 (HPS2) or *AP3b1* deficiency) (18–22) combine this predisposition with clinical manifestations of albinism and variable degrees of other immune cell and platelet dysfunction (23–28). From a pathophysiological viewpoint, this distinction is arbitrary. First, all genes mutated in these two groups of conditions are critically involved in the biogenesis, intracellular transport, release, and function of perforin-containing lytic granules of NK and T cells (1). Second, it becomes increasingly obvious that defects in platelets and other immune cells such as neutrophils or mast cells are also observed in diseases currently classified as FHL (29–33). Because the genetic predisposition to HLH is the dominant life-threatening clinical feature in all of these diseases, we prefer to classify them collectively as familial HLH syndromes (FHL syndromes).

While the overall pattern of clinical manifestations of HLH in patients with the different FHL syndromes is quite characteristic, onset of disease, severity of clinical symptoms, and duration of disease-free remission periods are highly variable (31, 34–36). This depends not only on the affected gene, but also on the nature

Table 1 | Diagnostic criteria for HLH.

Fever
Cytopenia in at least two cell lineages
Hyperferritinemia
High sCD25 (sIL-2Rα) concentration
Hypertriglyceridemia and/or hypofibrinogenemia
Splenomegaly
Hemophagocytosis
Low/absent natural killer (NK) cell cytotoxicity

of the mutation (null or hypomorphic) and the time point and nature of exposure to predominantly infectious triggers that can elicit HLH in predisposed individuals. In addition, in >60% of patients with FHL syndromes, no clear trigger for HLH can be identified and it is still a matter of debate whether an exogenous trigger is needed for disease induction at all (37–40). This variability makes it difficult to define the *a priori* risk of an individual patient to develop HLH in the different human FHL syndromes. A study of additional functional parameters may help to improve the predictability of HLH progression. For example, it is so far not clear, in what hierarchy the dysfunction of the different affected proteins becomes limiting for *in vivo* cytotoxicity.

In this context, animal models of FHL syndromes have proven useful to analyze the pathogenesis of HLH under more defined conditions. In 2004, Jordan et al. reported that following lymphocytic choriomeningitis virus (LCMV) infection as initial trigger, perforin-deficient (*PKO*) mice develop the full clinical picture of HLH as it is described for FHL2 patients (41). It was demonstrated that hyperactive cytotoxic T lymphocytes (CTL) and high levels of IFN-γ are the driving force behind the development of fatal HLH in *PKO* mice. Non-fatal HLH was observed after LCMV infection of *Jinx* mice (model for FHL3) (42), *STX-11*-deficient mice (model for FHL4) (43, 44), *ashen* mice (model for GS2) (45), *souris* mice (model for CHS) (46), and *pearl* mice (model for HPS2) (20). Although some of these strains were compared directly in these publications, the different mouse models were not analyzed in parallel under identical experimental conditions with standardized immunological and clinical criteria for HLH. Therefore, the relative risk for HLH development in these models in relation to the individual genetic defect and its consequences for cytotoxicity have not been fully defined. Moreover, the role of virus control and a potential contribution of the various proteins in processes other than cytotoxicity to the pathogenesis of HLH remain controversial.

In the present study we therefore performed a comprehensive comparative analysis of the clinical and immunological HLH phenotype in six different mouse models of FHL syndromes. In addition, recently published results on HLH severity (as determined by age at onset of HLH) in patients with FHL syndromes due to severe bi-allelic mutations were extended to additional genetic conditions (44). We discuss our results in the context of the overall value of LCMV-induced HLH in various murine cytotoxicity mutants for the understanding of human FHL syndromes and point out some key questions to be addressed in human and mouse models in the future.

RESULTS

HLH SEVERITY DIFFERS IN VARIOUS MOUSE MODELS OF FHL SYNDROMES

To analyze the impact of different defects in the cytotoxicity pathway of T and NK cells on HLH development, we assessed HLH parameters following intravenous LCMV infection in six established and previously described HLH-prone mouse models under identical experimental conditions with standardized read-out systems. The following mouse strains were used in this study: two mouse models for CHS carrying different mutations in the *Lyst* gene – *beige* and *souris* mice – (46), one mouse model for HPS2 deficient in AP-3 – *pearl* mice – (20), a *Rab27a*-deficient mouse model for GS2 – *ashen* mice – (45), one model for familial hemophagocytic lymphohistiocytosis (FHL) 2 deficient in perforin – *PKO* mice – (41, 47), and one model for FHL4 deficient in syntaxin-11 – *STX-11KO* mice – (43, 44). As previously described, none of these mutant mouse strains develop disease spontaneously. Infection with LCMV was used to induce disease.

Following LCMV infection, mice were weighed and ear temperature was taken daily. Mice were analyzed either at day 8 or at day 12 after infection and all eight criteria (**Table 1**) defined by the HLH study group of the Histiocyte Society for the diagnostic evaluation of patients with suspected HLH were determined (8). A drop in ear temperature due to circulatory centralization was taken as an equivalent of fever in humans. In addition, we measured lactate dehydrogenase (LDH) and glutamate dehydrogenase (GLDH) reflecting liver damage and IFN-γ serum levels, which have been shown to correlate well with HLH activity in mice (41, 48). Weight loss as a rough, but easily accessible measure of disease revealed a clear hierarchy of HLH severity in the six mutant mouse strains (**Figure 1**). While there was no weight loss in *wild-type* and *beige* mice, weight loss was transient until day 8 in *pearl* mice, progressive but moderate in *souris* mice and equally severe in *STX-11KO*, *ashen*, and *PKO* mice.

A similar hierarchy of disease severity was observed, when the formal diagnostic HLH criteria were evaluated on day 12 (**Table 2**). LCMV infection of *wild-type* mice led to splenomegaly and rare hemophagocytosis, but the other HLH criteria were not fulfilled. *Beige* mice, carrying a hypomorphic mutation in the *lyst* gene, in addition had low NK cell cytotoxicity, but no other HLH features (**Table 2**). *Pearl* mice in addition had cytopenia, elevated ferritin, liver enzymes, and IFN-γ and thus fulfilled 5/8 diagnostic criteria at day 8 after infection (**Table A1** in Appendix), but – as reported – disease was transient and the criteria were not fulfilled at day 12 (**Table 2**). All other strains fulfilled 7/8 (apart from elevated triglycerides) or 8/8 diagnostic criteria at day 12 and additionally had elevated liver enzymes and IFN-γ levels, which were more pronounced at day 8 after infection. Nevertheless, some notable differences were observed: first, the drop in temperature and alterations in hemoglobin, ferritin, sCD25, and triglycerides were less severe in *souris* mice than in the other three mouse strains – *STX-11KO*, *ashen*, *PKO* – although IFN-γ levels were similar or even higher. Second, alterations in platelet counts, ferritin, and sCD25 were less pronounced in *STX-11KO* than in *ashen* or *PKO* mice. Third, levels of ferritin and IFN-γ on day 8 after infection were higher in *PKO* than in *ashen* mice. Finally, previous experiments assessing survival beyond day 12 have shown that disease is usually

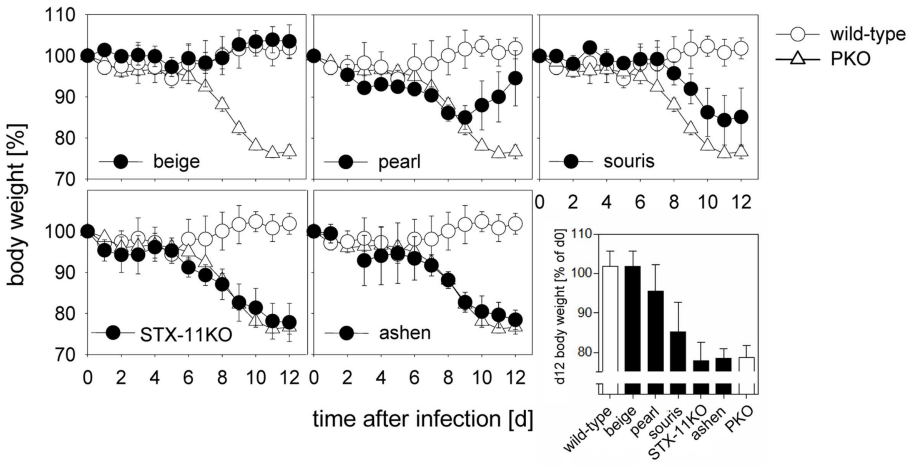

FIGURE 1 | Degree of weight loss after LCMV infection of different HLH-prone mouse models depends on the affected gene. Mice were infected with 200 pfu LCMV i.v. Percent weight loss of initial body weight is depicted over the course of 12 days. Body weight of mutant mice (*beige, pearl, souris, STX-11KO, ashen*/filled circles) in comparison with *wild-type* (open circle) and *PKO* (open triangle) mice are shown. The graph depicted on the lower right illustrates a direct comparison of body weight loss of all mouse groups on day 12 after LCMV infection.

lethal in *PKO* mice, sometimes lethal in *ashen* mice and not lethal in any of the other investigated mutant mouse strains (data not shown). Overall, HLH severity as assessed by weight loss, survival, and HLH criteria showed the following hierarchy: *wild-type* < *beige* (no HLH) < *pearl* (transient HLH) < *souris* < *STX-11KO* < *ashen* < *PKO* (full HLH).

DISEASE DEVELOPMENT IN HLH MOUSE MODELS CORRELATES WITH VIRUS PERSISTENCE

A comparison of virus titers in the spleen of the different HLH-prone mouse strains confirmed that virus persistence is one of the key characteristics of HLH development. Mice that showed no signs of disease such as *wild-type* and *beige* mice were able to reduce virus titers until day 8 and no virus was detectable at day 12 (**Figure 2**). In *pearl* mice, the transient HLH at day 8 was associated with a delay in virus control at this time point. When these mice recovered eventually, virus elimination was achieved. All of the mice fulfilling the criteria of HLH – irrespective of disease severity – failed to reduce or eliminate the virus until day 12 (**Figure 2**) and were persistently infected with similar titers in the spleen. Thus, virus persistence appears to be a prerequisite for the development of HLH in the various mouse models of impaired cytotoxicity, but disease severity does not correlate with titers of persisting virus.

GRADED DEFECTS IN CTL CYTOTOXICITY DETERMINE OUTCOME OF DISEASE

As extensively discussed before (47, 49–53), the cytotoxicity of CD8 T cells is the major factor in virus control following LCMV infection and the major determinant in preventing HLH development (41, 43, 45). Here, we directly compared CTL degranulation and CTL cytotoxicity in the six different HLH-prone mouse strains on day 8 after LCMV infection. First, we analyzed the degranulation capacity of virus-specific CTL in the different mouse strains by quantifying the expression of CD107a on IFN-γ positive CTL upon antigen-specific *in vitro* stimulation with gp33 peptide, the immunodominant epitope of LCMV. CTL from the four mutant mouse strains that developed the full picture of HLH – *souris*, *STX-11KO*, *ashen*, *PKO* – continuously produced high levels of IFN-γ even in the absence of stimulation (**Figure 3A**, upper panel), while this was not the case in the other strains. Interestingly, the grade of *ex vivo* IFN-γ expression of CTL correlated very well with IFN-γ levels in serum and disease severity (**Figure 3C**). In contrast, the grade of the degranulation defect did not completely reflect the observations on disease severity. The degranulation defect was more pronounced in *beige* (no HLH) than in *pearl* mice (transient HLH) and no difference could be found in the degranulation defect between *STX-11KO* and *ashen* mice. Unexpectedly, a mild reduction in degranulation was also observed in *PKO* mice (**Figure 3B**), although a role for perforin in the process of granule exocytosis has so far not been described. Analyzing the *ex vivo* cytolytic activities of CTL from the different mouse strains revealed a graded impairment of cytotoxicity from beige to *PKO* CTL that perfectly reflected distinct disease severity (**Figure 3D**). In addition, as reported previously, the cytotoxicity defect was more pronounced in *PKO* than in *ashen* or *STX-11KO* mice.

In a next step, we functionally evaluated the cytotoxic activity of CTL derived from the different HLH-prone mouse strains in an *in vivo* assay of virus control (50). For this, CTL were adoptively transfered into *wild-type* mice that had been infected with LCMV 10 h previously (**Figure 4**). In this assay, CTL from *wild-type* mice eliminated the virus from the spleen within 18 h, while CTL from the four strains that developed the full picture of HLH – *souris*, *STX-11KO*, *ashen*, *PKO* – had no impact on viral titers and failed to clear LCMV. Despite a more pronounced defect in degranulation – but not in cytotoxicity – *beige* CTL cleared the virus, while *pearl* CTL had an intermediate effect on virus clearance. Taken together, when CTL from the different HLH-prone mice failed to eliminate LCMV in this short-term protection assay, the mice developed HLH independent from their residual cytolytic activity

Table 2 | Analysis of HLH diagnosis parameters in various HLH-prone mouse models on day 12 after LCMV infection.

HLH parameter	Wild-type	Beige	Pearl	Souris	Syntaxin-11KO	Ashen	PKO
Fever/hypothermia	37.0 ± 0.4	37.0 ± 0.4	37.0 ± 0.4	**35.4 ± 0.8***	**34.7 ± 0.7***	**34.1 ± 0.3***	**34.4 ± 0.9***
WBC ($\times 10^3/\mu L$)	10.3 ± 3.4	14.3 ± 7.0	13.7 ± 5.5	**6.9 ± 3.5***	**3.84 ± 2.1***	**6.1 ± 3.5***	**4.1 ± 2.0***
HGB (g/dL)	11.3 ± 1.5	10.6 ± 1.2	10.7 ± 1.2	**6.5 ± 1.4*****	**3.8 ± 0.6*****	**3.0 ± 0.6*****	**4.6 ± 1.0*****
PLT ($\times 10^3/\mu L$)	839 ± 180	803 ± 213	**441 ± 11*****	**253 ± 75*****	**507.1 ± 156.5*****	**221 ± 151*****	**228 ± 114*****
Ferritin (ng/mL)	525.2 ± 199.4	508.6 ± 105.7	817.7 ± 296.3	1009.0 ± 158.2	**1624.3 ± 761.9*****	**3202.0 ± 568.5****	**2951.1 ± 2098.6*****
sCD25 (pg/mL)	271.9 ± 121.6	291.1 ± 46.0	321.3 ± 195.3	**631.7 ± 166.4*****	**709.1 ± 98.3*****	**833.3 ± 238.6*****	**823.5 ± 105.2*****
Triglycerides (mg/dL)	71.1 ± 18.7	75.3 ± 26.2	88.7 ± 12.3	82.5 ± 35.4	**216.0 ± 69.0*****	146.7 ± 37.8	111.4 ± 62.0
Hemophagocytosis (liver)	(+)	+	++	+++	+++	+++	+++
Splenomegaly	+	+	+	+	+	+	+
NK cell cytotoxicity	Normal	↓↓	↓	↓↓↓	↓↓	↓↓↓	↓↓↓
Additional parameter							
LDH (U/L)	502.7 ± 171.7	493.3 ± 161.2	1379.0 ± 1231.2	**1464.7 ± 422.5***	978.0 ± 692.8	**2053.3 ± 871.8***	**1944.2 ± 1535.7****
GLDH (U/L)	11.1 ± 3.7	15.8 ± 6.8	**121.9 ± 293.76***	**181.6 ± 136.6*****	**113.5 ± 98.9*****	**345.9 ± 93.9*****	**156.1 ± 103.1*****
IFN-γ (ng/mL)	b.d.	b.d.	b.d.	**23.6 ± 6.2*****	**23.5 ± 12.9*****	**38.0 ± 12.5*****	**26.2 ± 16.7*****
HLH	**NO**	**NO**	**Transient**	**YES**	**YES**	**YES**	**Lethal**

Red fields indicate fulfilled HLH criteria.

The red font accentuates the final disease phenotype.

****$p < 0.001$; **$p < 0.01$; *$p < 0.05$ (ANOVA; statistically significant differences compared to day 12 values of wild-type mice).*

b.d. below detection limit.

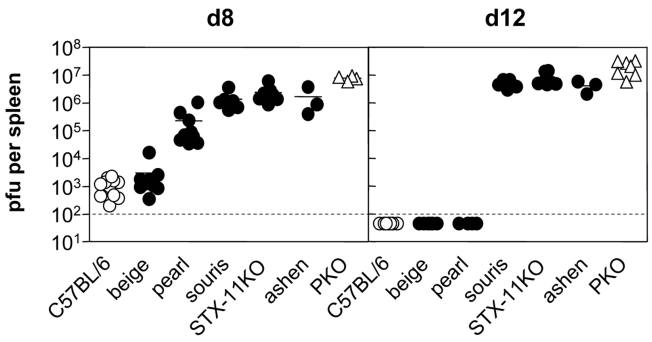

FIGURE 2 | Virus control following LCMV infection differs between various HLH-prone mouse models. Mice were infected with 200 pfu LCMV i.v. On day 8 and day 12 after LCMV infection, viral titers in spleen of *beige*, *pearl*, *souris*, *STX-11KO*, and *ashen* mice (filled circle) in comparison to *wild-type* (open circle) and *PKO* (open triangle) mice were determined.

as determined in a ^{51}Cr-release assay *in vitro* (**Figure 3D**). Thus, this *in vivo* virus protection assay is a better correlate for HLH susceptibility than cytolytic activity of CTL measured *in vitro*, since it translates a gradient of impaired cytotoxicity into the clinically observed "yes-no" decision for the development of HLH.

HLH DISEASE SEVERITY IN PATIENTS WITH FHL SYNDROMES

To assess how these observations in the murine cytotoxicity mutants relate to human patients, we intended to determine HLH disease severity in cohorts of patients carrying the respective mutations. Disease severity in humans is, however, not only determined by the affected gene, but also by the nature of the mutation (complete vs. partial loss-of function), genomic heterogeneity, and environmental factors including infections. Since disease severity in human cytotoxicity mutants correlates with the age at onset of HLH (54, 55), we used this as a surrogate parameter. To control in part for the nature of the mutation, we selected only patients

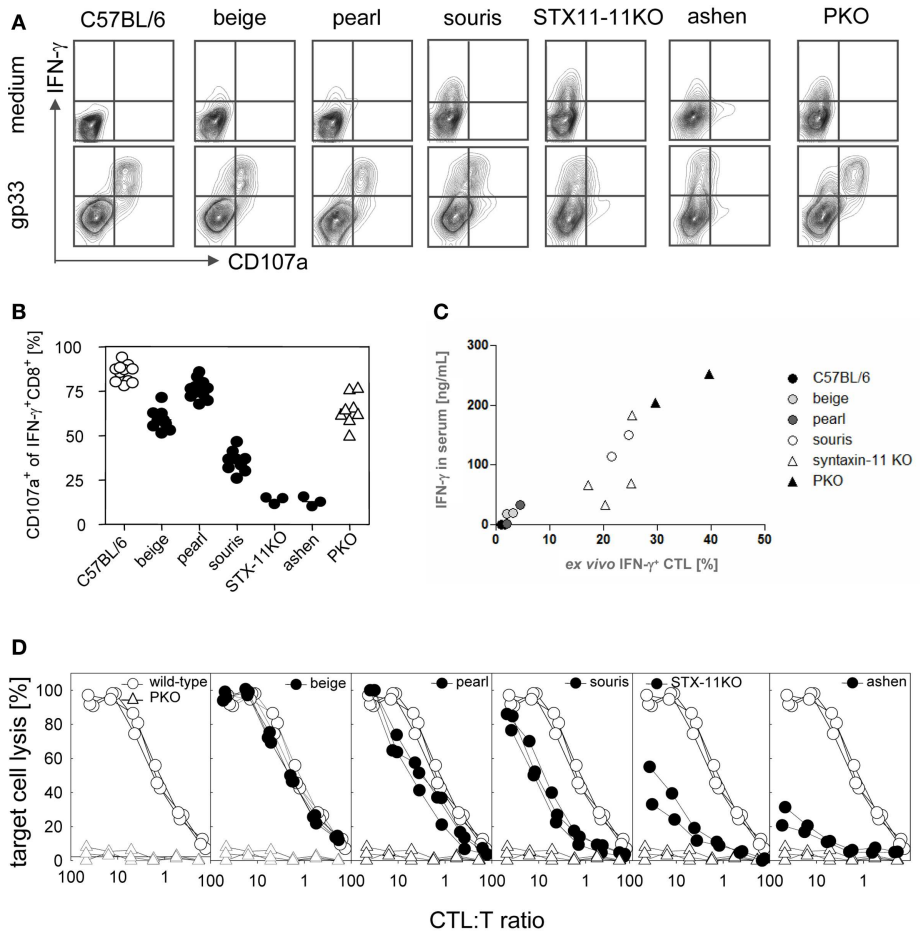

FIGURE 3 | Cytotoxic T lymphocytes degranulation and cytotoxicity are differently impaired in various HLH-prone mouse models. Mice were infected with 200 pfu LCMV i.v. On day 8 after LCMV infection, spleen cells of mutant mice (*beige*, *pearl*, *souris*, *STX-11KO*, *ashen*/filled circles) and *wild-type* (open circle) and *PKO* (open triangle) mice were restimulated with gp33 peptide or were left in medium without peptide. Surface expression of CD107a of IFN-γ$^+$CD8$^+$ T cells was determined by flow cytometry as illustrated by **(A)** representative FACS plots. **(B)** Percentage of CD107a expressing IFN-γ$^+$CD8$^+$ T cells after restimulation with gp33 peptide. **(C)** Correlation of percentage of CTL expressing IFN-γ without restimulation *ex vivo* with the IFN-γ levels in sera of the various HLH-prone mutant mice. **(D)** CTL cytotoxicity was determined in a ^{51}Cr-release assay by using LCMV-infected MC57 target cells.

with predicted severe impairment of protein expression due to a null mutation, a large gene deletion, the introduction of a stop codon or a frame shift mutations leading to a stop codon in the corresponding genes. We recently published data on patients with severe bi-allelic mutations in the *PERFORIN*, the *SYNTAXIN-11*, and the *RAB27A* genes (44). For this study we added observations in a cohort of patients with mutations in the *LYST* gene that was identified from the literature. Since only one HPS2 patient with an *AP3b1* mutation has been reported to have developed the full picture of HLH (at 5 years of age) (19), we did not include this group of HPS2 patients in our analysis.

As expected from our previous study, although there was a high variability in the age at onset for all four diseases, significant differences could be demonstrated between the different cohorts (**Figures 5A,B**). The mean age of HLH onset was 3.4 ± 5 months in *PERFORIN*-deficient patients, 13.4 ± 19 months in *RAB27A*-deficient patients, 27.3 ± 37 months in *SYNTAXIN-11*-deficient patients and 37.7 ± 41.9 months in *LYST*-deficient patients. Thus, this analysis reveals a gradient of HLH severity in humans that is identical to the mouse models: HPS2 < CHS < FHL4 < GS2 < FHL2.

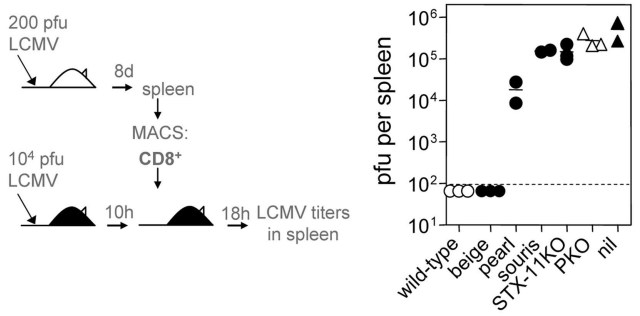

FIGURE 4 | Subtle differences in CTL cytotoxicity of various HLH-prone mouse models determine virus control. On day 8 after 200 pfu of LCMV infection, splenic $CD8^+$ T cells of mutant mice (*beige*, *pearl*, *souris*, *STX-11KO*, *ashen*/filled circles), *wild-type* (open circle), and *PKO* (open triangle) mice were MACS purified and 2×10^6 of MACS purified $CD8^+$ T cells were transferred into *wild-type* C57BL/6 mice that had been infected with 10^4 pfu LCMV 10 h before. After 18 h of $CD8^+$ T cell transfer, viral titers in spleen were determined (nil: infected *wild-type* C57BL/6 without cell transfer).

DISCUSSION

This study provides a comprehensive comparative analysis of HLH disease severity in mice and humans with mutations in different genes involved in cellular cytotoxicity. While the study builds on a number of previously published observations comparing individual strains of mice or patient cohorts, it is the first study to directly compare a large number of mutant mouse strains, in which HLH is induced and assessed with a single experimental protocol. Notably, we related the clinical HLH symptoms not only to the affected gene, but also to immunobiological parameters such as the degree of the degranulation/cytotoxicity defect of T cells and the ability to provide control of the triggering viral infection.

The overall result is a clear hierarchy of HLH severity among the different genetic defects predisposing to HLH that is surprisingly consistent between humans and mice. This is an important validation of using LCMV infection of murine cytotoxicity mutants for the study of human genetic defects in cytotoxicity and FHL development. While *beige* mice did not develop HLH upon LCMV infection, *pearl* mice showed transient HLH and *souris*, *STX-11KO*, *ashen*, and *PKO* mice exhibited the full picture of the disease. Moreover, among the latter four strains a gradient of disease severity could be established when considering the individual HLH associated parameters (**Table 2**). *Souris* mice had the least weight loss and drop in temperature, while *PKO* mice had the most severe and frequently lethal HLH progression (**Figure 1**). Finally, we could confirm our previous observation that the disease course was more severe in *ashen* (GS2) than in *STX-11KO* (FHL4) mice. Thus, the gradient of HLH severity in mice

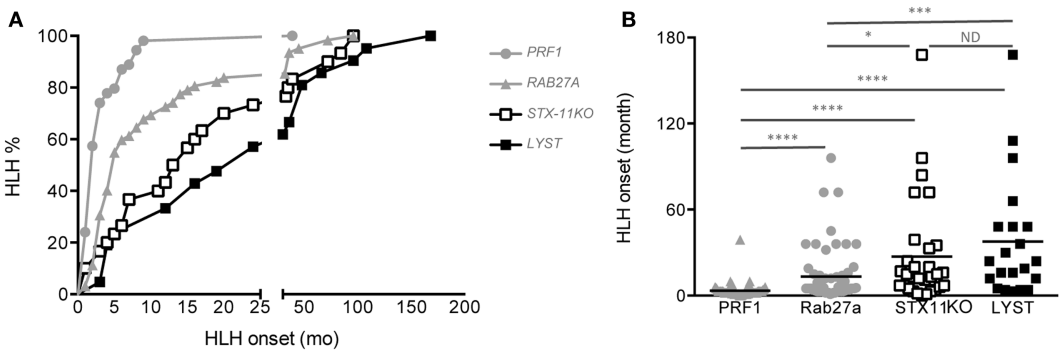

FIGURE 5 | Delayed HLH onset in patients with *SYNTAXIN-11* and *LYST* deficiency compared with *RAB27A*- and *PRF1*-deficient patients. **(A)** Cumulative incidence and **(B)** age at onset of HLH in FHL2 (PRF1 deficiency, gray circles; $n = 72$), GS2 (RAB27A deficiency, gray triangles; $n = 61$), FHL4 (STX11 deficiency, open squares; $n = 30$), and CHS (LYST deficiency, black squares; $n = 21$) patients carrying null bi-allelic mutations, as detailed in **Table 3**. The incidence of HLH was analyzed with a log-rank test; $*p < 0.05$ for FHL4 vs. GS2 patients; $***p < 0.001$ for GS2 vs. CHS patients; $****p < 0.0001$ for FHL2 vs. GS2, FHL2 vs. FHL4 and FHL2 vs. CHS; ND = no statistical differences were observed between FHL4 vs. CHS patients. The onset of HLH was analyzed with a one-way ANOVA. $*p < 0.05$; $***p < 0.001$; $***p < 0.0001$. Mutations in *PRF1*, *RAB27A*, and *STX11* are detailed in Ref. (44), and mutations in CHS1/*LYST* are detailed in **Table 3**.

was: *wild-type* < HPS2 < CHS < FHL4 < GS2 < FHL2 (**Figure 6**). Interestingly, the parameters best reflecting this gradient were ferritin and sCD25, while IFN-γ serum levels did not correlate as good, at least when analyzed at day 12 post infection.

In humans, disease severity as assessed by age at onset of HLH followed exactly the same pattern. However, in all cohorts, there were patients who developed HLH in the first months of life, confirming that the affected gene cannot predict disease onset in the individual patient. The different ages at onset probably rather reflect the likelihood of loosing control of perforin-mediated immune homeostasis in response to triggers of different intensity that are encountered by all children.

As expected, none of the mouse strains developed disease spontaneously. Infection with LCMV was required to trigger disease, while other infections including respiratory syncytical virus (RSV) and pneumonia virus of mice (PVM) failed to induce disease, even in the most severe mutant lacking perforin (unpublished observations). Of note, mouse cytomegalovirus (MCMV) is able to induce HLH in *PKO* (56, 57), but not in jinx mice (42). No data on HLH are so far available for the other HLH-prone mouse strains, although a higher susceptibility of *beige* mice to MCMV infection has been reported (58). The potency of LCMV to induce HLH might in part be explained by the fact that it infects antigen-presenting cells such as dendritic cells (63), leading to direct stimulation of the T cell response without the need for cross-presentation. This contributes to the fact that it is a better stimulator of CD8 T cell responses in the mouse than any of the other infections. Of note, this is a property that is also shared by EBV infection in humans, where infected B cells can also serve as APC and will lead to prolonged antigenic stimulation, if they are not eliminated by NK or T cells (64). Overall, a very potent CTL stimulation is required to provoke the impaired immune homeostasis characteristic of HLH in mice. This is in apparent contrast to early-onset HLH in patients with cytotoxicity defects, where in the majority of cases no pathogen trigger can be identified. This could reflect variable and incomplete infectious disease work-up of the patients, which may not only relate to known infections, but could also point to a role for so far unknown novel viruses. Alternatively, it may indicate that in humans, different from mice, perforin-mediated cytotoxicity also plays a role in T cell homeostasis under resting conditions, as it is described for the Fas/FasL pathway (65, 66). Thus, T cell–T cell interactions or T/NK cell–APC interactions involving perforin could be relevant for maintaining immune homeostasis even in the absence of infections or other obvious immune stimulations (67–73). Further research in HLH patients will be needed to address this important issue, since treatment of a triggering infection can be an important component of successful therapy for HLH (37, 74, 75).

What then is the role of the virus in the murine disease models? Our data and previous experiments suggest that it is not just a trigger of a pathogenetic sequence that – once initiated – becomes self-perpetuating and independent of the virus. This is probably best illustrated by the phenotype of *pearl* mice. Within the first 8 days after infection, these mice could not control virus replication and developed HLH symptoms. However, virus elimination was eventually achieved and this led to full recovery from HLH symptoms. Complementary to these findings, chronic antigen application (gp33 peptide injections in 12 h intervals) induced HLH-like symptoms in LCMV-infected *wild-type* mice indicating the critical role of antigen persistence and prolonged antigen presentation for disease development (41). Thus, persistence of cells presenting viral antigen (usually in the context of persisting virus) is the decisive factor for HLH induction in all animal models studied so far. A previous study has suggested that perforin may have an immunomodulatory function that is independent from its role in the clearance of virus and killing of APCs presenting viral antigen, but related to a role of perforin in modulating antigen presentation by DC (76). In this view, HLH development is not dependent on virus persistence *per se* but determined by an enhanced antigen presentation in the context of perforin deficiency. Further experiments are needed to decide whether it is possible to functionally separate antigen persistence from enhanced antigen presentation in this context.

While virus/antigen persistence was associated with perpetuated disease in all HLH-prone mouse strains analyzed, various factors may determine the hierarchy of disease severity in mice with different genetic defects in cellular cytotoxicity. First, early virus control based on differences in residual cytotoxic activity of T and NK cells may be an important factor in determining the severity of initial disease manifestation. The graded virus load in the spleen at day 8 after infection paralleled very well HLH severity in *beige*, *pearl*, *souris*, and *STX-11KO* mice. However, differences in splenic virus titers on day 8 could not explain the different HLH severity in *STX-11KO*, *ashen*, and *PKO* mice. Thus, a so far unsolved question is why differences in HLH severity are observed in these mice, although all of them exhibited virus persistence, which should lead to the same extent of chronic T cell stimulation by presented viral antigens. Early virus spread to other, non-lymphoid organs could be an important factor determining HLH severity. Differences in the spread of virus to key organs like the liver or the brain with subsequent recruitment of highly activated CTL may explain the fact that *PKO* mice die, while the other mutant mouse strains survive (77). Analysis of the early kinetics of virus spread to other organs may help to resolve this issue. Second, differences in the residual cytotoxic activity may not only affect early virus control and spread, but additionally influence effector cell homeostasis via a more or less efficient killing of distinct APC populations. The elimination of certain APC populations may critically determine the activation status and survival of the hyperreactive T cells and act as a rheostat to limit T cell responses (73, 78–81). As recently demonstrated, the elimination of a rare, antigen-presenting DC population by CD8 T cells in a negative feedback loop critically determines the magnitude of the T cell response in a perforin-dependent way after LCMV infection (82). Third, the proteins affected in the various cytotoxicity mutants are involved in different steps of lysosomal trafficking [as discussed for syntaxin-11 and Rab27a (26, 44)], which could also contribute to the quality of antigen presentation and hence indirectly determine T cell activity and HLH progression. This may also be the case for AP3b1 known to regulate several processes involved in antigen recognition/processing/presentation, i.e., CD1b presentation of phagocytosed antigens and TLR recruitment to phagosome (83, 84). Along the same line, defects in proteins involved in perforin-mediated cytotoxicity may have additional functions in other

immune cell types like platelets, neutrophils, and mast cells, which can be relevant for LCMV specific immune responses and thus modulate HLH pathogenesis (33, 85, 86). Fourth, different susceptibility to T cell exhaustion in the various cytotoxicity mutants can modify HLH progression and determine survival. As recently demonstrated, effector T cells chronically exposed to antigenic stimulation showed a variable extent of exhaustive differentiation in different HLH-prone mouse strains. Initially, *STX-11KO* mice developed all diagnostic symptoms of HLH after LCMV infection comparable to *PKO* mice. However, in *STX-11KO* mice with more extensive T cell exhaustion the HLH disease course was attenuated and the mice survived, whereas *PKO* mice developed lethal HLH (43). T cell exhaustion in *STX-11KO* mice was characterized by sustained expression of inhibitory receptors, step-wise loss of effector functions, and finally deletion of the disease-mediating T cells. Thus, T cell exhaustion can be an important disease-modifying parameter in HLH.

In summary, following a defined viral stimulus, the degree of impairment of CTL cytotoxicity was the best predictor of HLH development in the described animal models, but other host factors contributed. HLH appeared to be a threshold disease. Up to a certain degree of impaired cytotoxicity, disease was mild and transient, but resolved once the delayed virus control had been achieved. However, subtle differences in CTL cytotoxicity allowing the establishment of viral persistence led to the full picture of HLH and persistent disease (**Figure 6A**). Thus, the graded differences in cytotoxicity translated into a "yes-no" phenotype (**Figure 6B**) with respect to HLH. In HLH patients it is still a matter of debate to which extent CTL or NK cells contribute to disease induction. Hence, this concept may apply in a more general form to cytotoxic lymphocytes. Depending on the initial trigger CTL and/or NK cells may play the critical role in HLH induction and progression. While restoration of cytotoxicity and virus control appear to be key variables in the causal treatment of the disease, a further investigation of these factors in humans and mice may point to additional treatment approaches for this highly aggressive syndrome.

MATERIALS AND METHODS

PATIENTS

All patients with FHL or GS2 diagnosis were previously reported [summarized in Ref. (44)]. Patients with CHS diagnosis were previously published (as referenced in **Table 3**).

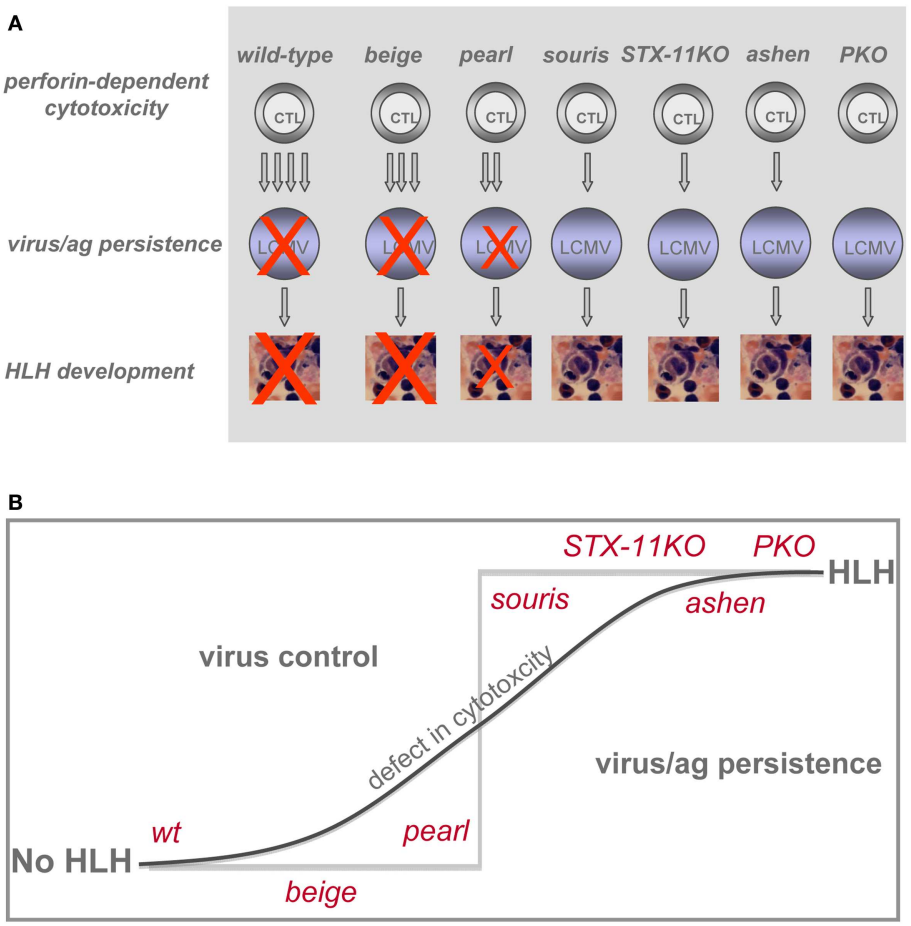

FIGURE 6 | Impact of various genetic defects on threshold of HLH development. **(A)** Differences in CTL cytotoxicity and their impact on LCMV control determine whether or not HLH develops. **(B)** Pronounced impairment of CTL cytotoxicity results in loss of virus control and development of HLH.

Table 3 | Genotype and age at HLH onset of previously published patients carrying severe bi-allelic mutations in *CHS1/LYST*.

LYST mutations	Predicted effect	*n*	HLH onset (months)	Reference
c.2620delT	p.F874Ffs25X	1	66	Certain et al. (59)
c.C3310T	p.R1104X	1	30	Certain et al. (59)
c.7555delT	p.Y2519Ifs9X	1	168	Certain et al. (59)
c.del7060-7066	p.delL2354_D2356Mfs15X	1	19	Certain et al. (59)
c.5317delA*/c.9228 + 10bp ins*	p.R1773Dfs12X*/p.H3076Hfs8X*	1	16	Certain et al. (59)
c.del9106-9161	p.delG3036_S3054Gfs15X	1	24	Certain et al. (59)
c.9590delA	p.Y3197Lfs61X	1	12	Certain et al. (59)
c.5004delA	p.G1668Gfs28X	1	12	Scherber et al. (60)
c.5519delC	p.S1840Yfs1X	1	108	Scherber et al. (60)
c.9590delA	p.Y3197Lfs61X	1	3	Scherber et al. (60)
c.3622C > T*/c.11002G > T*	p.Q1208X*/E3668X*	1	16	Scherber et al. (60)
c.5506C > T	p.R1836X	1	48	Kaya et al. (61)
c.5506C > T	p.R1836X	1	4	Kaya et al. (61)
IVS24 c.7060-1G > A	Exon25fsX	1	4	Jessen et al. (46)
c.10551_10552del2	p.Y3517X	1	5	Jessen et al. (46)
c.5506C > T	p.R1836X	1	4	Jessen et al. (46)
c.2374_2375 delGA	p.D792FX6	1	96	Jessen et al. (46)
c.4508C > G	p.S1483X	1	48	Jessen et al. (46)
c.4508C > G	p.S1483X	1	36	Jessen et al. (46)
c.5506C > T	p.R1836X	1	48	Jessen et al. (46)
10395delA	p.K3465Kfs2X	1	24	Karim et al. (62)

All of the selected genetic anomalies were either large deletion, null mutations, introduce a stop codon or affect the first base of an intron predicted to induce a frameshift, and a consecutive stop codon.

**Heterozygous mutations; n = number of patients.*

MICE AND VIRUS

C57BL/6 (*wild-type*, wt) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). C57BL/6J-*Lystbg*^{J}/J (*beige*^{J}; stock no. 000629) and B6Pin.C3-*Ap3b1*^{pe}/J (*pearl*, stock no. 003215) mice were purchased from the Jackson Laboratory (Bar Harbor, USA), and C57BL/6-*Lyst*$^{bg\text{-}Btlr}$/Mmcd (*souris*; stock no. 010470-UCD) mice originally generated by Dr. B. Beutler and colleagues (Scripps Research Institute, La Jolla, CA, USA) were obtained from the Mutant Mouse Regional Resource Center (University of California, Davis, CA, USA). Syntaxin-11-deficient (*STX-11KO)* mice were generated by Dr. U. zur Stadt (Hamburg) on a C57BL/6 background by deletion of the only coding exon. C3H/HeSn-Rab27a^{ash}/J mice were purchased from the Jackson Laboratory and backcrossed to the C57BL/6 background for 10 generations (C57BL/6J-Rab27a^{ash}/j; *ashen*). Perforin-deficient C57BL/6-Prf1^{tm1Sdz} (*PKO*) mice were obtained from Dr. H. Hengartner (Zurich). Mice were kept under specific pathogen-free conditions. All mouse experiments were approved by the Regierungspraesidium Freiburg. The lymphocytic choriomeningitis virus WE (LCMV-WE) was grown on MC57G fibroblasts and stored at −80°C until use. Mice were injected intravenously with 200 pfu (*plaque forming units*). To quantify virus in organs from infected mice a focus forming assay was used as described (87). Temperatures were obtained using a digital infrared ear thermometer (Braun, ThermoScan type 6022).

HLH BIOMARKERS IN MICE

Blood counts were determined by a Sysmex KX-21 hematology analyzer. Serum levels of ferritin, triglycerides, LDH, and GLDH were analyzed by the Department of Clinical Chemistry using the Roche Modular Analytics Evo. Levels of sCD25 were determined by using the mouse IL-2Ralpha DuoSet kit (R&D systems) according to the instructions of the manufacturer. The IFN-γ ELISA was performed as described before (46).

HISTOLOGY

To evaluate hemophagocytic macrophages, immunohistochemistry on paraffin-embedded liver sections was performed as previously described (46).

ANTIBODIES, INTRACELLULAR STAINING, DEGRANULATION, AND CYTOTOXICITY ASSAY

Antibodies were purchased from eBioscience or BD Biosciences. Surface expression of CD107a and intracellular IFN-γ of $CD8^{+}CD3^{+}$ CTL was determined after 4 h of restimulation with the immunodominant CTL epitope gp33-41 (PolyPeptide) or medium in the presence of monensin (BD Biosciences). For fixation and permeabilization of spleen cells the Cytofix/Cytoperm kit (BD Biosciences) was used. CTL cytotoxicity was determined in a 5 h ^{51}chromium-release assay by incubating spleen cells as effectors with LCMV-infected MC57 target cells. In order to calculate the CTL to target ratio, CD8 T cells were quantified by antibody staining and flow cytometry.

ADOPTIVE TRANSFER EXPERIMENT

Splenic CD8 T cells from mice that had been infected with 200 pfu LCMV-WE 8 days earlier were MACS purified using the MACS $CD8a^{+}$ T Cell Isolation Kit II (Miltenyi Biotec). Purity

was determined by flow cytometry and was above 90% in all experiments. About 2×10^6 purified CD8 T cells were transferred intravenously into C57BL/6 *wild-type* mice that had been infected with 10^4 pfu LCMV 10 h before. After 18 h of adoptive cell transfer, splenic virus titers were determined.

STATISTICAL ANALYSIS

Tests were performed using the GaphPad InStat software version 3.06. The comparison between data was evaluated with a one-way ANOVA (Analysis Of Variance) with posttest. Differences were considered significant at a p-value below 0.05.

AUTHORS CONTRIBUTION

Birthe Jessen, Tamara Kögl, and Fernando E. Sepulveda performed experiments; Stephan Ehl, Peter Aichele, Genevieve de Saint Basile designed the study and supervised the project; Stephan Ehl, Peter Aichele, Genevieve de Saint Basile, and Birthe Jessen wrote the manuscript.

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APPENDIX

Table A1 | Analysis of HLH biomarker on day 8 after LCMV infection.

HLH parameter	Wild-type	Beige	Pearl	Souris	Syntaxin-11KO	Ashen	PKO
Ferritin (ng/mL)	393.8 ± 244.0	493.9 ± 181.1	**892.6 ± 424.6*** **	481.8 ± 259.4	365.0 ± 163.1	578.3 ± 79.7	**1280.7 ± 1212.9*** **
sCD25 (pg/mL)	545.7 ± 206.2	508.0 ± 261.5	805.2 ± 294.5	816.4 ± 234.5	707.1 ± 389.7	916.7 ± 415.3	**1060.4 ± 314.1***
Triglycerides (mg/dL)	58.3 ± 18.0	97.1 ± 21.6	97.3 ± 93.3	63.5 ± 54.2	**187.5 ± 97.0***	174.0 ± 107.9	**164.1 ± 90.1*** **
Additional parameter							
IFN-γ (ng/mL)	0.4 ± 1.2	8.45 ± 9.9	**20.5 ± 16.5***	**114.8 ± 26.0*** **	**79.7 ± 60.6****	**70.3 ± 42.3***	**156.7 ± 56.8*** **
HLH	**NO**	**NO**	**Transient**	**YES**	**YES**	**YES**	**Lethal**

Gray fields indicate fulfilled HLH criteria.

The red font accentuates the final disease phenotype.

****p < 0.001; **p < 0.01; *p < 0.05 (ANOVA; statistically significant differences compared to day 8 values of wild-type mice).*

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An N-terminal missense mutation in *STX11* causative of FHL4 abrogates syntaxin-11 binding to Munc18-2

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Familial hemophagocytic lymphohistiocytosis (FHL) is an often-fatal hyperinflammatory disorder caused by autosomal recessive mutations in *PRF1*, *UNC13D*, *STX11*, and *STXBP2*. We identified a homozygous *STX11* mutation, c.173T > C (p.L58P), in three patients presenting clinically with hemophagocytic lymphohistiocytosis from unrelated Pakistani families. The mutation yields an amino acid substitution in the N-terminal Habc domain of syntaxin-11 and resulted in defective natural killer cell degranulation. Notably, syntaxin-11 expression was decreased in patient cells. However, in an ectopic expression system, syntaxin-11 L58P was expressed at levels comparable to wild-type syntaxin-11, but did not bind Munc18-2. Moreover, another N-terminal syntaxin-11 mutant, R4A, also did not bind Munc18-2. Thus, we have identified a novel missense *STX11* mutation causative of FHL type 4. The syntaxin-11 R4A and L58P mutations reveal that both the N-terminus and Habc domain of syntaxin-11 are required for binding to Munc18-2, implying similarity to the dynamic binary binding of neuronal syntaxin-1 to Munc18-1.

Keywords: familial hemophagocytic lymphohistiocytosis, syntaxin-11, Munc18-2, N-peptide

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory disorder clinically diagnosed based on fulfillment of five out of eight criteria including fever, splenomegaly, bicytopenia, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis, low/absent natural killer (NK) cell activity, hyperferritinemia, and high soluble interleukin (IL)-2 receptor levels (1). Familial hemophagocytic lymphohistiocytosis (FHL) typically presents in infancy (2, 3). The incidence of FHL has been estimated to 1 in 50,000 live births (4). Chemo- and immunotherapy succeeds in controlling the disease in the majority of patients, but persistent remission is rarely obtained. At present, hematopoietic stem cell transplantation (HSCT) is the only cure for FHL (5).

Familial hemophagocytic lymphohistiocytosis is associated with autosomal recessive mutations in genes including *PRF1*, *UNC13D*, *STX11*, and *STXBP2* (6–10). In addition, Griscelli syndrome type 2 and Chediak Higashi syndrome, associated with autosomal recessive mutations *RAB27A* and *LYST*, respectively, may also present with HLH and are in addition characterized by hypopigmentation. These genes encode proteins required for cytotoxic granule biogenesis, secretion, and target cell death (11). *STX11*, associated with FHL type 4 (FHL4), has the shortest coding sequence among these genes and accounts for only a small fraction of FHL patients. Relative to other FHL subtypes, patients with *STX11* non-sense mutations or *Stx11*-deficient mice typically display less severe disease (12, 13). Although syntaxin-11 (Stx11)-deficiency abrogates degranulation by both cytotoxic T cells and NK cells (14, 15). The exact molecular mechanisms are not clear. Stx11 has been shown to bind Munc18-2, as well as the SNARE domain-containing proteins SNAP-23 and Vti1b (9, 10, 16, 17). Missense mutations can be informative in elucidating how Stx11 acts to facilitate exocytosis. To date, only two *STX11* missense mutations have been reported (18).

In this study, we report a novel *STX11* missense mutation in three unrelated Pakistani families. The autosomal recessive mutation abrogated NK cell degranulation. Interestingly, biochemical analyses of this N-terminal mutation, in addition to another mutation at the conserved N-terminus of Stx11, revealed binding of the N-terminal Habc domain of Stx11 to Munc18-2, stabilizing Stx11 expression, and facilitating cytotoxic lymphocyte exocytosis.

MATERIALS AND METHODS

PATIENTS AND CONTROLS

The studies were approved by the ethics committee at the Karolinska Institutet. Written consent was obtained from the patients' families.

CELLS AND ANTIBODIES

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by density gradient centrifugation (Lymphoprep, Axis-Shield) and maintained in complete medium (RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine; all Invitrogen). LAK cells were generated as previously described (19). The human erythroleukemia K562 and mouse mastocytoma P815 cell lines were maintained in complete medium. HEK-293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS. Rabbit polyclonal anti-Stx11 and Munc18-2 (Proteintech Group) as well as mouse monoclonal anti-HA (clone 16B12, Covance) and anti-actin (C4, Fischer Scientific) antibodies were used for Western blotting. Mouse monoclonal anti-FLAG (M2, Sigma) was used for immunoprecipitation.

FUNCTIONAL ASSAYS

For assessment of NK cell-mediated cytotoxicity, a standard 4-h ^{51}Cr assay was used (14). Cytotoxic lymphocyte exocytosis was assessed by flow cytometry, as previously described (15). Samples were acquired on a Calibur instrument (BD Biosciences) and analyzed using Flowjo 9.4 software (Tree Star).

PLASMIDS AND SEQUENCE ANALYSES

Sequences encoding human Stx11 and Munc18-2 were cloned into a pDisplay vector backbone (Invitrogen) for expression on N-terminally tagged proteins. Stx11 mutations were generated by site-directed mutagenesis. Sequence analyses, alignments, and phylogenetic trees were performed and created with CLC Main Workbench software (v.6).

BIOCHEMICAL ANALYSES

Patient and control PBMC or LAK cells were lysed in lysis buffer [20 mM Tris, pH 7.4, 2 mM EDTA, 1% Triton-X-100, 10% glycerol, 100 mM NaCl, protease inhibitors (Roche)]. The protein concentration in nuclei-depleted lysates was determined using Bradford assay (Thermo Scientific). Proteins were separated by SDS-PAGE (NuPAGE, Invitrogen), transferred to PVDF membranes (Millipore). The membranes were blocked with 5% skimmed milk, and blotted with specific antibodies. HEK-293T cells were transfected (Lipofectamine, Invitrogen) with plasmids encoding wild-type or mutated FLAG-tagged Stx11 (FLAG-Stx11) constructs, wild-type HA-tagged Munc18-2 (HA-Munc18-2, the empty vector, or combinations thereof). Twenty-four hours following transfection, the cells were lysed and the protein concentration was determined by Bradford assay (Thermo Scientific). For pull-down experiments, protein G-beads (Invitrogen) were pre-incubated with anti-FLAG mAb, washed in lysis buffer, and incubated with lysates from different FLAG-Stx11 transfected cells for 2 h at 4°C. Subsequently, FLAG-Stx11-loaded beads were washed and incubated with lysates from vector or HA-Munc18-2 transfected cells for 4 h at 4°C.

RESULTS

CLINICAL AND IMMUNOLOGICAL CHARACTERIZATION OF PATIENTS WITH A HOMOZYGOUS *STX11* MISSENSE MUTATION

Here, we describe two infants and one 5-year-old child born to unrelated Pakistani families that presented with HLH (**Table 1**). Patient A and B presented with a laboratory parameters consistent with a clinical diagnosis of HLH at the Aga Khan Hospital, Karachi. Patient C also presented with a hyperinflammatory syndrome and was later referred to the Aga Khan Hospital. For patient C, it has not been possible to retrieve laboratory parameters at initial presentation.

Due to suspicion of FHL, NK cell cytotoxicity, degranulation, and intracellular expression of granule constituents was assessed. All patients displayed defective lysis of K562 target cells and degranulation by NK cells in response to K562 target cells or engagement of the Fc receptor CD16 (**Figures 1A,B**). Notably, cytotoxicity and degranulation were partially restored by IL-2 stimulation (**Figures 1C,D**). Moreover, expression of cytotoxic granule constituents' perforin, granzyme B, and CD107a was normal in patient NK cells, suggesting that granule integrity was not impaired (**Figure 2**). On the basis of these functional and phenotypic assessments, mutations in genes required for lymphocyte exocytosis and associated with FHL were suspected.

Sequencing of the coding regions and splice-sites of *UNC13D*, *STX11*, and *STXBP2* revealed that all three patients were homozygous for a novel *STX11* mutation, c.173T > C (p.L58P) (**Table 1**). The L58P localizes to an α-helical strand of the predicted Stx11 Habc domain. The parents were heterozygous for this *STX11* mutation, but did not have any recorded history of inflammatory disease. In addition, patient A was heterozygous for a rare *UNC13D* c.811C > T (p.P271S; frequency 0.001 in a Caucasian population of 4294 individuals) variant inherited from the father and homozygous for an uncommon *UNC13D* c.2782C > T (p.R928C, frequency 0.01 in a Caucasian population of 4294 individuals) variant inherited from either parent. As no hypopigmentation was evident in the patients, *RAB27A* and *LYST* were not sequenced.

A HOMOZYGOUS *STX11* MISSENSE MUTATION RESULTS IN SELECTIVE LOSS OF SYNTAXIN-11 EXPRESSION IN PATIENT NK CELLS

To gain insights into how the Stx11 L58P missense mutation may cause disease, we analyzed Stx11 expression in PBMCs from patient C and controls. Stx11 levels were found to be greatly reduced in the patient (**Figure 3A**). PBMCs from the patient's mother displayed low Munc18-2 expression as well as slightly decreased syntaxin-11 expression. Although Munc18-2 levels were comparable between the patient and controls, the loss of Stx11 expression may reflect differences in the distribution of immune cell subsets or the inflammatory state between the patient and controls. Thus, we generated LAK cells from patient C and controls. LAK cells from the patient also displayed a selective loss of Stx11 expression, whereas Munc18-2 expression was similar to that of control LAK cells (**Figure 3B**). LAK cells from the patient's mother displayed syntaxin-11 and Munc18-2 levels similar to those of control LAK cells. Thus, Stx11 L58P might either be poorly expressed or be destabilized and degraded in the patient cells.

SYNTAXIN-11 R4A AND L58P MUTATIONS DISRUPT BINDING TO MUNC18-2

Stx11 interacts with Munc18-2 and loss of Munc18-2 expression has previously been shown to result in loss of Stx11 expression as well, suggesting a requirement for Munc18-2 in stabilization of Stx11 (9, 10). With respect to N-terminal peptide sequences,

Table 1 | Clinical, laboratory, and genetic findings in patients.

	A	B	C
Ethnical origin	Pakistan	Pakistan	Pakistan
Familial disease	No	Yes	No
Parental consanguinity	Yes	Yes	Yes
Sex	Male	Male	Female
STX11	173T > C, Leu58Pro hmz	173T > C, Leu58Pro hmz	173T > C, Leu58Pro hmz
STXBP2	None detected	None detected	None detected
UNC13D	c.811C > T p.Pro271Ser htz c.2782C > T p.R928C hmz	None detected	None detected
Age at diagnosis-HLH	2 months	5 years	48 months
Fever	Yes	Yes	nd
Splenomegaly	Yes	Yes	nd
Hepatomegaly	Yes	Yes	nd
Hb (g/L)	55	71	nd
Neutrophils (10^9/L)	0.3	0.4	nd
Platelets (10^9/L)	13	8	nd
Triglycerides (mmol/L)	5.1	5.5	nd
Fibrinogen (g/L)	0.28	0.16	nd
Hemophagocytosis	No	No	nd
Ferritin (μg/L)	8636	1929	nd
sCD25 (U/mL)	nd	nd	nd
NK cell activity[a]	Deficient	Deficient	Deficient
NK cell degranulation	Deficient	Deficient	Deficient
Neurological manifestations[b]	None	None	nd
Pathological CSF	nd	nd	nd
Treatment active disease	Dexa, CsA, etoposide	Dexa, CsA, etoposide	nd
Remission at 2 months	Yes	Yes	Lost to follow-up
Age at HSCT	15 months	Not done	nd
Outcome	Deceased	Deceased	nd

[a] *Defective: 10 lytic units or less.*

[b] *Reported at some point during the course of the disease; nd = no data; Dexa = dexamethasone; CsA = cyclosporine A; HSCT = hematopoietic stem cell transplantation.*

human Stx19, Stx1A, Stx1B, Stx2, Stx3, and Stx4 represent close homologs to human Stx11 (**Figure 4A**). Remarkably, the N-peptide as well as the sequence surrounding the Stx11 L58 residue in the Habc are highly conserved between Stx11 and Stx1 isoforms, as well as other related Stxs (**Figures 4B,C**). Interestingly, studies of neuronally expressed Stx1A and Munc18-1 have revealed that the N-terminal residues (N-peptide) as well as the N-terminal Habc domains of Stx1 mediate interactions with Munc18-1 (20–22). As both the N-peptide and Habc domain of Stx1A are closely conserved to those of Stx11, we evaluated whether a Stx11 R4A mutation as well as the patient-derived Stx11 L58P mutation located to the Habc domain would interfere with binding of Munc18-2. Constructs for ectopic expression of FLAG-tagged wild-type and mutant Stx11 were transfected into HEK-293T cells. In transfection experiments, both Stx11 R4A and L58P mutants were expressed at levels comparable to Stx11 wild-type (**Figure 4D**). Notably, in pull-down experiments using beads loaded with FLAG-tagged Stx11 wild-type and Stx11 mutants neither FLAG-tagged Stx11 R4A nor L58P mutants bound HA-tagged Munc18-2 (**Figure 4E**). In contrast, the C-terminal Stx11 Q268X mutation previously associated with FHL4 (14), did not display impaired binding of Munc18-2 (**Figure 4E**). Together, the data show that mutations in the N-peptide or Habc domain of Stx11 can disrupt interactions with Munc18-2, demonstrating a critical role for both the N-peptide and Habc domain of Stx11 in binding of Munc18-2.

DISCUSSION

We describe a novel autosomal recessive missense *STX11* c.173T > C (p.L58P) mutation causative of FHL4 in three children from different Pakistani kindreds. Stx11 expression was absent in NK cells from a patient homozygous for this *STX11* mutation. Consistent with previous studies, the *STX11* mutation was associated with defective degranulation by resting NK cells (14, 23). Of note, whereas only *STX11* mutations were detected in the other patients, patient A also carried *UNC13D* variants. By comparison, this patient presented at an earlier age than the patient B and C, and displayed less of a restoration of NK cell degranulation upon IL-2 stimulation. Thus, although an abrogation of NK cell degranulation is expected in Munc13-4 deficient patients, it is possible that the *UNC13D* variants contribute to the severity of disease presentation in patient A.

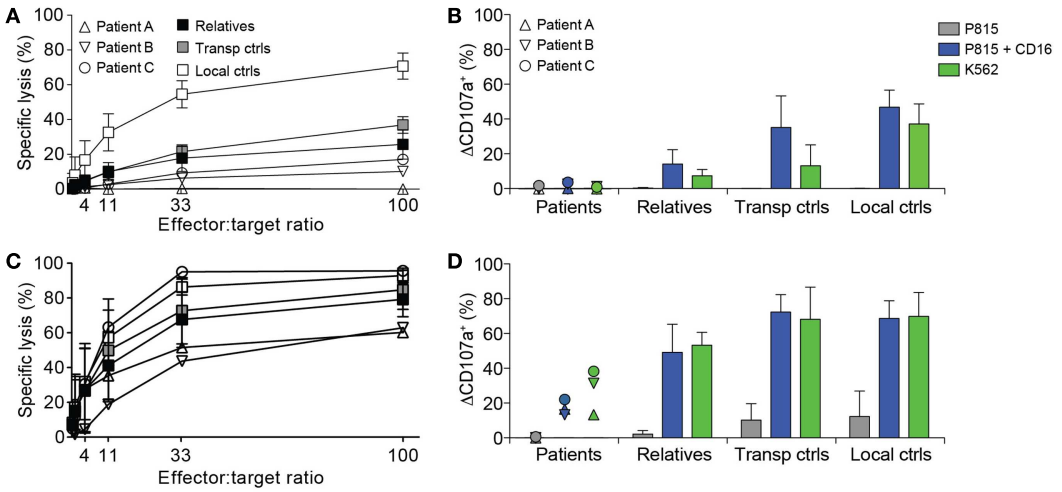

FIGURE 1 | Defective NK cell degranulation in patients with homozygous *STX11* c.173T > C mutations. **(A–D)** PBMC were isolated from three patients with homozygous *STX11* c.173T > C, p.L58P mutations, relatives ($n = 10$, all six parents in addition to four siblings) as well as transport ($n = 3$) and local controls ($n = 5$). PBMC **(A)** freshly isolated or **(C)** stimulated with IL-2 overnight were mixed with ^{51}Cr labeled K562 target cells. After 4 h, ^{51}Cr-release was quantified in supernatants and specific lysis was calculated. Symbols indicate individual values for patients or mean values for relatives and controls. Error bars indicate SD. PBMC **(B)** freshly isolated or **(D)** stimulated with IL-2 overnight were mixed with target cells and antibodies, as indicated. The cells were stained with antibodies to lineage markers and CD107a. The frequency of cells expressing surface CD107a was determined by flow cytometry. Symbols indicate values for patients and bars mean for relatives and controls. Error bars indicate SD.

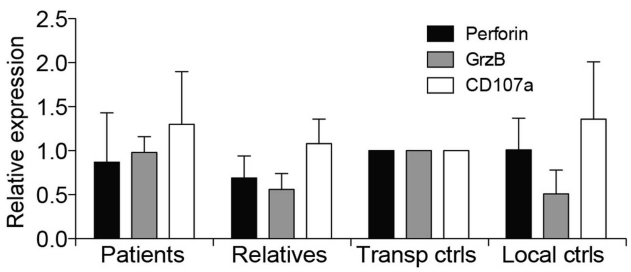

FIGURE 2 | Intracellular expression of CD107a, perforin, and granzyme B in patients with homozygous *STX11* c.173T > C mutations. Intracellular expression of perforin, granzyme B, and CD107a was examined in patient B and C, in addition to relatives ($n = 8$, all four parents in addition to four siblings) as well as transport ($n = 2$) and local controls ($n = 4$). Expression of granule constituents was normalized relative to that of the transport control. Bars indicate the mean value, Error bars indicate SD.

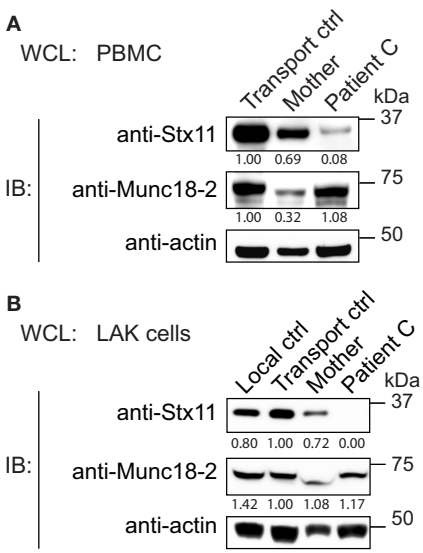

FIGURE 3 | Low expression of syntaxin-11 L58P in patient lymphocytes. Whole cell lysates (WCL) prepared from **(A)** PBMC or **(B)** LAK cells from Stx11 L58P patient C and controls, as indicated, were analyzed by Western blotting for Stx11 and Munc18-2. β-actin was probed as a loading control. Densitometry values normalized to β-actin for each individual are indicated.

By sequence homology to Stx1A, the Stx11 L58P mutation is located in the first α-helix of the conserved Habc domain of Stx11 (20). Substitution to a proline residue at this position likely disrupts the conformation of the Stx11 Habc domain. Interestingly, biochemical analyses examining ectopically expressed, tagged Stx11 in a cell line revealed that the Stx11 L58P mutation disrupted interactions with tagged Munc18-2. Mutations in *STXBP2* that lead to loss of Munc18-2 expression also cause loss of Stx11 expression in lymphocytes (9, 10). Thus, conversely, it is tenable that Stx11 mutations that disrupt Munc18-2 binding might reduce Stx11 expression through a similar mechanism, explaining the low expression of mutant Stx11 in patient cells.

With respect to neuronal Stx1 binding of Munc18-1, the very N-terminal residues as well as the Habc domain of Stx1 mediate a binary interaction with Munc18-1 (20–22). Our data suggest that Stx11 binding to Munc18-2 has similar molecular requirements as either mutation of the conserved Stx11 N-peptide (R4A) or

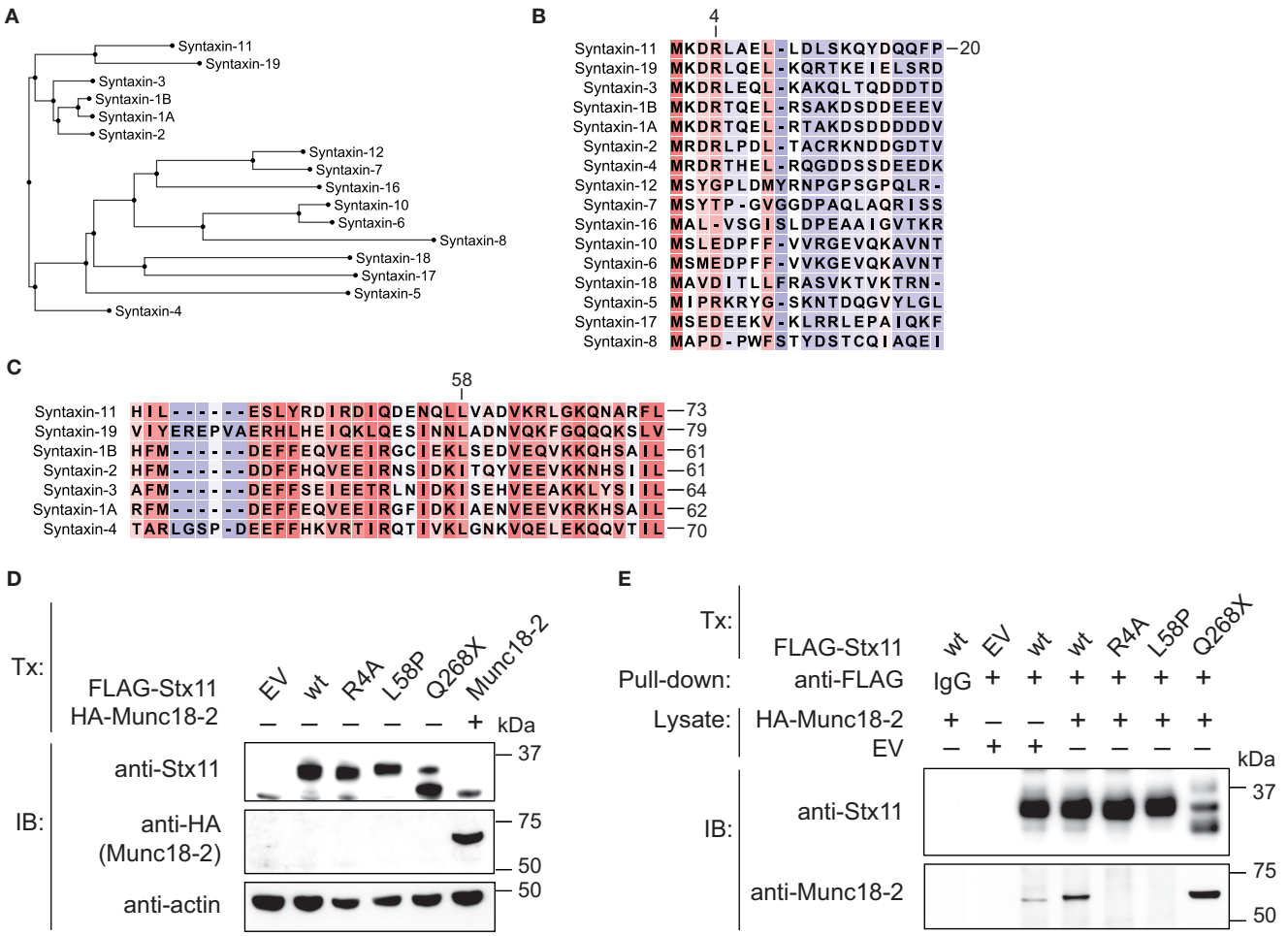

FIGURE 4 | Abrogated binding of Munc18-2 to syntaxin-11 N-terminal R4A or Habc-domain L58P mutations. **(A)** Dendrogram comparing human syntaxins based on peptide sequence alignments of the N-termini (N-peptide and Habc domain; residues 1-167 of Stx11). **(B)** Amino acid alignments of human syntaxin **(B)** N-terminus and **(C)** Habc domain sequences. Red shade indicates a high degree of sequence homology, whereas blue shade indicates low homology. **(D)** HEK-293T cells were transfected with empty vector (EV), or constructs encoding FLAG-Stx11 wild-type (wt), R4A, L58P, Q268X, or HA-Munc18-2 wild-type. Twenty-four hours after transfection, WCL were prepared and analyzed for expression of Stx11, Munc18-2, and actin. **(E)** HEK-293T cells constructs as indicated. Twenty-four hours after transfection, WCL were prepared from Stx11 transfectants and Stx11 was immunoprecipitated to beads. The beads were washed extensively and thereafter incubated with WCL from EV or HA-Munc18-2 wt transfected HEK-293T cells. Beads were washed, proteins eluted, and analyzed by Western blotting with antibodies as indicated.

of the Habc domain (L58P) abrogated Munc18-2 binding. These observations are supported by recent publications demonstrating that mutations in the hydrophobic pocket of Munc18-2, which can bind the N-peptide of Stx11, abrogate Stx11 binding and mast cell degranulation (24). During preparation of this manuscript, the Munc18-2 crystal structure was reported (25). The crystal structure, as well as studies of how Munc18-2 mutations associated with FHL5 impact Stx11 binding, similarly suggest a requirement for the N-peptide and Habc domains of Stx11 for binding to Munc18-2 (25). With respect to neuronal exocytosis, it has recently been shown that mutations in the Habc domain of Stx1 abrogate interactions with Munc18-1, which usually keep Stx1 in a closed conformation, leading to reduced Mun18-1 expression (26). In contrast, mutations of the Stx1 N-peptide more specifically interfere with vesicle fusion (26). It will be interesting to further determine how the N-terminus and Habc domain of Stx11 regulate Stx11 conformation, trafficking, and granule exocytosis.

Stx11 has been shown to interact with additional proteins involved in vesicle exocytosis, including SNAP-23, VAMP2, and Vti1b (16, 17). In addition, the priming factor Munc13-4 has been shown to interact with different Stxs (27). These proteins interact with the Stx11 C-terminal SNARE domain, with preferential binding to the open conformation of Stx11. Speculatively, although other more direct mechanisms for degradation of Stx11 due to protein misfolding also may explain low Stx11 expression, disruption of the Stx11 – Munc18-2 interaction in patient NK cells may lead to Stx11 degradation based on a mechanism dependent on such facilitators and regulators of vesicle exocytosis. Thus, it is of interest to perform a more comprehensive screen of how

different Stx11 mutations impact interactions with other proteins implicated in facilitating and regulating granule exocytosis.

In conclusion, we demonstrate that both the N-terminus and Habc domain of Stx11 are involved in binding to Munc18-2. In the patients homozygous for a Stx11 L58P mutation, it is quite possible that the abrogated interaction between Stx11 and Munc18-2 leads to destabilization of Stx11 expression. Further studies of Stx11 mutants may provide insights into mechanisms, specificity, and redundancy governing SNARE complex formation for lytic granule exocytosis by cytotoxic lymphocytes.

AUTHOR CONTRIBUTIONS

Martha-Lena Müller designed research, performed biochemical experiments, analyzed and interpreted data, and drafted the manuscript; Samuel C. C. Chiang designed research, performed functional evaluations of lymphocytes, analyzed and interpreted data, and drafted the manuscript; Marie Meeths designed research, performed targeted sequencing of FHL genes, and drafted the manuscript; Bianca Tesi designed research and performed targeted sequencing of FHL genes; Miriam Entesarian, Daniel Nilsson, and Stephanie M. Wood designed experiments and interpreted data; Magnus Nordenskjöld and Jan-Inge Henter designed research and interpreted data; Ahmed Naqvi identified and cared for patients, collected clinical data, and drafted the manuscript; Yenan T. Bryceson designed research, interpreted data, and drafted the manuscript.

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Functional significance of CD57 expression on human NK cells and relevance to disease

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Historically, human NK cells have been identified as $CD3^{-}CD56^{+}CD16^{\pm}$ lymphocytes. More recently it has been established that CD57 expression defines functionally discrete sub-populations of NK cells. On T cells, CD57 expression has been regarded as a marker of terminal differentiation and (perhaps wrongly) of anergy and senescence. Similarly, CD57 expression seems to identify the final stages of peripheral NK cell maturation; its expression increases with age and is associated with chronic infections, particularly human cytomegalovirus infection. However, $CD57^{+}$ NK cells are highly cytotoxic and their presence seems to be beneficial in a number of non-communicable diseases. The purpose of this article is to review our current understanding of CD57 expression as a marker of NK cell function and disease prognosis, as well as to outline areas for further research.

Keywords: CD57, NK cells, HCMV infection, ageing, chronic infection, cancer, autoimmune diseases, T cells

CD57 IS A MARKER OF NK CELL DIFFERENTIATION

CD57 was first identified on cells with natural killer activity using the mouse monoclonal antibodies Human Natural Killer-1 (HNK-1) (1) and Leu-7 (2) and was subsequently assigned the cluster of differentiation (CD) designation, CD57, at the fourth International Workshop of Human Leukocyte Antigens in 1989. HNK-1/Leu-7/CD57 was initially believed to be uniquely expressed on NK cells – and was used to define this population (1, 3) – although it was soon apparent that CD57 was expressed only on a subset of functionally distinct NK cells (4). CD57 was subsequently identified on $CD8^{+}$ T cells (5–7) as well as cells of neural crest origin (1, 8–13). Indeed, it was the neuroscience community that ultimately defined CD57 as a terminally sulfated carbohydrate epitope (glucuronic acid 3-sulfate) (14–16). In neural cells, the CD57 epitope is predominantly restricted to adhesion molecules (17) but little attention has been paid to the precise identity of the molecules expressing the CD57 epitope on NK cells and T cells, precluding a full understanding of the relationship between CD57 expression and lymphocyte function. Although one study identified the CD57 epitope on the IL-6 receptor gp130 of resting lymphocytes (18), the cells expressing CD57/gp130 were not identified and no comprehensive analysis of CD57-expressing molecules on T cells or NK cells has been reported.

While first characterized as an NK cell marker, CD57 has been most widely explored as a marker of replicative senescence on T cells (19). Under conditions of persistent immune stimulation, memory T cells convert from $CD28^{+}CD57^{-}$ to $CD28^{-}CD57^{+}$ (20); $CD57^{+}$ cells have short telomeres, low telomerase activity, low expression of cell-cycle associated genes and limited proliferative capacity (20, 21). However, $CD57^{+}CD28^{-}CD8^{+}$ T cells can proliferate given an appropriate cytokine milieu (22), their sensitivity to apoptosis is disputed (23, 24), they are highly cytotoxic (25, 26) and express natural killer receptors (27). $CD57^{+}CD8^{+}$ T cells should thus be regarded as terminally differentiated, oligoclonal populations of cytotoxic cells generated in response to chronic antigen stimulation.

In light of the T cell data it was suggested that CD57 may also be a marker of NK cells with poor proliferative capacity and, perhaps, a degree of immunosenescence (21, 23, 28). Indeed, acquisition of CD57 on NK cells – following stimulation with IL-2 or coculture with target cells – correlates with maturation of the $CD56^{dim}$ NK cell subset, with lower expression of NKp46, NKp30, NKG2D, and NKG2A, and higher expression of CD16, LIR-1, and killer cell immunoglobulin-like receptors (KIRs) (29). Similarly, in hematopoietic stem cell transplant recipients exposed to human cytomegalovirus (HCMV) infection, differentiation of $CD56^{dim}$ NK cells involves acquisition of CD57, loss of NKG2A, gain of KIRs, and changing expression of homing molecules (30). These studies, together with experiments in $Rag2^{-/-}\ \gamma cR^{-/-}$ mice reconstituted with human hematopoietic stem cells and treated with IL-15 (30), and the observation that fetal and newborn NK cells lack CD57 (31), indicate that $CD57^{+}$ NK cells differentiate from $CD56^{dim}CD57^{-}$ NK cells in an irreversible process with highly stable expression of CD57 likely being the final step in maturation (30, 32). This differentiation is accompanied by functional changes (29, 30): compared with $CD57^{-}$ cells, $CD57^{+}$ NK cells proliferate less well in response to IL-2 and IL-15 and produce less IFN-γ in response to IL-12 and IL-18, consistent with their lower levels of IL-12Rβ mRNA (29) and reduced surface expression of IL-2Rβ and IL-18Rα (30). On the other hand, $CD57^{+}$ NK cells retain their cytolytic potential (30) and a proportion of $CD57^{+}$ NK cells are able to produce IFN-γ after crosslinking of CD16 [Ref. (29); White et al. submitted] indicating that $CD57^{+}$ NK cells are intrinsically able to produce IFN-γ but that they may have different activation requirements.

In summary, therefore, progression from $CD56^{bright}$ to $CD56^{dim}CD57^{-}$ to $CD56^{dim}CD57^{+}$ reflects a maturation pathway for NK cells (33, 34) and rather than being a marker of anergy or

immunosenescence, acquisition of CD57 represents a shift toward a higher cytotoxic capacity, greater responsiveness to signaling via CD16 and natural cytotoxicity receptors (NCRs) and decreased responsiveness to cytokines (29, 35). The extent to which CD57 expression *per se* drives these changes in function, as opposed to being a marker for cells with altered expression of other attributes of a mature NK cell, is not entirely clear and may represent a fertile area for further research. In addition, a much better characterization is required of the cell surface molecules that express the CD57 epitope, the mechanisms by which CD57 is induced on them, and its functional consequences.

CD57 EXPRESSION AND CANCER

Both $CD8^+$ T cells and NK cells are able to kill tumor cells through mechanisms including perforin/granzyme-mediated cytolysis and TRAIL- or FAS-mediated apoptosis (36). Accumulation of $CD57^+CD8^+$ T cells is seen frequently in individuals with various forms of cancer (37) and has been associated with reduced survival in those with renal cell carcinoma (38), melanoma (39), gastric carcinoma (40), multiple myeloma (41), lymphomas, acute and chronic myeloid, and lymphocytic leukemias (42), among many other examples. CD57 expression on $CD4^+$ T cells has also been associated with Hodgkin's lymphoma (43) and chronic lymphocytic leukemia (44). This association between malignancy and expanded populations of $CD57^+$ T cells is likely explained by persistent stimulation of these cells by tumor-associated antigens in the absence of effective tumor clearance (45).

NK cells were initially identified by their ability to kill malignant cells (46–48) and a large body of clinical and experimental evidence now supports their crucial role in cancer immunosurveillance (49). Reduced MHC Class I expression (50) and *de novo* expression of stress related molecules (such as B7-H6, MICA, MICB, RAE-1, MULT1, and members of the ULBP family) in malignant cells alter the balance of inhibitory (via KIRs and NKG2-CD94 heterodimers) and activating (via NCRs and NKG2D homodimers) signals for NK cells (51), leading to their activation. High frequencies of peripheral or tumor-associated $CD57^+$ NK cells are reported in cancer patients and – in sharp contrast to what has been seen for $CD8^+$ T cells – have frequently been linked to less severe disease and better outcomes (**Table 1**). This would be consistent with enhanced tumor surveillance/cytotoxicity of the mature, $CD57^+$ NK cell subset (29); whether these associations are confounded by HCMV infection status (see below) is currently unclear. In the case of advanced gastrointestinal stromal tumors treated with the chemotherapeutic agent imatinib mesylate, NK cell secretion of IFN-γ after IL-12/IL-2 stimulation was correlated with improved long-term survival (52). Since $CD57^-$ NK cells are the major subset producing IFN-γ in response to cytokines, this suggests that a heterogeneous NK cell population comprising both $CD57^-$ and $CD57^+$ subsets may be optimal for combating neoplasia. Clearly further studies, ideally longitudinal in nature and accompanied by data on potentially confounding factors, are needed to determine the roles of different NK cell subsets in combating different types of malignancies.

CD57 EXPRESSION AND AUTOIMMUNITY

Autoimmune diseases tend to be highly antigen-specific and mediated by autoantibodies or autoreactive T cells. In general, expanded populations of autoreactive $CD57^+$ T cells are associated with more severe disease – Wegener's granulomatosis (65), pars planitis (25), multiple sclerosis (MS) (66), type I diabetes mellitus (67), Graves' disease (68), and rheumatoid arthritis (RA) (69), amongst others. This likely reflects killing of vital host cells by these highly cytotoxic lymphocytes (68), although the loss of T cells with immunosuppressive potential may also play a role (67).

Perhaps surprisingly, autoimmune disease is consistently associated with reduced frequencies or absolute numbers of circulating $CD57^+$ NK cells and/or impaired NK cell cytotoxicity (**Table 2**) (70–78), suggesting that cytotoxic $CD57^+$ NK cells may play a regulatory role, preventing or suppressing autoimmune disease. In MS, peripheral NK cells lose expression of FAS during relapse and regain it during remission (70) and FAS^+ NK cells can inhibit myelin basic protein-specific T cell IFN-γ responses (79), suggesting that NK cells may regulate autoreactive T cells. On the other hand, chronic NK cell lymphocytosis (which is associated with peripheral neuropathy, arthritis, and vasculitis) is characterized by increased absolute numbers of circulating immature NK cells with low cytotoxicity (80, 81). Similarly, NK cells have been found in the inflammatory infiltrates of psoriatic skin lesions (82), in synovial fluid of joints affected by RA (83), and in pancreatic islets of type I diabetes patients (84). NK cells in the synovial fluid of patients with RA, and those infiltrating psoriatic skin lesions, are immature $CD56^{bright}$ or $CD57^-$ and able to secrete IFN-γ and TNF (85, 86), suggesting that they may contribute to the inflammation rather than suppress it (84).

Taken together, these data are consistent with the hypothesis that immature $CD57^-$ NK cells may contribute to autoimmune inflammation and tissue damage whereas more highly differentiated, cytotoxic, $CD57^+$ NK cells may fulfill an immunoregulatory role, possibly deleting chronically activated T cells, as in viral hepatitis (103).

CD57 EXPRESSION DURING INFECTION

Chronic viral infections such as HCMV (104), human immunodeficiency virus (HIV) (105), hepatitis C virus (106), and Epstein–Barr virus (EBV) (107) infections offer some of the clearest examples of expansion of $CD57^+CD8^+$ T cells, presumably as a result of persistent antigenic stimulation, and increased proportions of $CD57^+CD8^+$ T cells have also been reported in those infected with human parvovirus (108), measles (109), pulmonary tuberculosis (92), and toxoplasmosis (93). The majority of these $CD57^+CD8^+$ T cells, at least in HCMV infection, appear to be antigen-specific and their presence is associated with a low incidence of reactivation (94, 95). Similar skewing of NK cells toward the $CD57^+$ phenotype is now reported in a variety of viral infections (**Table 2**).

Increased frequencies of $CD57^+CD16^+$ NK cells were first reported in HCMV-infected individuals by Gratama et al. (110) and have been repeatedly confirmed (99, 111, 112). Studies of hematopoietic stem cell transplantation (HSCT) have been particularly informative, allowing detailed comparison of stem cell differentiation into NK cells in HCMV-infected and uninfected transplant recipients (111, 112) with rapid and persistent expansion of $CD57^+$ NK cells that are also $NKG2C^+$, KIR^+, $CD158b^+$, and potent producers of IFN-γ after stimulation with MHC Class I-deficient target cells, only in the HCMV-infected group (111). We now know that HCMV drives expansion of $NKG2C^+$ NK cells and

Table 1 | Associations between cancer prognosis and CD57 expression by NK cells.

Cancer type	Observations	Reference
Acute lymphoblastic leukemia	Increased NK cell activity and increased numbers of $CD57^+$ and $CD16^+$ NK cells in bone marrow associated with complete remission	Sorskaar et al. (57)
Hodgkin's disease	Absence/low number of $CD57^+$ NK cells in tumor tissue (by immunohistochemistry) associated with relapse	Ortaç et al. (58)
Non-Hodgkin's lymphoma	Higher numbers of intratumoral $CD57^+$ NK cells are associated with relapse free survival in pediatric cases	Ortaç et al. (58)
Metastatic tumors in the brain	$CD57^+$ NK cells infiltrate brain metastases of various origins (lung, breast, and renal carcinomas; melanoma) but no correlation between numbers of infiltrating $CD57^+$ NK cells and apoptosis of malignant cells	Vaquero et al. (59)
Colorectal cancer	Increased $CD57^+$ NK cells in germinal centers of draining lymph nodes, but rarely in primary or metastatic lesions; $CD57^+$ NK cells may prevent establishment of tumor in lymph nodes?	Adachi et al. (60)
Bladder carcinoma	Lower frequency of $CD56^+$ and $CD57^+$ PBMC in patients with invasive and non-invasive tumors is correlated with reduced cytotoxicity against T24 bladder cancer cell line	Hermann et al. (61)
Breast carcinoma	Survival is positively correlated with the number of tumor infiltrating $CD57^+$ NK cells and with expression of CX3CL1 (a known NK cell chemoattractant) by the tumor cells	Park et al. (62)
Gastric carcinoma	$CD57^+$ NK cell infiltration associated with a lower clinical grade tumor, reduced venous invasion, fewer lymph node metastases, less lymphocytic invasion, and increased 5 year survival outcome	Ishigami et al. (63)
Oral squamous cell carcinoma	Low density of tumor infiltrating $CD57^+$ NK cells and high numbers of TNF^+ cells associated with higher clinical staging	Turkseven and Oygur (64)
Esophageal squamous cell carcinoma	Tumor infiltrating $CD57^+$ NK cells positively associated with increased survival over 80 months	Lv et al. (87)
Squamous cell lung carcinoma	Tumor infiltrating $CD57^+$ NK cells positively correlated with increased survival 2 years after surgery	Villegas et al. (88)
Pulmonary adenocarcinoma	Higher absolute numbers of tumor infiltrating $CD57^+$ NK cells correlated with tumor regression	Takanami et al. (89)
Various	Low numbers of $CD57^+$ NK cells in peripheral blood are associated with carcinomas of colon, lung, breast, and neck; no association was with melanoma or sarcoma	Balch et al. (90)

that these cells preferentially acquire CD57 (97–99, 111, 112). In HCMV-uninfected donors, there are roughly equal proportions of $CD57^+NKG2C^+$ and $CD57^-NKG2C^+$ NK cells whereas the ratio of $CD57^+NKG2C^+$ to $CD57^-NKG2C^+$ NK cells ranges from <1 to >60 in HCMV-infected donors (99); whether this variation reflects varying duration of HCMV infection is not known. HCMV reactivation after HSCT is associated with a threefold increase in the ratio of $CD57^+NKG2C^+$ to $CD57^-NKG2C^+$ NK cells within one year (111). Yet, in the absence of HCMV infection, $NKG2C^+$ NK cells are no more likely to acquire CD57 than are $NKG2C^-$ NK cells (112), suggesting that either binding of NKG2C to specific HCMV ligands or chronic viral infection *per se* drives NK cell differentiation. Importantly, $CD57^+CD16^+$ NK cells can kill HCMV-infected target cells (96) and this may be dependent upon, or enhanced by, α-HCMV antibodies (113).

While HCMV remains the clearest example of infection driving NK cell differentiation, other viral infections may cause a similar effect. For example, there is a three to fourfold expansion of the NK cell pool during acute hantavirus infection; NK cell numbers peak approximately 10 days after the onset of symptoms and remain above baseline for at least 60 days (114). This expansion is restricted to the $NKG2C^+$ NK cell subset and the majority of these cells are $CD57^+$, KIR^+ and highly responsive to MHC Class I-deficient target cells. Hantavirus-infected endothelial cells express high levels of the NKG2C ligand HLA-E and expansion of the $NKG2C^+$ NK cell subset is seen only in HCMV seropositive hantavirus patients, suggesting that hantavirus-induced HLA-E expression and/or inflammatory cytokines released during infection may drive the expansion and subsequent maturation of $NKG2C^+$ NK cells that have been induced or "primed" by HCMV infection (114). Similarly, transient expansion of the $CD57^+$ $NKG2C^+$ NK cell population during acute chikungunya virus infection is also associated with HCMV seropositivity (115).

Expansion of the $NKG2C^+CD57^+$ NK cell subset has also been reported in $HCMV^+$ individuals with chronic hepatitis B and hepatitis C infections, although the proportions of these cells did not differ markedly from previous reports in HCMV-infected but hepatitis virus-uninfected donors, leading the investigators to conclude that HCMV, rather than viral hepatitis, is the underlying driver of NK cell differentiation (97). In line with this, no

Table 2 | Associations between autoimmune diseases or infections and CD57 expression by NK cells.

	Observations	Reference
AUTOIMMUNE DISEASE		
Alopecia areata	$CD57^+$ NK cells are significantly reduced in peripheral blood of patients with multiple foci of alopecia	Imai et al. (91)
Atopic dermatitis	Reduced frequencies of $CD57^+$ NK cells in peripheral blood of patients compared to healthy controls, with greatest reduction in the most severe cases	Wehrmann et al. (126) and Matsumura (127)
Sjögren's syndrome	Decreased numbers of $CD57^+$ NK cells observed in peripheral blood of patients compared to controls	Struyf et al. (128)
IgA nephropathy	Decreased proportion of $CD57^+$ $CD16^+$ lymphocytes in the peripheral blood of patients compared to healthy controls	Antonaci et al.(129)
Psoriasis	NK cells infiltrating skin lesions – but also unaffected skin – are predominantly CD57low	Batista et al. (85)
INFECTION		
HCMV	Increased proportions of $CD57^+$ NK cells in infected individuals; CD57 expression limited to the $NKG2C^+$ subset	Gratama et al. (110), Lopez-Vergès et al. (99) and Foley et al. (111, 112)
HIV	In chronic infections, there is a loss of CD57-/dim NK cells, but the absolute number of $CD57^+$ NK cells remains constant	Hong et al. (100)
Chikungunya virus	Increased proportions of $CD57^+$ NK cells after infection in $HCMV^+$ patients	Petitdemange et al. (115)
Hantavirus	$NKG2C^+$ NK cell subset expanded during infection in $HCMV^+$ patients and the majority of these cells are $CD57^+$	Björkström et al. (114)
Hepatitis B and Hepatitis C	$NKG2C^+$ NK cell population is expanded in chronic infections, and these are predominantly $CD57^+$, but co-infection with HCMV appears to be the driver of this effect	Béziat et al. (97)
Lyme disease	Conflicting evidence on whether chronic disease leads to a reduced proportion of $CD57^+$ NK cells in peripheral blood	Stricker et al. (117), Stricker and Winger (118), and Marques et al. (119)

association was found between expansion of the $NKG2C^+CD57^+$ NK cell subset and clinical indicators of hepatitis such as viral load or liver enzyme concentrations (97).

In HIV-infected individuals, the absolute number of $CD57^+$ NK cells is stable and comparable to HIV-negative individuals but the ratio of $CD57^+$ to $CD57^-$ NK cells is higher than in uninfected individuals due to a gradual loss of $CD57^-$ cells (which are highly dependent on monocyte and T cell-derived cytokines for their survival) (100). Unfortunately, the HCMV status of these subjects was not reported and may confound the comparison between the HIV^+ and HIV^- individuals. Indeed, in another study, the positive association between frequency of $NKG2C^+$ NK cells and HIV-1 infection disappears when adjusted for HCMV status (101). Nonetheless, it is also the case that the frequency of $NKG2C^+(CD57^+)$ NK cells is higher in HCMV seropositive donors with HIV-1 infection than in HCMV seropositive donors without HIV-1 infection (102), suggesting either that – as for hantavirus or chikungunya virus – HIV-1 infection drives expansion of the HCMV-induced $NKG2C^+$ population or that HIV-1 infected individuals experience more frequent reactivation of HCMV which then expands the $NKG2C^+$ population. Significantly, $CD57^+$ NK cells of HIV^+ individuals retain a highly differentiated phenotype ($CD16^+KIR^+perforin^+$) but have defects in degranulation (100) suggesting that they may have reduced cytotoxic potential. Finally, although no association was seen between accumulation of $CD57^+$ NK cells and recurrence of genital herpes lesions due to herpes simplex virus 2 (HSV-2) infection (116), interpretation of this study is hindered by the lack of an HSV-2-uninfected control group.

There have been very few studies of NK cell subsets in the context of bacterial or parasitic infections. Patients with chronic Lyme Disease (*Borrelia burgdorferi*) have lower proportions of peripheral blood $CD57^+$ NK cells compared to those with acute disease and uninfected controls and this phenotype was maintained for over 10 years in one person with persistent infection (117, 118). In contrast, no significant differences in numbers of peripheral blood $CD3^-CD57^+$ cells were noted between patients with post-Lyme disease syndrome, individuals recovered from Lyme disease and healthy controls (119). The suggestion (118) that high frequencies of $CD57^+$ NK cells may be a biomarker of Lyme disease progression thus seems premature, especially given the potential impact on NK cell phenotype of HCMV and other infections.

In summary, viral infections are important drivers of NK cell differentiation with HCMV playing a primary role in selecting for $NKG2C^+CD57^+$ cells and other viruses driving their expansion and differentiation.

CD57 EXPRESSION AND AGING

Given the enormous impact of infection on NK cell maturation and differentiation, it is not surprising that NK cell populations change with age, which is a proxy for cumulative exposure to infection and other physiological insults. At birth virtually no T cells express CD57 (120) but the proportion rises with age, reaching 20–30% in young adults (20); by 80 years of age 50–60% of $CD8^+$ T cells are $CD28^-$ (and thus likely $CD57^+$) (20, 121). Similarly, with increasing age, increasing numbers of circulating NK cells are achieved by an expansion of the $CD56^{dim}$ and $CD57^+$ subsets and an absolute, as well as a proportional, decline in $CD56^{bright}$ cells (35, 53–55, 122–125). At birth, all $CD56^{dim}$ NK cells are $CD57^-$; among European adults (18–60 years of age) 25–60% of $CD56^{dim}$ NK cells are $CD57^+$ and this continues to increase slightly, but significantly, after the age of 80 years (124). Interestingly, $CD56^{dim}CD57^+$ NK cells accumulate very rapidly in an African (Gambian) population reaching adult levels (20–70%) by the age of 5 years (Goodier et al. unpublished); this may reflect very high HCMV seroprevalence rates in this age group in this community.

The increased proportion of $CD56^{dim}CD57^+$ NK cells in the elderly likely explains the maintenance of NK cell cytotoxic responses despite reduced responsiveness to cytokine stimulation [reviewed in Ref. (56)], however, the significance of these changes in terms of overall immune competence is poorly understood. The gradual loss of the $CD56^{bright}$ NK cell population, and the consequent decline in NK-derived cytokines that activate dendritic cells and monocytes, has been assumed to contribute to age-associated declines in immune competence but the potential counterbalancing effect of an increased proportion of highly cytotoxic $CD57^+$ NK cells has received little attention (123). Comprehensive studies are now needed to assess the cytokine-producing and cytotoxic function of individual NK cell subsets in response to cytokine stimulation as well as activation via CD16 and NCRs and the extent to which this changes with age and HCMV status.

CONCLUSION AND FUTURE DIRECTIONS

CD57 is a very useful marker of NK cell maturation, identifying cells with potent cytotoxic potential but decreased sensitivity to cytokines and reduced replicative potential. $CD57^+$ NK cells appear to be a stable sub-population, increasing with age and exposure to pathogens (especially, but not exclusively, HCMV) and their presence is consistently associated with better outcomes in cancer and autoimmune disease. However, the majority of clinical studies have been cross-sectional, with limited follow up and data on crucial confounding factors such as HCMV infection are typically lacking. Recent studies of HSCT (111, 112) demonstrate the power of prospective and longer term studies in beginning to assign causality in terms of NK cell phenotype, function, and disease. Nevertheless, precise understanding of the role of CD57 expression on NK cells requires a detailed dissection of the underlying biology of CD57, about which very little is known. Given that there is no evidence that CD57 is expressed on murine NK cells, this is not a simple task. Possible approaches in human NK cells might include conducting a comprehensive analysis of NK cell molecules expressing CD57, blocking CD57 in *in vitro* functional NK cell assays, or manipulating expression or enzymatic activity of B3GAT1 (the key enzyme in the biosynthesis of CD57) using RNA interference or specific inhibitors.

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Combination immune therapies to enhance anti-tumor responses by NK cells

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Natural killer (NK) cells are critical innate immune lymphocytes capable of destroying virally infected or cancerous cells through targeted cytotoxicity and further assisting in the immune response by releasing inflammatory cytokines. NK cells are thought to contribute to the process of tumor killing by certain therapeutic monoclonal antibodies (mAb) by directing antibody-dependent cellular cytotoxicity (ADCC) through FcγRIIIA (CD16). Numerous therapeutic mAb have been developed that target distinct cancer-specific cell markers and may direct NK cell-mediated ADCC. Recent therapeutic approaches have combined some of these cancer-specific mAb with additional strategies to optimize NK cell cytotoxicity. These include agonistic mAb targeting NK cell activating receptors and mAbs blocking NK cell inhibitory receptors to enhance NK cell functions. Furthermore, several drugs that can potentiate NK cell cytotoxicity through other mechanisms are being used in combination with therapeutic mAb. In this review, we examine the mechanisms employed by several promising agents used in combination therapies that enhance natural or Ab-dependent cytotoxicity of cancer cells by NK cells, with a focus on treatments for leukemia and multiple myeloma.

Keywords: NK cells, immunotherapy of cancer, antibodies, monoclonal, ADCC, multiple myeloma

INTRODUCTION

Natural killer (NK) cells are generally known for the ability to mediate spontaneous "natural" cytotoxicity of major histocompatibility complex class I (MHC-I)-deficient tumor or virus-infected cells. NK cells kill target cells through the release of perforin and granzymes from cytolytic granules or the surface expression of Fas ligand or TNF-related apoptosis-inducing ligand (TRAIL). Activated NK cells are also a potent source of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and a variety of other cytokines and chemokines that contribute significantly to early immune responses.

The activation of NK cells is controlled by a balance of signals emanating from a collection of germline-encoded activating and inhibitory receptors. Several inhibitory receptors, including CD94/NKG2A, members of the highly polymorphic killer cell-Ig-like receptor (KIR) family and ILT2/LIR-1/CD85j, play critical roles in tolerizing NK cells toward healthy cells by binding to MHC-I ligands [human leukocyte antigen (HLA)-A, -B, and -C allotypes] expressed on virtually all healthy cells (1). When NK cells engage with MHC-I-expressing healthy cells, the inhibitory receptors transduce negative signaling that efficiently abrogate stimulatory signals from co-engaged activating receptors at the immunological synapse (2–4). Many tumors and virus-infected cells down-regulate their surface expression of MHC-I to avoid recognition by the antigen receptor on cytotoxic T cells, but these abnormal cells inherently become susceptible to NK cell-mediated attack in the absence of the tolerizing MHC-I ligands.

Activating receptors expressed on human NK cells include FcγRIIIA (CD16), activating forms of KIR, 2B4, NKG2D, and the natural cytotoxicity receptors (NCR), which are also known as NKp30, NKp44, and NKp46 (5, 6). Of these, the NCR and NKG2D are particularly important receptors for triggering NK cell responses toward tumor target cells (7). In contrast to inhibitory receptors, triggering of the activating receptor NKG2D is a key mechanism by which NK cells recognize stressed or diseased cells and destroy them (8). NKG2D specifically recognizes MHC chain-related (MIC) A, MICB, and UL16-binding proteins (ULBPs) in humans, which are HLA-related molecules lacking peptide presentation capacity that are not expressed on normal cells but are up-regulated on stressed cells, such as tumors (8, 9).

Thus, NK cells are important effectors in immune responses to tumors and viral infections, and increased understanding of the mechanisms controlling NK cell activation has led to the development of therapeutic agents that can improve their responsiveness. While these agents show promise, results are inconsistent between patients due to inherent differences in activity/function of an individual's immune system, and expression of distinct biomarkers on cancers that can differentially influence NK cell responsiveness. A growing variety of treatment options can improve outcome for individual patients. Novel treatment regimens combining new and old therapies are even showing promise among relapsed and refractory patients. Here, we review some of the currently available therapies that are known to stimulate NK cell functions and how they are being used in combination with other agents to boost anti-tumor responses in cancer patients.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

FcγRIIIA (CD16) provides NK cells with the capacity to mediate antibody-dependent cellular cytotoxicity (ADCC) upon recognition of the Fc segment of IgG bound to cell surfaces. This

antigen-specific NK cell targeting mechanism appears to play an important role in tumor eradication by several therapeutic tumor-specific mAb, such as herceptin, rituximab, and elotuzumab. Interestingly, Deguine et al. recently suggested that NKG2D engagement might be crucial for NK cell-mediated ADCC responses. In that study, mouse tumors expressing NKG2D ligands induced enhanced ADCC degranulation, while responses were reduced toward tumors lacking NKG2D ligand. The results indicate that the mAb-FcR interaction mainly stabilized adhesion with the target cell to contribute contact stability, while NKG2D triggering provided the activation threshold needed to trigger NK cell degranulation (10). This concept is supported by earlier work in a mouse model of renal cancer that showed NKG2D ligation does not stimulate strong adhesion with tumor cells. In that report, target cells that did not express ICAM-1 were not killed in an NKG2D-dependent manner, but transfection of ICAM-1 into these cells restored NKG2D-mediated cytotoxicity (11).

An emerging strategy to enhance NK cell-mediated ADCC responsiveness is to inhibit the shedding of CD16 that occurs after NK cell activation (12, 13). Specifically, CD16 can be cleaved by metalloproteinases after being triggered by an IgG-opsinized target cell or treatment with IL-2, but if metalloproteinases are inhibited, CD16 is not shed. In fact, CD16 signaling is sustained and target cell killing and cytokine production are enhanced in the presence of metalloproteinase inhibitors (14). Furthermore, combining metalloproteinase inhibition with a bispecific antibody (against CD16 and CD33) resulted in a sustained and very specific anti-tumor response (15). Such a combination strategy has the potential to substantially increase NK cell responses to cancer.

An additional immunoglobulin-based strategy to target more immune responses toward tumor cells is to create bispecific or trispecific antibodies. The engineering of bispecific monoclonal antibodies (mAb) that create surrogate bridging between activating receptors on T or NK cells with tumor-specific antigens have shown therapeutic promise for decades (16). A common bispecific design theme to target NK cells has employed fusing antibody Fv domains that bind CD16 with Fv domains that bind tumor cell markers, such as CD30 (17), ErbB2 (18–20) or CD19 (21, 22). These constructs can trigger ADCC responses by NK cells and monocytes/macrophages through the CD16 FcR without the need for an incorporated Fc domain. As a cautionary tale for appropriate design, a bispecific incorporating a CD16-binding Fv domain in combination with a CD16-binding Fc domain was found in an early phase I study to induce toxicity through dimeric engagement of the FcR on the surface of monocytes and NK cells in the blood, which induced rapid release of cytokines, including TNF-α, IL-6, and IL-8 (18). Modern recombinant approaches fuse Fv domains into single chain constructs that have been termed bispecific or trispecific killer cell engagers (BiKEs or TriKEs), which lack Fc domains entirely. Importantly, these BiKEs and TriKEs can be highly effective at inducing NK cell ADCC and cytokine responses (22). In another approach, von Strandmann et al. created a bispecific protein (ULBP-BB4) that fused the NKG2D ligand, ULBP2, onto a single chain Fv targeting CD138, which is highly expressed on a number of malignancies, including multiple myeloma (MM). In xenograft mouse models injected with human tumor cells and human PBLs, ULBP-BB4 demonstrated potent anti-tumor activity while not significantly harming healthy cells (23). Many researchers have also developed trivalent antibodies composed of two tumor antigen-recognition domains and one monovalent domain that recognizes NK cells (19–21, 24). If properly manipulated for safety, bispecific and trispecific antibodies offer a very targeted approach to effective tumor therapy that can directly involve NK cell effector function.

THE USE OF NK CELL-RELATED IMMUNE THERAPIES TO TREAT MULTIPLE MYELOMA

A common proving ground for testing immune therapies that stimulate NK cell activity has been MM. MM is a deadly hematologic cancer characterized by clonal expansion of malignant plasma cells that reside in the bone marrow and thrive on interactions with the bone marrow microenvironment (25). Despite advances in treatment strategies, MM remains an incurable disease, with about 20,000 newly diagnosed cases and over 10,000 deaths per year in the U.S. (26). Novel therapies have improved survival over the last decade, including autologous hematopoietic stem cell transplantation (HSCT) and the use of new drugs (27). Allogeneic stem cell transplantation can be curative, but is often associated with high transplantation-related mortality (28). Despite these advanced therapeutic options, median survival remains around 4–5 years in adults (29) and the development of better treatments is essential. Interestingly, evidence is accumulating that NK cells may play a prominent role in immune responses toward MM and can also contribute to graft-versus-myeloma responses in haploidentical HSCT (30), It is becoming clear that NK cells can elicit potent allogeneic and autologous responses to myeloma cells *in vitro* and in patients (30, 31). Given the importance of NK cells in immune responses toward MM, combination therapies that enhance NK cell functions are showing promise in treating this deadly disease, as will become evident in the following discussion.

IMMUNOMODULATORY DRUGS (IMiDs®)

Thalidomide, lenalidomide, and pomalidomide form a new class of immunomodulatory drugs, referred to as IMiDs, which can broadly stimulate the functions of NK cells and T cells to treat cancer (32). Thalidomide is a glutamic acid derivative with a dark history as a therapeutic agent, since it caused severe birth defects when used to treat morning sickness in pregnant women in the late 1950s. Nonetheless, it was subsequently found to have anti-inflammatory, anti-angiogenic, anti-proliferative, and immunomodulatory properties that fostered further investigation (33–35). The anti-inflammatory properties of thalidomide are at least partially due to potent inhibition of the production of TNF-α by activated monocytes (35). Lenalidomide and pomalidomide are more potent thalidomide analogs that have since emerged (36), and pomalidomide is even more potent at co-stimulating T cells than lenalidomide (37). Since these IMiDs can enhance the functions of T cells and NK cells, suppress angiogenesis, inhibit TNF-α production, and directly repress tumor cell growth, they are potentially beneficial in treating cancer. To date, both lenalidomide and pomalidomide have been used to treat MM and a variety of other cancers.

The mechanism of immune stimulation by IMiDs is complex and not entirely established (32). Treatment of patients with lenalidomide has been shown to increase the overall frequency of NK cells in peripheral blood, suggesting that they either proliferate or migrate into the bloodstream (38–40). Lenalidomide does not appear to stimulate NK cells directly, however, but instead functions through effects on other leukocytes in peripheral blood (40). Stimulation of T cells by lenalidomide overcomes the need for signals from antigen presenting cells and induces increased proliferation and enhanced production of the type 1 cytokines, IL-2, and IFN-γ (37, 41, 42). At least part of the stimulatory effects of IMiDs on NK cells appears to be due to the T cell production of IL-2, which is a potent growth factor for NK cells (43, 44). Both lenalidomide and pomalidomide have also been shown to increase ADCC activity by NK cells (44, 45). At least part of this effect may result from an increased frequency of the $CD56^{dim}$ NK cells expressing CD16 and LFA-1 in peripheral blood, which are responsible for mediating ADCC (46). This ability of IMiDs to augment ADCC has been borne out in clinical studies, particularly in combination with the CD20-targeting antibody rituximab, where significant activity has been seen in relapsed/refractory B-cell lymphomas and chronic lymphocytic leukemia (47, 48). In MM, lenalidomide is usually used in combination with steroids (49, 50). However, the enhanced NK cell-mediated responses by lenalidomide can be reversed in combination with dexamethasone (40), suggesting that using steroids long-term in combination with lenalidomide may be counterproductive to its immune-stimulatory effects, and that steroid-free combinations should be explored. It should also be noted that tumor cell lines cultured in lenalidomide become more susceptible to NK cell-mediated lysis, due to their increased expression of ligands for NK cell activating receptors (38–40, 51). Taken together, NK cell-mediated anti-tumor responses can be stimulated in a variety of ways by IMiDs, and this enhanced function can be beneficial in treating cancer.

BORTEZOMIB

Bortezomib is an inhibitor of the 26S proteasome that is currently used to treat MM and lymphoma. Inhibition of the proteasome has several direct negative impacts on tumor cells, including inhibiting proliferation and inducing apoptosis, but bortezomib-treated tumor cells also become more susceptible to attack by NK cells (52). Upon inhibition of the proteasome, tumor cells are incapable of processing and presenting proteolytic peptide fragments on MHC-I molecules on the plasma membrane. Consequently, bortezomib down-regulates the surface expression of MHC-I on tumor cells *in vitro* and *in vivo* (53), thereby reducing the levels of this important protein for NK cell tolerance and enhancing susceptibility to NK cell-mediated natural and antibody-dependent cytotoxicity (54, 55). Bortezomib treatment can augment the efficacy of adoptively transferred NK cells in murine tumor models (56), and this approach has now been translated to the clinic for cancer patients (57).

ELOTUZUMAB

A promising new monoclonal antibody candidate for treatment of MM is elotuzumab (formerly HuLuc63). Elotuzumab is a fully humanized antibody that recognizes the SLAM family member CS1 (CRACC, SLAMF7, CD319), a surface glycoprotein normally expressed on NK cells, monocytes, mature dendritic cells, a subset of T cells, and stimulated B cells (58, 59). Normal plasma cells express high levels of CS1, which correlates with high expression on MM cells (60). CS1 is an attractive therapeutic ADCC target because the available clinical data indicate that expression persists on MM cells even after conventional treatments (61–63). CS1 was originally found to engage in homotypic interactions as a self-ligand, and pretreatment of a NK cell line with recombinant CS1-Ig fusion protein was shown to stimulate killing of K562 target cells, apparently by directly activating the NK cells via homotypic interaction (64). Two separate reports found high CS1 expression in most MM cases studied, and elotuzumab was found to significantly increase NK cell-mediated ADCC of primary MM cells (65, 66).

While initiation of NK cell-mediated ADCC upon engagement with CD16 is the best characterized function of elotuzumab, the exact mechanism(s) of action is unclear (67). Importantly, CS1 is also considered a co-stimulatory receptor on NK cells (64, 68), and recent evidence indicates that elotuzumab may also stimulate NK cells directly through direct interactions with CS1 on the NK cell surface (69). As an additional potential mechanism, elotuzumab may interfere with interactions of MM cells with the bone marrow microenvironment, which is a key requirement for tumor survival and proliferation (60, 67). MM cell interactions with bone marrow stromal cells, osteoclasts, and osteoblasts lead to bone deterioration, angiogenesis, and MM cell survival and proliferation (70), so disrupting the ability of MM cells to interact with the microenvironment could provide multiple benefits. All of these potential mechanisms of action continue to be explored, though so far the data continue to indicate that NK cells contribute to the therapeutic efficacy of this anti-CS1 monoclonal antibody.

Clinically, elotuzumab used alone was well-tolerated and led to disease stabilization in a subset of relapsed/refractory myeloma patients (63). More promising clinical activity was seen, however, when it was used in combination with lenalidomide and dexamethasone, with over 80% of relapsed patients responding in phase I and II trials, and progression-free survival significantly longer (median 26.9 months at the 10 mg/kg dose) than that previously observed for lenalidomide/dexamethasone alone (62). Elotuzumab-mediated ADCC of MM targets by NK cells can be enhanced *in vitro* by pretreatment with a proteasome inhibitor (54, 66), and a small combination study of elotuzumab and bortezomib in relapsed/refractory myeloma patients showed this combination to be safe, with preliminary efficacy observed (61). Currently, three randomized phase III trials are underway adding elotuzumab to either bortezomib or lenalidomide/dexamethasone, in both newly diagnosed and relapsed/refractory MM patients. These studies will more definitively assess if there is a benefit to adding elotuzumab to these standard therapies.

KIR-BLOCKING MONOCLONAL ANTIBODY

Allogeneic HSCT has emerged as an effective treatment option for a variety of hematological cancers after chemotherapeutic ablation of the recipient's immune cells (71). Because of the high polymorphic variability of KIR and MHC-I in the human population, variability of these receptor/ligand pairs should be considered in

the context of transplantation. Velardi and colleagues first reported that donor allogenicity of NK cells in HSCT to treat acute myeloid leukemia (AML) resulted in a double benefit by enhancing anti-leukemia responses, while reducing graft-versus-host disease (72). In the absence of an HLA-identical sibling donor, haploidentical HSCT is commonly used, in which a mismatch exists in at least one HLA allele. This mismatch improves the odds that at least one inhibitory KIR in the donor NK cells lacks an MHC-I ligand in the transplant recipient. In this scenario, the donor-derived NK cells are less inhibited and considered to be more capable of triggering a graft-versus-leukemia effect. KIR/HLA mismatch in HSCT has resulted in improved outcomes by several groups, specifically in treating AML (73–75), and may play a role in myeloma as well (30, 76). It is believed that NK cell-mediated autoimmunity does not occur in these patients because healthy cells are less likely to up-regulate the stress ligands that trigger NK cells (77). It is important to note that recent evidence indicates that donors expressing activating KIR (especially donors expressing KIR2DS1, but lacking its ligand, HLA-C2) also contribute significantly to improved outcomes in HSCT to treat AML (78, 79). These results indicate that certain engineered mismatches of KIR/HLA interactions in the context of HSCT can significantly influence NK cell responses in AML patients and perhaps other cancers.

In addition to exploiting KIR/HLA ligand mismatch in the context of HSCT, monoclonal antibody-mediated blockade of the KIR/HLA interaction has emerged as a potential cancer immunotherapy to lower the threshold of NK cell activation. The *in vitro* use of mAb to block KIR function was first found to increase cytokine production in T cells in 1996 (2). To further test this concept *in vitro*, Binyamin et al. potentiated NK cell responses to autologous EBV-transformed B cells by combining a panel of mAbs to block numerous NK cell inhibitory receptors (KIR, CD94/NKG2A, and ILT2/LIR-1/CD85j) in combination with the B cell-specific anti-CD20 mAb rituximab to simultaneously reduce inhibitory signals and trigger ADCC, respectively (80). Importantly, NK cell-mediated cytotoxicity of the transformed target cells was not elevated by inhibitory receptor blockade alone, suggesting that other tolerizing mechanisms effectively prevent the attack of normal cells in the context of these inhibitory receptor-blocking conditions.

Based on this concept, a humanized KIR-blocking mAb IPH2101 (formerly 1-7F9) has been developed and is currently being tested in clinical trials. IPH2101 is a pan-specific anti-KIR antibody that binds KIR2DL1, -2 and -3, which are the most relevant inhibitory KIR family members, due to their combined capacity to recognize all allotypes of HLA-C. The antibody was shown to block the interaction between these inhibitory KIR2DL and HLA-C and also binds the activating receptors KIR2DS1 and KIR2DS2, although the functional impacts of these interactions have not been formally tested (77). *In vitro* preclinical studies demonstrated that IPH2101 mAb augments NK cell cytotoxicity of HLA-C-expressing tumor cells without targeting normal blood mononuclear cells, which is critical to assure that NK cells remain tolerant in treated patients (77). This was confirmed in a preclinical mouse model that was engineered to co-express KIR2DL3 and its ligand, HLA-Cw3, which was able to license or educate the mouse NK cells. When KIR was then blocked *in vivo* with IPH2101, the mouse NK cells were able to destroy HLA-Cw3-positive target cells without development of autoimmunity (81).

In view of the capacity of NK cells to respond to myeloma cells, phase I clinical trials have been initiated to treat MM patients with IPH2101 and the modified variant, IPH2102. When used alone in patients, the side effects of IPH2101 were minimal with no evidence of autoimmunity, and *ex vivo* functional assays showed enhanced NK cell cytotoxicity (82, 83). A trial of IPH2101 in combination with lenalidomide has since been initiated, based upon a variety of effects by these reagents that can potentially synergize to enhance NK cell responses. IPH2101 is expected to enhance NK cell killing by blocking inhibitory signals, while lenalidomide can stimulate general NK cell function and may even up-regulate triggering ligands on MM cells (51).

Importantly, the addition of KIR blocking mAbs may prove to be an asset in treating a variety of cancers, as a way to boost the potential of NK cells to kill stressed or cancerous cells, while retaining general NK cell tolerance toward normal cells. Nonetheless, it is possible that optimal clinical responses may require a combination therapy of multiple antibodies blocking a wider variety of inhibitory receptors expressed on NK cells, such as KIR3DL family members, CD94/NKG2A, and ILT2/LIR-1/CD85j, as well as the addition of an ADCC targeting mAb to stimulate tumor-specific cytotoxicity, as demonstrated by the *in vitro* studies of Binyamin et al. described earlier (80).

AGENTS PROMOTING THE EXPRESSION OF NKG2D LIGANDS ON TUMOR CELLS

The human NKG2D ligands MICA, MICB, and ULBPs are commonly up-regulated on stressed or infected cells and thereby provide a key recognition element for NK cell-mediated attack of many cancer cells (8, 9). Several cancer types are able to shed NKG2D ligands into the sera as an immune evasive mechanism, and these soluble ligands have been shown to cause down-regulation of NKG2D on NK cells, which leads to a stunted immune response (84–87). Demonstrating the high frequency of shed ligands, Hilpert et al. recently found at least one soluble NKG2D ligand in the sera of 183 leukemia patients analyzed, and culture of NK cells in leukemia patient sera resulted in down-regulation of NKG2D expression (88). Shedding of the NKG2D ligand, MICA, by chronic lymphocytic leukemia cells can be induced upon translocation of the endoplasmic reticulum-resident proteins ERp5 and GRP78 to the tumor cell surface (89). Additionally, shedding of MICA/B has been attributed to proteolytic activity of the ADAM10 and ADAM17 metalloproteinases in some tumor cell lines (90). The MICA*008 allele can also be released into exosomes, which can subsequently down-regulate NKG2D expression and reduce NK cell-mediated cytotoxicity (90, 91). On the other hand, the expression of CEACAM1 in cancer cells can cause the intracellular retention of NKG2D ligands, thereby limiting NK cell detection through NKG2D (92). These observations have made the retention/upregulation of NKG2D ligands on tumor cells an attractive goal for cancer therapy.

Many drugs that were first considered for cancer therapy because they can alter gene expression in tumor cells were subsequently found to also increase the susceptibility of tumor cells to cytotoxicity by NKG2D-expressing NK cells. These drugs include

those that promote gene upregulation (93–96), differentiation (97–99), and DNA or protein damage (100–103). For example, HDAC inhibitors cause the upregulation of NKG2D ligands, MICA/B and ULBPs, in tumor cells but not healthy cells. Treatment with the HDAC inhibitor valproic acid (VPA, valproate) leads to higher expression of NKG2D ligands at the transcriptional and translational levels by inducing acetylation of the histones bound to MICA and MICB gene promoters (104, 105). The relatively low toxicity and low occurrence of off target effects of VPA make it a reasonable means of boosting effector cell function. Furthermore, treating cultured cells with VPA and the DNA methylation inhibitor hydralazine was shown to increase dimethylated MICA/B gene promoters, thereby further stimulating transcription (106, 107). The addition of a metalloproteinase inhibitor has also been shown to block the proteolytic cleavage of NKG2D ligands, as a means to further decrease shedding into the sera when used in conjunction with VPA treatment (108). Lastly, spironolactone, a diuretic commonly used to treat heart failure and high blood pressure, has very recently been found to also upregulate NKG2D ligands and therefore increase NK cell cytotoxicity of colon cancer cell lines (109).

Glycogen synthase kinase (GSK)-3 has recently been discovered as a new target to promote MICA/B upregulation. GSK3 inhibitors have been widely used to suppress the proliferation of malignant lymphoid cells, but Fionda et al. recently showed that GSK3 inhibition also increased MICA expression at the protein and mRNA levels in MM cells (110). In conjunction with the increased MICA expression, the addition of the GSK3 inhibitor significantly enhanced NK cell-mediated cytotoxicity of tumor cells. Mechanistically, GSK3 inhibition correlated with the down-regulation of STAT3, which is a negative regulator of MICA expression (110). In addition, the combination of lenalidomide with GSK3 inhibition further enhanced MICA expression, which further supports the combined mechanistic benefit of these agents with current anti-tumor therapies.

ANTI-CD137 MONOCLONAL ANTIBODIES

CD137 (4-1BB, TNFRSF9) is an inducible member of the TNF receptor superfamily that functions as a co-stimulatory signaling molecule on the surface of activated T and NK cells. CD137 ligation further augments activation of these cells, increasing their proliferation, cytokine secretion, and effector function, and preventing activation-induced cell death (111, 112). Treatment with agonist anti-CD137 mAb can mimic this co-stimulatory signal, leading to regression of large tumors in multiple murine models, including B-cell lymphoma and myeloma (111, 113–115). This effect requires $CD8^+$ T cells, but is also dependent upon the presence of NK cells (116, 117), implying an impact on these cells as well. Many diverse types of tumors express elevated levels of the CD137 ligand, CD137L, accompanied by increased expression of CD137 on immune cells within the tumor environment, while expression of either is negligible on healthy cells (115, 118–120).

There are conflicting data regarding the effects of anti-CD137 mAb on NK cells. CD137 engagement on mouse NK cells consistently results in activation, but this can be either activating or inhibitory in humans, depending on the setting or model used. In human leukemia cells, the CD137/CD137L interaction can also result in bidirectional signaling to suppress NK cell-mediated responses; CD137 recognition of CD137L on leukemic cells transmits inhibitory signals into the NK cell to impair cytokine production and cytotoxicity responses and CD137L engagement by CD137 induces the production of TNF and immunosuppressive interleukin (IL)-10 by the tumor cells (119). Inhibiting this interaction using soluble CD137-Fc or a "blocking" anti-CD137 mAb can restore NK cell cytotoxicity (119). Taking this rationale one step further, Buechele et al. suggested the potential merits of a dual strategy of blocking CD137/CD137L interaction and neutralizing immunosuppressive TNF (121). Lin et al. demonstrated that human NK cells up-regulate CD137 *in vitro* following Fc-receptor engagement, which promotes release of pro-inflammatory cytokines but decreased cytotoxicity against K562 targets. This implies a negative impact of CD137 expression and signaling in human NK cells, though the direct impact of CD137 ligation was not tested in this model (122, 123). In contrast, Kohrt et al. have shown that following FcR-induced CD137 up-regulation on NK cells, adding an agonist anti-CD137 mAb known to induce CD137 signaling actually enhances NK cell-mediated ADCC toward rituximab- or trastuzumab-coated target cells (124, 125). Whether this antibody may also be working by preventing CD137 on immune cells from binding to CD137L and inducing "reverse" signaling within the tumor cell was not explored in these studies. Nonetheless, these data have led to an ongoing clinical trial (NCT01775631) combining rituximab with agonist anti-CD137 mAb (BMS-663513, urelumab) in patients with relapsed/refractory B-cell malignancies.

These inconsistent findings on the impacts of manipulating CD137 in NK cells are likely the result of signaling differences between mice and men, the use of different CD137-targeting reagents, and even between different hematopoietic versus non-hematopoietic cancer models (120). For clinical relevance, it will be important to better understand the human mechanistic impact of CD137/CD137L interaction in order to properly exploit the potential treatment options. The role of CD137/CD137L interactions on interplay between NK cells and $CD4^+$, $CD8^+$, and regulatory T cells (Tregs) is also incompletely understood (126). For instance, the depletion of immunosuppressive Tregs has been shown to enhance the anti-tumor activity of anti-CD137 mAb (115). However, CD137 engagement on $CD4^+$ and $CD8^+$ T cells is believed to always be activating in mice and men (126). Nonetheless, while the exact mechanism is still unclear, the addition of the CD137 mAb along with neutralization of immunosuppressive cells and signals shows potential to boost immune function when added to current cancer therapies (121, 127).

ANTI-GITR MONOCLONAL ANTIBODIES

Glucocorticoid-induced TNF receptor (GITR, TNFRSF18) is another co-stimulatory member of the TNF receptor superfamily expressed on T cells, NK cells, and B cells, among other hematopoietic cell types (126). GITR expression is generally low in resting T and NK cells, is up-regulated after activation, and the receptor is expressed constitutively at high levels in $CD4^+Foxp3^+$ Tregs. GITR is an activating receptor in T cells, since *in vitro* or *in vivo* engagement with GITR ligand or agonist anti-GITR mAb has been reported to support the expansion of $CD4^+$ and $CD8^+$ T

cells and renders T cells resistant to suppression by Tregs (128, 129). Treatment with agonist anti-GITR mAb leads to enhanced vaccine-induced and endogenous effector T cell responses and tumor rejection in multiple murine tumor models, and is associated with a marked reduction in Treg frequency within the tumor microenvironment, though the exact mechanism(s) by which GITR ligation modulates Tregs remains controversial (115, 130–133). Based on these preclinical findings, the first clinical trial of an agonist anti-human GITR mAb (TRX518) in patients with advanced melanoma or other solid tumors is now underway (NCT01239134).

Similar to CD137, there is conflicting evidence about whether GITR is activating or inhibitory in human NK cells, which may also reflect differential NK cell responses to GITR engagement between mice and men (120). While agonist anti-GITR mAb can augment murine NK cell proliferation and cytotoxicity, GITR engagement on human NK cells has been reported to block NF-κB activation, cause release of anti-inflammatory cytokines, suppress NK cell proliferation, and increase NK cell apoptosis (134). Furthermore, Baltz et al. found that soluble GITR ligand (sGITRL) is released by several hematologic malignancies, detectable in patient sera, and these patients display reduced NK cell cytotoxicity and IFN-γ production (135). In CLL, bidirectional GITR/GITRL signaling can support tumor cell growth by causing release of survival factors, such as IL-6, IL-8, and TNF, and interfering with rituximab-induced ADCC responses (136). However, an antagonistic anti-GITR mAb can block GITR-GITRL interaction, and restore NK cell-mediated ADCC responses. Finally, a dual strategy has been developed by Schmiedel et al. that has potential to enhance existing therapies for AML and CLL (137). An Fc-optimized GITR-Ig fusion protein was found to block the GITR/GITRL interaction and target GITRL-bearing cells for ADCC, thus enhancing NK cell-mediated cytotoxicity of cancer cells. Hence, like anti-CD137 mAb, anti-GITR mAb, and other GITR-targeting therapies have the potential to boost the effectiveness of current cancer therapies by virtue of pro-inflammatory and cytotoxic effects involving NK cells.

PD-1 OR PD-L1 BLOCKING MONOCLONAL ANTIBODIES

Programed death 1 (PD-1; CD279) is a member of the B7 family of co-signaling receptor that is up-regulated on activated T cells, NK cells, B cells, dendritic cells, and monocytes (138). The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), which can recruit the protein tyrosine phosphatases SHP-1 and SHP-2 to mediate inhibitory signaling (139). The engagement of PD-1 by its ligands, PD-L1 or PD-L2, blocks the immune response in both T and NK cells by inhibiting PI3K/Akt and Ras activation signaling (140–142). PD-1 expression also marks "exhaustion" in T cells, and engagement of PD-1 can cause apoptosis of $CD8^+$ T cells and the differentiation of $CD4^+$ T cells into immunosuppressive Tregs (143–146). In NK cells, PD-1 engagement impairs activation, conjugate formation, cytotoxicity, and cytokine production (145, 147, 148). In healthy tissue, PD-L1-induced inhibitory signaling minimizes damage to bystander cells and prevents excessive immune responses during acute infections (149). However, many tumor and viral models express PD-1 ligands as an immune evasion mechanism. In contrast, PD-L1 is expressed at low levels on healthy tissue, and resting NK cells express low levels of PD-1 (144, 145, 147, 148). IFN-γ can potently up-regulate PD-L1 expression (150), suggesting that localized cytokine production by NK cells and Th1 cells may actually promote PD-1-based immune evasion by tumors.

The disruption of PD-1/PD-1 ligand interactions can significantly potentiate immune responses to viral infections and cancer, and antibody-mediated blockade of these interactions has emerged as a prime target for immune therapies. In the case of viral persistence in the liver, the abrogation of PD-L1 by siRNA was shown to enhance the number of intrahepatic NK cells and CTL, thereby increasing cytotoxicity, cytokine production, viral clearance, and memory (148). During HIV infection, PD-1 levels increase on patient NK cells, and this has been shown to diminish NK cell proliferation (145). PD-L1 expression on tumors and PD-1 expression on tumor-infiltrating lymphocytes have been associated with poor outcome in renal cell carcinoma patients (151, 152). We have recently reported significantly increased expression of PD-1 on cytolytic NK cells in renal cell carcinoma patients, suggesting that these tumors can directly suppress tumor-infiltrating NK cells by this mechanism (153). Remarkably, the PD-1 expression on NK cells and other leukocytes was rapidly reduced after surgical resection of the primary renal tumor. Anti-PD-1 or anti-PD-L1 mAbs block the interaction of PD-1 on T and NK cells with its ligand, PD-L1 and this restores the function of exhausted cytolytic T cells, augments T cell proliferation, and enhances NK cell cytokine production and cytotoxicity responses, leading to enhanced anti-tumor effector responses and tumor regression in multiple murine models (146, 149). In clinical trials of anti-PD-1 and anti-PD-L1 antibodies to treat a variety of solid tumors, objective, often durable responses were seen in up to a third of patients, demonstrating proof of principle for this approach, and patients that responded to the treatment were shown to express PD-L1 in their tumors (154, 155). Further clinical trials of several candidate antibodies in both solid and hematologic cancers are ongoing.

In studies of MM patients, Benson et al. found upregulation of PD-1 on NK cells in conjunction with PD-L1 expression on MM cells, and *in vitro* treatment with an anti-PD-1 mAb enhanced NK cell conjugation with PD-L1-expressing MM target cells, resulting in enhanced cytotoxicity and IFN-γ production (147). In the same study, *in vitro* treatment with lenalidomide was shown to further enhance NK cell responsiveness by lowering PD-L1 expression on MM cells. In a mouse model of MM, Hallett et al. also saw increased levels of PD-L1 on MM cells, along with an exhausted phenotype in T cells, release of immunosuppressive IL-10 and expansion of Treg cells accompanied by increased levels of PD-1 on T and NK cells (144). Blockade of the PD-1/PD-L1 interaction with a PD-L1-specific mAb increased mouse survival by 40%. Like CS1, PD-1 expression persists after stem cell transplant and prior treatment, so PD-1 is a reliable target in relapsed or refractory cancer. Although PD-1 upregulation on human NK cells has only been reported in MM and renal cell carcinoma to date, its expression may be elevated in a variety of cancers as a mechanism to suppress anti-tumor responses. Therefore, the addition of PD-1/PD-L1 blocking mAb to an existing treatment regimen shows encouraging promise in boosting anti-tumor and

Table 1 | Summary of some major therapeutic agents discussed in the text.

Agent	Examples	NK cell-specific mechanism	Disease studied	Reference
Immuno-modulating drugs (IMiDs)	Thalidomide, lenalidomide, pomalidomide	Stimulate NK and T cells to release cytokines, kill tumor cells, block angiogenesis	MM, leukemia, NHL, pancreatic, esophageal, prostate	Sampaio et al. (33), D'Amato et al. (34), (35) Hayashi et al. (44), Wu et al. (45), Quach et al. (36), clinicaltrials.gov
Proteasome inhibitors	Bortezomib	Induce tumor cell death, increase NKG2D ligand expression	MM, leukemia, lymphoma, hepatocellular	Armeanu et al. (160), Shi et al. (53), (55) Moreau (156), clinicaltrials.gov
Anti-CS1 antibody	Elotuzumab	Triggers NK cell ADCC	MM	Hsi et al. (65), Tai et al. (66), (54) Benson and Byrd (67), Moreau (156)
Anti-KIR2DL antibody	IPH2101, IPH2102 (Lirilumab)	Blocks inhibitory KIR	MM, leukemia	Binyamin et al. (80), Sola et al. (81), Benson et al. (51), Vey et al. (83), clinicaltrials.gov
HDAC inhibitors	Valproic acid, panobinostat, vorinostat	Increase NKG2D ligand expression	MM, leukemia, Hodgkin's lymphoma, hepatocellular	Armeanu et al. (93), Yamanegi et al. (104), Moreau (156), Yang et al. (105), clinicaltrials.gov
Anti-CD137 antibodies	BMS-663513 (urelumab)	Augment NK cell ADCC, co-stimulatory in T cells	NHL, melanoma, breast cancer	Baessler et al. (119), Buechele et al. (121), Kohrt et al. (125), clinicaltrials.gov
Anti-GITR antibodies	TRX518	Block GITR/GITRL in NK cells, co-stimulatory in T cells, neutralize Tregs	Solid tumors, melanoma	clinicaltrials.gov
Anti-PD-1 antibodies	BMS-936558 (nivolumab), CT-011, MK-3475	Block PD-1 receptor, block PD-1/PD-L1 interaction in NK and T cells	Hepatitis C, renal, prostate, melanoma, MM, colorectal, NHL, NSCLC	Benson et al. (147), Brahmer et al. (154), Rosenblatt et al. (161), Gardiner et al. (162), Lipson et al. (163), clinicaltrials.gov
Anti-PD-L1 antibodies	BMS-936559, MSB0010718C, MPDL3280A	Block PD-L1 interaction with receptor PD-1 in NK and T cells	Solid tumors, melanoma, leukemia, MM, breast, NHL	Sznol and Chen (149), clinicaltrials.gov

HDAC, histone deacetylase; ADCC, antibody-dependent cellular cytotoxicity; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; NSCLC, non-small cell lung cancer.

anti-viral responses by several immune cell types, including NK cells, through counteracting a potent mechanism of immune evasion.

CONCLUSION

A variety of new therapeutic agents have recently become available to potentiate NK cell responses in cancer patients, and many of these drugs are either approved or undergoing clinical trials (see **Table 1**). Here we have discussed a representation of this expanding toolbox of agents that allows clinicians to potentiate NK cell functions and thereby enhance anti-tumor therapy. Major future goals will be to expand upon these therapeutic options, better define mechanisms of action for these agents, identify specific combination therapies that are most effective at boosting NK (and T) cell function, and identify biomarkers (e.g., PD-L1 expression on tumor cells) that may better predict which patients are most likely to respond to these immune therapies. Furthermore, while MM has served as an appropriate proving ground for testing the therapeutic efficacy of several of these agents (156), they will need to be tested on other cancers, as well as viral infections and other disease states, to expand their usefulness.

Many of these agents are already being tested in conjunction with other immune therapies; especially in combination with ADCC-inducing mAbs that target NK cells to attack tumors. Certainly any new therapy utilizing immunostimulatory mAbs must be carefully evaluated in a stepwise manner to avoid possible adverse side effects, such as autoimmunity (113). However, combination therapies allow clinicians to take advantage of mechanistic synergies that can effectively boost NK cell function using agents that have limited impacts when used alone. An appropriate starting platform combines strategies that boost the immune system and block immune suppression (157), and the hard wiring of NK cells makes them particularly receptive to this strategy. Of particular interest, several phase I clinical trials are currently in progress that combine antibodies designed to block multiple inhibitory immune receptors on NK cells and other leukocytes simultaneously. These include combining the anti-CTLA antibody, ipilimumab with the anti-KIR antibody, IPH2102 (lirilumab) to treat advanced tumors (NCT01750580) and an anti-PD-1 antibody (nivolumab) in combination with either ipilimumab to treat melanoma (NCT01024231) or with lirilumab to treat solid tumors (NCT01714739). Combination therapies may also allow the use

of more toxic conventional anti-tumor therapies at lower doses when new therapies are added. Also, patients who have weakened immune function, due to prior radiotherapy or chemotherapy, may particularly benefit from NK cell boosting therapies.

In using these immune therapies, it is important to consider the phenotype of individual patients (158, 159). It is possible that most of these agents can be used together in various combinations or with conventional therapies, depending on the biomarkers present in a particular tumor environment. Altogether, there are several new tools in the medicine cabinet that offer the possibility to improve patient outcomes through boosting NK cell functions. While no one-size-fits-all solution is available to universally improving anti-tumor therapy, proper patient screening should allow the application of personalized combination therapies that harness the beneficial attributes of NK cell-mediated anti-tumor responses to systematically improve overall patient survival.

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Impact of HCMV infection on NK cell development and function after HSCT

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Natural Killer (NK) cell function is regulated by an array of inhibitory and activating surface receptors that during NK cell differentiation, at variance with T and B cells, do not require genetic rearrangement. Importantly, NK cells are the first lymphocyte population recovering after hematopoietic stem cell transplantation (HSCT). Thus, their role in early immunity after HSCT is considered crucial, as they can importantly contribute to protect the host from tumor recurrence and viral infections before T-cell immunity is fully recovered. In order to acquire effector functions and regulatory receptors, NK cell precursors undergo a maturation process that can be analyzed during immune reconstitution after HSCT. In this context, the occurrence of human cytomegalovirus (HCMV) infection/reactivation was shown to accelerate NK cell maturation by promoting the differentiation of high frequencies of NK cells characterized by a KIR^+NKG2A^- and $NKG2C^+$ mature phenotype. Thus, it appears that the development of NK cells and the distribution of NK cell receptors can be deeply influenced by HCMV infection. Moreover, in HCMV-infected subjects the emergence of so called "memory-like" or "long-lived" NK cells has been documented. These cells could play an important role in protecting from infections and maybe from relapse in patients transplanted for leukemia. All the aspects regarding the influence of HCMV infection on NK cell development will be discussed.

Keywords: human NK cells, HCMV infection, NKG2C, KIR, hematopoietic stem cell transplantation

INTRODUCTION

Natural killer (NK) cells represent crucial effectors in innate immune response to viral infections and tumors. NK cell function is regulated by an array of germline-encoded surface receptors, that, upon interaction with their ligands, transmit either inhibitory or activating signals (1–3).

Most human NK cells express inhibitory receptors specific for HLA-class I molecules, including the Killer Ig-like Receptors (KIRs), able to distinguish among different HLA-A, -B, and -C allotypes (4), and the CD94/NKG2A heterodimer, specific for HLA-E (5). These receptors allow NK cells to spare HLA-class I^+ autologous normal cells and to kill cells in which HLA class I expression is down-regulated (e.g., by tumor transformation or viral infection) or cells expressing non-self HLA class I alleles unable to engage inhibitory KIRs (e.g., in the allogeneic transplant settings).

Activating counterparts of the inhibitory receptors specific for HLA class I molecules can be expressed by NK cells. In particular, activating KIRs, including KIR2DS1, KIR2DS2, and KIR3DS1, are highly homologous to their inhibitory counterparts in the extracellular domain, but are characterized by a short cytoplasmic tail lacking Immunoreceptor Tyrosine-Based Inhibitory Motifs (ITIM). On the other hand, activating KIRs interact with DAP-12, an adaptor signaling molecule carrying an Immunoreceptor Tyrosine-Based Activating Motif (ITAM) that can induce NK cell activation (6). So far, the HLA class I specificity of activating KIRs has been clearly demonstrated only for KIR2DS1 and KIR2DS4 (6–8). KIR genes are located on chromosome 19 and are inherited as haplotypes. Two basic KIR haplotypes can be found in the human genome: group A haplotypes, which have a fixed number of genes that encode inhibitory receptors (with the exception of the activating receptor KIR2DS4) and group B haplotypes, which have variable gene content, including additional activating KIR genes (4, 9).

Another HLA class I-specific activating receptor is represented by the HLA-E-specific CD94/NKG2C heterodimer. At variance with its inhibitory counterpart CD94/NKG2A, which contains an ITIM in the NKG2A cytoplasmic domain, NKG2C, like the activating KIRs, lacks ITIM and is associated with DAP-12 (9).

Human NK cells mainly differentiate in the bone marrow (BM) from $CD34^+$ hematopoietic stem cells (HSCs) through discrete stages of development. However, recent studies suggest that different sites, such as secondary lymphoid compartments (SLCs) (10), human decidua (11), thymus (12), or fetal and adult liver (13) can be involved in this process. Two main subsets of mature NK cells with distinct functional and phenotypic properties have been described: the $CD56^{bright}$ $CD16^{-/low}$ and the $CD56^{dim}$ $CD16^+$ subsets. $CD56^{bright}$ NK cells typically express high levels of the receptor CD94/NKG2A, but low levels of KIR molecules. They are

not abundant in peripheral blood, while dominate in SLCs for which they express specific homing receptors. $CD56^{bright}$ NK cells produce high levels of immunoregulatory cytokines, but are poorly cytotoxic. In contrast, the $CD56^{dim}$ subset is characterized by high surface expression of KIR receptors, is largely represented in peripheral blood and is highly cytotoxic against tumor and virus-infected targets (14). A recent study shows that $CD56^{dim}$ NK cells may also release high amounts of IFN-γ very early after activation (15). In addition, a third subset, characterized by a $CD56^{-}CD16^{+}$ surface phenotype, exists, but is rare in healthy individuals and represents a small percentage of total NK cells. However, expansion of $CD56^{-}$ NK cells have been described in HIV and HCV chronically infected subjects (16, 17) and also in recipients of hematopoietic stem cell transplantation (HSCT) (18, 19).

The developmental relationship between $CD56^{bright}$ and $CD56^{dim}$ has been long debated; however, recent reports suggest that $CD56^{dim}$ derive from $CD56^{bright}$ cells. Thus, it has been shown that in $CD56^{bright}CD16^{-}$ cells telomeres are significantly longer than in $CD56^{dim}CD16^{bright}$ cells (20). In addition, when $CD56^{bright}$ NK cells were infused in NOD/SCID mice, they progressed toward the $CD56^{dim}$ phenotype (21). In line with these observations the first wave of NK cells that reconstitute after HSCT is represented by the $CD56^{bright}$ NK cell subset. Only later on $CD56^{dim}$ NK cells appear in the blood and are initially characterized by high CD94/NKG2A expression. The complete maturation of $CD56^{dim}$ NK cells involve the progressive loss of CD94/NKG2A and the acquisition of combinations of KIR molecules, in order to form a relatively stable repertoire which is mainly genetically determined, but influenced by the HLA class I genotype (4, 22–25). During their late stages of differentiation, $NKG2A^{-}KIR^{+}$ $CD56^{dim}$ NK cells progressively acquire a $CD57^{+}$ $CD94^{low}$ $CD62L^{neg}$ phenotype (26–29). In order to become fully competent NK cells have been shown to require recognition of self HLA class I molecules during maturation, a phenomenon referred to as "licensing" or "education" (30–32). However, unlicensed NK cells lacking inhibitory receptors specific for self HLA molecules (either KIR or NKG2A/CD94) do exist, but are hyporesponsive (31, 33). Thus, in a self environment the NK cell receptor repertoire will ensure self tolerance as each functional NK cell expresses at least one inhibitory receptor specific for self HLA-I molecules. In contrast, in allogeneic settings, NK cells may kill allogeneic cells expressing HLA-I non-self molecules. The existence of "alloreactive" NK cells can be particularly important in HSCT (34). Since HSCT is a widely employed treatment used to cure malignant disorders such as acute leukemia, these alloreactive NK cells can greatly contribute to the eradication of residual tumor cells (Graft vs. Leukemia effect), prevent Graft vs. Host Disease (GvHD), and improve engraftment by the killing of recipient dendritic cells (DC) and T-cells, respectively (35, 36).

NK CELL RESPONSES TO HCMV INFECTION

Natural killer cells can importantly contribute to immune responses against viral infections. Indeed, in patients with NK cell deficiency a higher susceptibility to herpesvirus infections has been observed (37–39).

Human cytomegalovirus (HCMV) is a β-herpesvirus that establishes a lifelong persistent infection (40). In immunocompetent hosts, HCMV infection is usually asymptomatic, but reactivation becomes an important cause of morbidity in primary or acquired immunodeficiencies and immunosuppressed patients particularly in transplant recipients. In healthy individuals both T-cell and NK cells are involved in controlling HCMV infection (41).

During the host-HCMV interplay, NK cells are likely to receive stimuli from infected cells or other immune cells that can modulate their phenotype and function. In particular it has been shown that HCMV is capable of shaping the NK cell receptor repertoire inducing the expansion of an NK cell subset expressing the activating NKG2C receptor. Remarkably, this expanded $NKG2C^{+}$ NK cell subset found in HCMV seropositive individuals is also characterized by a mature phenotype, mostly $KIR^{+}NKG2A^{-}$ (42). This finding was observed both in healthy individuals and in patients with different pathological conditions. Indeed, increased proportions of $KIR^{+}NKG2A^{-}NKG2C^{+}$ NK cells have been described in subjects who experienced different viral infections, including HIV (43, 44), Chikungunya virus (45), Hantavirus (46), HBV and HCV (47). However, it is conceivable that HCMV infection/reactivation, which may occur in chronically infected subjects, may be responsible for the induction of such NK cell phenotype in these patients. $NKG2C^{+}KIR^{+}$ NK cell expansions have been described also in congenital immunodeficiencies where this NK cell subset has been proposed to play a relevant role in the resolution of HCMV infection (48). Together, these data suggest that the NKG2C receptor could play a crucial role in HCMV recognition and in promoting the expansion and/or maturation of $NKG2C^{+}$ cells, as well as in the control of infection.

The mechanism responsible for the NK cell expansion described is still unclear. It has been suggested that NK cells could be stimulated by HCMV-infected targets, through the heterodimer CD94/NKG2C. In this context, HCMV-infected fibroblasts have been shown to favor the expansion of $NKG2C^{+}$ NK cells, cultured in the presence of either IL-15 or IL-2. MAb-mediated masking of CD94 could inhibit this selective expansion (49). Other studies reported that $NKG2C^{+}$ NK cells can undergo proliferation when co-cultured with HLA-E-transfected 721.221 lymphoblastoid cells (50, 51) or K562 cell lines (46). It is possible that HCMV-infected targets may express ligands interacting with $NKG2C^{+}$ NK cells, thus inducing their activation and proliferation. Remarkably, the signal peptide of the HCMV UL40 protein stabilizes HLA-E expression on HCMV-infected fibroblasts, while other HCMV-derived peptides (including US2, US3, US6, US10, and US11) dampen the surface expression of classical HLA class I molecules (52). These observations suggest that, during the interaction with HCMV-infected cells, the expansion of mature NK cells expressing inhibitory self KIRs could be favored because of the lack of inhibitory interactions with classical HLA class I molecules. Moreover, the stabilization of HLA-E, while favoring the expansion of $NKG2C^{+}$ cells, would inhibit that of cells expressing NKG2A. This is in line with a recent study showing that a past HCMV infection in healthy individuals is strongly correlated with expansion of $NKG2C^{+}$ NK cells expressing inhibitory self KIRs (i.e., educated NK cells expressing KIRs specific for self HLA class I molecules) (51).

However, direct evidence for the specificity of NKG2C for HLA-E molecules loaded with viral peptides, or for an unknown ligand of either host or viral origin expressed by HCMV-infected cells is missing or limited. It would be important to analyze the peptides that *in vivo* are bound to HLA-E in HCMV-infected cells and to verify their role in inducing NKG2C-mediated NK cell recognition. In this context, by the analysis of HCMV UL40 sequences isolated from HSCT recipients undergoing HCMV reactivation, it has been shown that UL40 is characterized by a certain degree of polymorphism that could modulate NK cell-mediated recognition of HCMV-infected targets. In particular, some UL40 peptides derived from the signal sequence (i.e., HLA-E-binding peptides) encoded by HCMV isolates, are capable of both inhibiting NK cell lysis by NKG2A engagement and inducing NK cell activation through NKG2C triggering, whereas other forms of UL40 peptides do not stimulate NKG2C$^+$ cells, but are still capable of inducing inhibitory responses via NKG2A (53). Whether such UL40 polymorphisms can affect the expansion of NK cells expressing NKG2C and/or virus clearance in HCMV-infected HSCT recipients is unknown.

Human NK cell responses to HCMV can also be elicited through direct recognition of HCMV virions by NK cells (54). After exposure to HCMV, NK cells become activated and produce IFN-γ. This anti-HCMV response involves the engagement of TLR2 on NK cells by viral particles and the endogenous release of IFN-β. However, direct recognition of HCMV is not sufficient to induce stable changes in NK cell receptor repertoire.

HCMV DRIVES NK CELL MATURATION TOWARD HIGHLY DIFFERENTIATED STAGES IN HSCT RECIPIENTS

The imprinting on NK cell phenotype induced by HCMV infection results particularly dramatic when T-cell immunity is impaired in the infected host, such as in chronically infected HIV patients (43, 44), congenitally immunodeficient individuals (48, 55) and patients undergoing HSCT.

In this context, two recent studies have shown that HCMV reactivation can promote a rapid NK cell development after umbilical cord blood transplantation (UCBT) (56, 57). NK cells achieved a full maturation more rapidly in HCMV-reactivating patients as compared to non-infected ones. In particular, NK cells isolated from HCMV-reactivating patients show low percentages of CD56^{bright} NK cell and high proportions of mature CD56^{dim} NK cells expressing the NKG2C$^+$ NKG2A$^-$ KIR$^+$ Siglec-7$^-$ CD57$^+$ signature, at variance with non-reactivating patients that display a more immature phenotype (57). NKG2C$^+$ CD56^{dim} NK cells were characterized by the expression of self-KIR and displayed full competence in terms of cytolytic activity and cytokine production. The frequency of mature KIR$^+$NKG2C$^+$ NK cells persisted and continued to increase after 1 year from HSCT in recipients who reactivated HCMV. This expanded and long-living NKG2C$^+$ NK cell subset is clearly reminiscent of a population of Ly49H$^+$ NK cells which expands in murine CMV (MCVM) infected mice and is responsible for disease clearance through the induction of a "memory-like" NK cell response. Thus, NKG2C$^+$ NK cells, expanding after HCMV infection, could represent the human counterpart of murine "memory-like" NK cells. However, a clear recall response against HCMV-infected targets, mediated by human NKG2C$^+$ NK cells, has not been shown yet.

Importantly, in mouse, the Ly49H receptor has been demonstrated to bind to the MCMV protein m157 which is expressed by infected cells (58).

The mechanisms lying behind this accelerated maturation and expansion of KIR$^+$NKG2C$^+$ NK cells after HSCT are not completely understood. As already discussed, developing NK cells could be stimulated by HCMV-infected targets, possibly through the heterodimer CD94/NKG2C. HCMV infection could exert its influence on NK cell development at different sites and/or stages of maturation (**Figure 1**). First, NK cell precursors could be stimulated by HCMV while differentiating in the BM where they may interact with cells of the myeloid lineage that can be infected by HCMV and that represent a reservoir of latent virus (41). Second, CD56^{bright} NK cells, that are characterized by the expression of the lymph node homing chemokine receptor CCR7 (59), can reach SLC (e.g., lymph node) and interact with infected antigen-presenting cells, DC, or macrophages, that would promote NK cell differentiation toward more mature CD56^{dim} stages. A third possibility is that circulating CD56^{dim} NK cells could migrate to peripheral tissues (59) (e.g., mucosal tissues) where HCMV infection can affect different cell types (fibroblasts, endothelial cells, epithelial cells) (41). In this microenvironment, NK cells would receive, directly or indirectly, proliferating and maturing signals from infected targets that would drive NK cells toward highly differentiated stages of maturation, characterized by the KIR$^+$ NKG2C$^+$ NKG2A$^-$ signature. This last scenario better corresponds to *in vitro* data showing that CD56^{dim} NK cells proliferate in response to HCMV-infected fibroblasts (49). However, it is not clear so far whether this expanded NKG2C$^+$KIR$^+$NKG2A$^-$Siglec-7$^-$ NK cell subset, developed in response to HCMV-derived stimuli, represents a long-living cell subset or is rather continuously replenished by novel mature NK cells.

Remarkably, in HCMV-reactivating patients a unusual CD56$^-$CD16$^+$Siglec-7$^-$ NK cell subset was detected (57) that was reminiscent of that described in viremic HIV-infected patients undergoing HCMV reactivation (60). The similarity between CD56$^-$ NK cells found in HCMV-infected UCBT recipients and those present in HIV-infected patients was also confirmed by a marked impairment in functional capabilities. This may be consequent, at least in part, to the low expression on CD56$^-$ cells of several activating receptors such as NCRs and NKG2D, as also reported in chronically HIV-infected patients. It is conceivable that these hyporesponsive CD56$^-$ NK cells are generated after HCMV infection when T-cell immunity is impaired. The accumulation of these phenotypically aberrant and hypofunctional NK cells could also support the concept that HCMV has a role in immunosenescence (61). However, it should be noted that, in HSCT recipients, the hypofunctional state of CD56$^-$ NK cells could be reversed by exposure to exogenous cytokines, such as IL-2. This observation would suggest that the generation of these cells can be consequent to chronic stimulation together with lack of appropriate T-cell responses and cytokine production rather than to an irreversible process of aging induced by HCMV.

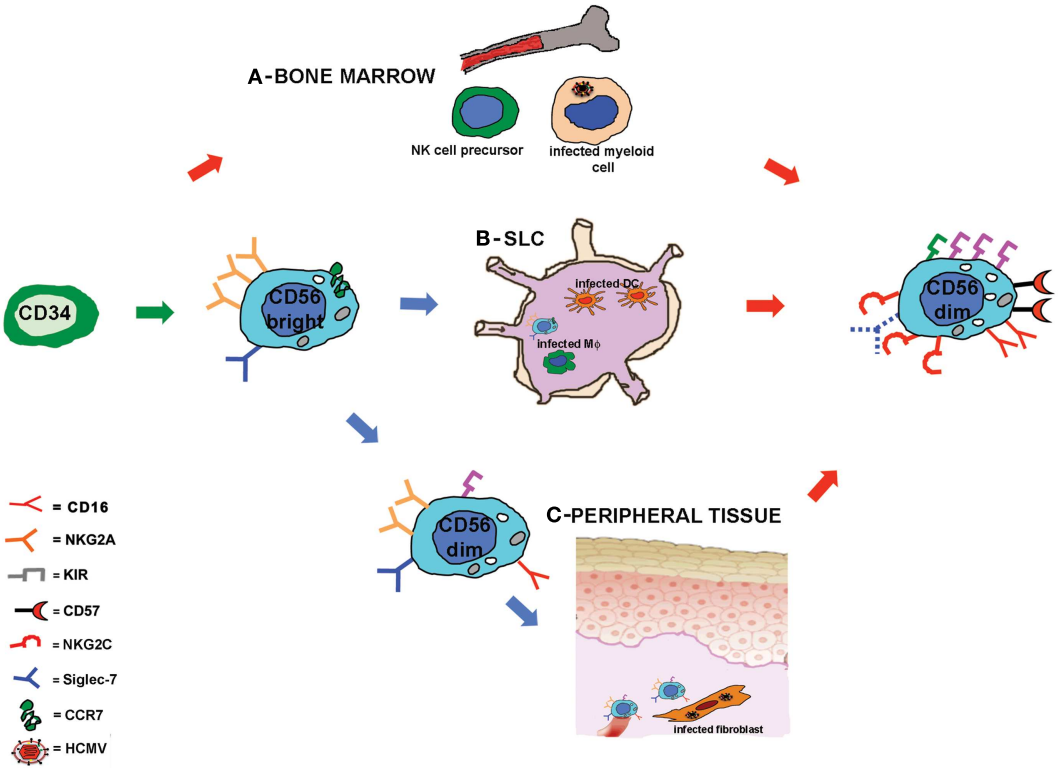

FIGURE 1 | Human cytomegalovirus accelerates NK cell maturation after HSCT: possible sites of NK cell-HCMV interplay. Schematic representation of NK cell maturation from CD34$^+$ HSC in recipients reactivating HCMV. NK cell development is rapidly driven toward a mature stage of differentiation characterized by a KIR$^+$NKG2A$^-$NKG2C$^+$CD57$^+$ Siglec-7$^-$ surface phenotype. The signals driving NK cell maturation might be provided at different developmental stages in different sites: in **(A)** it is hypothesized that during NK cell differentiation in the bone marrow, NK cell precursors could interact with HCMV-infected myeloid cells; in **(B)** CD56^{bright} NK cells that express CCR7 can reach the secondary lymphoid compartment (SLC) where they could interact with HCMV-infected stromal cells, dendritic cells, or macrophages (Mϕ); in **(C)** circulating CD56^{dim} NKG2A$^+$ NK cells could migrate to peripheral tissues and interact with HCMV-infected fibroblasts or endothelial cells.

A distinguishing feature of both CD56^{dim} and CD56$^-$ NK cells observed in HCMV-infected recipients is the dramatic down-regulation of Siglec-7. Although the mechanism/significance of this event is unknown, it represents, together with NKG2C up-regulation, the most typical marker of NK cell expansions promoted by HCMV infection. Whether Siglec-7 down-regulation could be a marker of "memory-like" NK cells has still to be investigated.

Human cytomegalovirus-induced NK cell populations with a "memory-like" surface phenotype may contribute not only to the control of virus infection, but also to the protection from leukemia relapses after HSCT. In this context, a recent study reported a correlation between early HCMV reactivation and reduction of leukemia relapse after allogeneic HSCT in adult patients (62). The HCMV-induced rapid maturation of functional NK cells could favor an NK cell-mediated anti-leukemic activity, especially in the case of a KIR-mismatched haplo-HSCT in which the fast differentiation of mature KIR$^+$NKG2A$^-$ NK cells could promote the emergence of anti-leukemic alloreactive NK cells.

"Memory-like" NKG2C$^+$ NK cells have been also demonstrated to be transplantable employing unmanipulated adult grafts from HCMV seropositive donors (63). These donor-derived NK cells, contained in the graft, expanded not only in recipients undergoing HCMV reactivation, but also in seropositive recipients in the absence of detectable viremia. These NKG2C$^+$ NK cells were capable of producing higher levels of IFN-γ as compared to NKG2C$^+$ NK cells infused in seronegative recipients, suggesting that a subsequent exposure to viral antigens in the recipient can increase cytokine production, by inducing a "memory-like" response that might contribute to control of HCMV reactivation in these HCMV$^+$ recipients. Of note, a recent paper suggested that NK cells exposed to multiple cytokines (a circumstance which likely occurs during an anti-viral immune response) can acquire a "memory-like" phenotype and would release higher amounts of IFN-γ following restimulation with cytokines or target cells (64).

NKG2C thus seems to play a central role in HCMV-induced responses by NK cells; however, recent reports indicated that also other NK receptors may be involved and that NKG2C could be dispensable. For example, in a cohort of children with or without congenital HCMV infection the deletion of one or two copies of NKG2C did not correlate with a higher incidence of HCMV infection (65). Further supporting the concept that NK receptors other than NKG2C can contribute to shape NK cell receptor repertoire, following HCMV infection, is a study reporting the expansion

of NKG2A$^-$NKG2C$^-$ NK subsets expressing activating KIRs in a cohort of HCMV seropositive healthy individuals (51). In this context, a number of studies suggested that the presence of activating KIRs is protective against viral infections (66–69).

CONCLUDING REMARKS

The development of NK cells reconstituting after transplantation can be profoundly affected by HCMV infection/reactivation, which is a common event in immunocompromised HSCT recipients. Although HCMV infection is cause of morbidity and mortality in such patients, it can also promote NK cell differentiation by accelerating the acquisition of a fully mature KIR$^+$ NKG2A$^-$ phenotype. Notably, in the case of KIR-mismatched haplo-HSCT, this phenomenon may be of particular benefit, since it results in rapid expansion of alloreactive NK cells (characterized in all instances by the KIR$^+$NKG2A$^-$ phenotype). These mature NK cells can display not only anti-leukemia activity but also important antiviral activity that could be beneficial to HSCT recipients. Finally, the strong imprinting induced by HCMV on developing NK cells could be (cautiously) harnessed to design new adoptive NK cell based therapies.

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