Acquired Natural Killer Cell Dysfunction in the Tumor Microenvironment of Classic Hodgkin Lymphoma

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An understanding of interactions within the tumor microenvironment (TME) of classic Hodgkin lymphoma (cHL) has helped pave the way to novel immunotherapies that have enabled dormant and tumor-tolerant immune cells to be reactivated. The immunosuppressive nature of the TME in cHL specifically inhibits the proliferation and activity of natural killer (NK) cells, which contributes to tumor immune-escape mechanisms. This deficiency of NK cells begins at the tumor site and progresses systemically in patients with advanced disease or adverse prognostic factors. Several facets of cHL account for this effect on NK cells. Locally, malignant Reed–Sternberg cells and cells from the TME express ligands for inhibitory receptors on NK cells, including HLA-E, HLA-G, and programmed death-ligand 1. The secretion of chemokines and cytokines, including soluble IL-2 receptor (sCD25), Transforming Growth Factor-β, IL-10, CXCL9, and CXCL10, mediates the systemic immunosuppression. This review also discusses the potential reversibility of quantitative and functional NK cell deficiencies in cHL that are likely to lead to novel treatments.

Keywords: natural killer cells, Hodgkin disease, tumor microenvironment, immunologic cytotoxicity, killer cell immunoglobulin-like receptor, interleukin-2, immunotherapy

INTRODUCTION

Despite improvements in the therapy of classic Hodgkin lymphoma (cHL), over 1,000 deaths per year in North America and 10,000 worldwide result from the failure of effective management (1, 2). cHL comprises four histological subtypes and a unique, heterogeneous phenotype (3, 4). Given that 99% of cHL tumor tissue is composed of inflammatory cells (4), the study of the tumor microenvironment (TME) and its interactions with antitumor immune cells has gained increasing relevance. The most promising recent results in patients with relapsed cancers including cHL have come from the use of immunotherapies (5–7), underscoring the notion that tumor-tolerant cytotoxic cells can be reactivated to kill cancer cells (8). Most immunotherapeutic advances against refractory and relapsed cHL have focused on T-cells, while studies with natural killer (NK) cells remain scanty.

Natural killer cells, as a key component of innate anticancer immunity, deserve further investigation in the context of the cHL TME (9, 10). Exploring the interactions between NK cells and cHL TME that drive NK cell-escape mechanisms will provide a better understanding of the targets needed to reverse NK cell anergy, thereby directing treatment strategies.
IMMUNE CELLS IN HL TME

While only 1% of tumor tissue is composed of malignant Reed-Sternberg (RS) cells, the remaining 99% of cHL tissue comprises the TME and includes numerous inflammatory cells including B-cells, T-cells [CD4+ T-helper cells, regulatory T-cells (Tregs), and cytotoxic CD8+ T-cells], macrophages, eosinophils, neutrophils, plasma cells, dendritic cells, and fibroblasts, all meticulously orchestrated by the dysregulated secretion of chemokines and cytokines from both TME and RS cells (11).

Many of the cells, including tumor-tolerant Th2 T-helper cells and Tregs, are recruited for the growth and survival of RS cells (12, 13). Cytokines responsible for the recruitment comprise IL-7, IL-10, Transforming Growth Factor-β (TGF-β), and galectin-1, known to promote tumor expansion, stimulate the differentiation of Tregs, and enhance immunosuppressive interactions between RS cells and cytotoxic T- and NK cells (14–17). Despite the dominance of Tregs and Th2 cells, tumor-antagonizing cells, including NK cells, CD8+ T-cells, and Th1 T-helper cells, are still a part of the TME infiltrate. They are attracted by chemokines and cytokines, including CXCL9 (Mig-1), CXCL10 (IP-10), and interferon (IFN)-γ (18–20). These antitumor efforts fail, yielding to tumor-tolerant cells and disease progression (21).

IMPORTANCE OF NK CELLS IN THE ELIMINATION OF CANCER CELLS

Natural killer cell effector functions are tightly controlled by the balance between inhibitory and activating signals, as recently reviewed (9, 22). Activating receptors include NKGD2, natural cytotoxicity receptors (NCRs), DNAM1, and FcγRIIIa CD16, among others. Inhibitory signals are mainly mediated by killer cell immunoglobulin-like receptors (KIRs), CD96, and the immune checkpoint receptors, programmed cell death protein-1 (PD-1), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), T-cell immunoglobulin and mucin-3 (TIM-3), and lymphocyte-activation-gene-3 (LAG-3). Not only are activating and inhibitory receptors important for stimulating NK cell cytotoxicity but they also strictly control cytokine and chemokine secretions that further drive antitumor reactions (23). In addition to stimulation by activating receptors, NK cell activity can be enhanced by certain cytokines, notably IFN-γ, interleukin (IL)-2, IL-12, IL-15, IL-18, and IL-21 (24–29).

Two broad subsets of NK cells displaying different forms of anticaner activity are commonly recognized. CD56bright–CD16dim precursor NK cells play an immune regulatory role via chemokine and cytokine secretions that attract and activate antitumor cells from both innate and adaptive arms of the immune system, comprising CD8+ T-cells, dendritic cells, and Th1 cells (30–32). Cytokines, including IFN-γ, TNF-α, and GM-CSF, work individually to recruit, activate, and stimulate the proliferation of antitumor immune cells and induce the presentation of MHC class II molecules on antigen-presenting cells (33). Later stages of NK cell maturation and activation are characterized by a CD56dim–CD16bright phenotype, with antibody-dependent cell cytotoxicity (23, 30, 34, 35). More recently, these classical categories have been put into question, where further activation of CD56dim–CD16dim NK cells has demonstrated the functional reversibility of these cells to a predominantly IFN-γ-secreting role with lesser cytotoxicity, described as CD56bright–CD16dow/negative NK cells. Such a phenomenon has been coined “split anergy” (36, 37). Additional phenotypical analysis of tissue-resident NK cells and NK cells in peripheral blood and bone marrow has provided insight on a broad spectrum of NK cells (38). The differential role of these subsets in mediating a regulatory versus cytotoxic function against cancer continues to be investigated.

In cHL, the infiltration and activation of NK cells confers a favorable prognosis. Naranjo et al. found that a lower number of infiltrating activated CD56dim–CD16bright–CD57+ NK cells in cHL patients were associated with adverse prognostic factors, including the presence of B symptoms and advanced clinical stage (39). Nonetheless, NK cells remain largely decreased in cHL TME and fail to kill RS cells (40, 41).

CHL INDUCES A QUANTITATIVE AND QUALITATIVE NK CELL DEFICIENCY

Early studies of biopsies from cHL patients show a significant deficiency in NK cell numbers, with functional impairment in cytotoxicity. By looking at the in situ quantification of immune cells in cHL-affected lymphoid tissues, Gattringer et al. found NK cell density in cHL-affected tissues to be five times less compared to that of normal tissues and non-Hodgkin lymphoma (HL)-affected tissues, regardless of histological subtype (40). In addition, using the chromium release assay to measure cytotoxicity against the leukemic cell line K562, others have shown NK cells from spleens of cHL patients to be significantly less active than those of healthy donors (42). This impairment was amplified when cHL patients had B symptoms, suggesting a systemic response.

Concomitantly, a quantitative decrease in peripheral blood NK cells in cHL patients has also been observed, without correlation to adverse prognosis or advanced clinical stage (43). More importantly, peripheral blood NK cells in cHL patients are less cytotoxic, regardless of the stage or histological subtype (44–50).

Most recently, additional details on mechanisms behind the functional deficiency of NK cells in cHL patients have emerged. Reiners et al. observed feeble cytolysis of cHL-derived NK cells against the cHL cell line L428, in contrast to efficient killing by healthy donor NK cells (51). They found a significant reduction in NKGD2 expression on untreated cHL-patient NK cells, without changes in other activating receptors or the markers, CD25 and CD69.

CHL MECHANISMS FOR NK CELL INHIBITION

Several factors contribute to the quantitative and functional deficiency of NK cells in cHL, including molecules and surface ligands produced and expressed by RS cells and the surrounding inflammatory milieu. We address those with evidence that
directly and specifically promotes NK cell dysfunction in cHL as summarized in Table 1 and Figure 1.

**Cytokines and Chemokines: IL-2, IL-10, TGF-β, IL-15, CXCL9, and CXCL10**

One significant mechanism of NK cell evasion that explains the persistent failure of NK cell lysis in cHL is the inhibition of IL-2, necessary for NK cell proliferation and activation (24). IL-2 is produced mainly by CD4+ T-cells, but also by activated CD8+ T-cells, dendritic cells, and NK cells themselves (52). However, IL-2 has been found to be largely absent from cHL TME (53–56). Moreover, cHL-patient-derived NK cells are unresponsive to exogenous IL-2 administration. cHL-derived NK cells and healthy donor NK cells fail to respond to IL-2 in the presence of cHL-patient serum, while observing expected enhancement effects in the absence of the serum (51). These findings can be attributed to the production of soluble IL-2Rs (sCD25) by RS cells, which bind IL-2 and prevent IL-2 interaction with T- and NK cells (57, 58). The observations are consistent with studies showing that higher levels of sCD25 in cHL are associated with poorer prognosis and advanced disease (59, 60).

In addition to IL-2, the immunosuppressive cytokines, IL-10 and TGF-β, are actively secreted by RS and TME cells (61). Both favor Treg recruitment and expansion (62) and reduce lymphocyte production of IFN-γ (63) involved in attracting NK cells (18). TGF-β can also diminish the expression of NKG2D ligands (MICA, ULBP2, and ULBP4) and downregulate activating receptors NKG2D and Nkp30 (NCR) (64–66). TGF-β has been shown to directly mediate the transformation of NK cells into tumor-tolerant type 1 innate lymphoid cells in TME (67). IL-15 remains a surprising evasion mechanism in cHL due to its expected role in the differentiation and survival of NK cells (27). Ulrich et al. found that cHL cell lines upregulate IL-15 and corresponding receptors, demonstrating that RS cells utilize IL-15 for growth and apoptosis resistance in an autocrine fashion, thereby competing with NK cells (68).

CXCL9 and CXCL10 are ligands for CXCR3 and attract CXCR3-expressing NK cells (18, 69). Both chemokines are upregulated in cHL tissues and expressed at even higher levels in Epstein–Barr Virus (EBV)+ cHL (19, 70). However, because CXCR3 expression remains limited mostly to the less cytolytic NK cell subset (71), CXCL9 and CXCL10 favor the attraction of CD56bright–CD16neg NK cells. TGF-β further increases CXCR3 expression, enabling an increased attraction of the CD56bright subset toward CXCL9 and CXCL10 (72). Although the failure of CD56bright–CD16neg NK cells to kill RS cells is not entirely understood, recent studies on the genetic and molecular characteristics of RS have provided evidence of intrinsic resistance to cytokine-mediated apoptosis. Using flow sorting and exome sequencing of primary RS cells, Reichel et al. demonstrated that tumor necrosis factor alpha-induced protein 3, normally responsible for inhibiting NFXB survival pathway and mediating TNF-α-induced apoptosis, is the second most common mutation (73).

**HLA-G and HLA-E**

Reed–Sternberg cells have long been known to lack the expression of MHC class I proteins (41, 73), putting them at risk of NK cell lysis due to loss of "self" ligands for inhibitory KIRs. Thus, RS cells evade NK cell cytotoxicity by upregulating HLA-G and HLA-E. HLA-G is a ligand for the inhibitory receptors, immunoglobulin-like transcript (ILT) 2, ILT4, and killer immunoglobulin-like receptor KIR2DL4 (p49) (74, 75), while HLA-E interacts with CD94/NKG2A (76). Notably, HLA-G has been found to stain positive in more than 50% of cHL-lymph node specimens (41), while HLA-E tests positive in 70 and 62.5% of RS cells and TME lymphocytes, respectively (76). HLA-E also correlates with advanced clinical stage.

**Soluble Ligands**

Two NK cell-activating signals often interrupted in cHL are surface receptors NKP30 and NKG2D (51). MICA, ligand for NKG2D, and BAG6/BAT3 ligand for NKP30, are only expressed on cell surfaces upon stress or damage to alert and activate immune cells (77, 78). RS cells overcome this threat by releasing both soluble ligands, predominantly using protein disulfide isomerase ERp5, and disintegrins and metalloproteinases ADAM10 and ADAM17 (79). In fact, MICA and BAG6/BAT3 can be significantly elevated in the serum of untreated cHL patients (51). Not only are these soluble ligands ineffective in activating NKG2D and NKP30 but they also result in receptor endocytosis and degradation (80).
**Physical Barriers**

Another evasion mechanism is the formation of a protective barrier around RS cells using Tregs, Th2 cells, and macrophages, termed “rosetting” (81). This rosetting exhibits physical binding characteristics that are not easily destroyed. Using immunostaining, Hartmann et al. detected CD4+ T-cell and CD163 macrophage rosetting encompassing RS cells to be present in 14 of 15 cHL cases examined (82). The maintenance of a close contact between RS cells with CD4+ T-cells and CD163 macrophages suggests their role in physically shielding malignant cells from NK cell attack.

**Fas/Fas ligand (FasL)**

Although RS cells largely express extrinsic death receptor Fas, they avoid extrinsic apoptosis induced by FasL-expressing NK cells by overexpressing cellular FLICE-inhibitory protein (83). In addition, RS cells overexpress FasL in 87% of cases, making them capable of inducing apoptosis on Fas-expressing NK cells (84, 85).

**Immune Checkpoints**

A near universal expression of immune checkpoints has been demonstrated among TME and RS cells (86, 87). A group of
genetic alterations in the loci of PD-1 ligands, PD-L1 and PD-L2, were found in 97% of cHL cases (88). Of significance, the amplification of locus 9p24.1 correlated with advanced clinical stages and worse progression-free survival. PD-1 and PD-L1 expression in patient biopsies are also of prognostic significance: a high expression of PD-1 and PD-L1 correlated with a lower event-free survival, while a high expression of PD-L1 correlated with a lower overall survival (89). NK cells and CD8+ T-cells in cHL TME can, therefore, be directly inhibited upon the expression of PD-1. The development of the anti-PD-1 monoclonal antibodies, Nivolumab and Pembrolizumab, was aimed to prevent such inhibition. A majority of relapsed and refractory cHL patients showed responses to treatments (65 and 87%, respectively) (89). The role of NK cell-immune checkpoints, LAG-3, TIM-3, and TIGIT, remains unknown in cHL.

EBV+ cHL
Classic Hodgkin lymphoma cells are infected with EBV in approximately 40% of cases (90). EBV+ cHL TME is characterized by the predominance of CD8+ T-, Th1, and NK cells, significantly contrasting the EBV-negative phenotype. Despite the presence of additional cytotoxic cells in EBV+ cHL, this has minimal influence on prognosis (91, 92), suggesting effective evasion. EBV induces an upregulation of MHC class I molecules in approximately 70% of cases, encouraging self-tolerance in NK cells through inhibitory KIRs (41). In addition, EBV+ Tregs secrete twofold more IL-10, increasing immunosuppression in TME (92).

THE REVERSIBILITY OF EVASION MECHANISMS
Despite numerous evasion mechanisms, observations have been made on their reversibility after achieving remission with chemotherapy and/or radiotherapy (47, 93). NK cell cytotoxicity is significantly diminished at cHL diagnosis, independent of clinical stage. This functional deficiency of NK cells normalized 6 weeks after completion of the treatment protocol, contrasting with the long-lasting cellular immune suppression of T-cells. Moreover, the failure to respond or the evidence of early relapse does not appear to improve NK cell function, suggesting that NK cell activity is a biomarker of clinical response and prognosis.

Immune reconstitution after autologous hematopoietic cell transplantation (AHCT) for relapsed or refractory cHL also demonstrates the reversibility of immune suppression. Patients with early recovery (day 15 post AHCT) of the absolute lymphocyte count defined as greater than 500 × 10^9/L in one study, and greater than 667 × 10^9/L in another, had a significantly higher progression-free (in both studies) and overall survival (one study) (94, 95). NK cell counts can rapidly return to normal as early as 2 weeks post AHCT (96), while T- and B-cells remain deficient for months to years. This not only implies early reversibility of NK cell evasion mechanisms after treatment but also highlights the importance of NK cells in preventing relapse early after transplant.

POTENTIAL FOR NK-TARGETED IMMUNOTHERAPIES IN cHL
One approach to reactivate silenced NK cells in cHL is to employ the currently clinically available monoclonal antibodies, Nivolumab and Pembrolizumab, to block immune checkpoint PD-1 on activated CD4+ and CD8+ T-cells, and NK cells (97). Several other molecules targeting NK cell reactivation are under investigation. Heat shock protein-90 inhibitor, BIIB021, is effective against cHL in preclinical studies in vitro and in vivo (97). It acts by directly blocking the NFκB pathway and potentiating NK cell-directed lysis through downregulating MHC class I molecules and upregulating NKG2D ligands MICA, MICB, and ULBP2. A different approach to NK cell immunotherapy has been the study of ADAM10/17 inhibitors. Although still in preclinical testing, ADAM10/17 inhibitors have shown high specificity in their activity and with high affinity (IC50 40 nM for ADAM10) (98, 99). More recently, the tetravalent bispecific CD30/CD16A tandem antibody, AFM13, has been developed to boost autologous NK cells against RS cells (51, 100). A phase I clinical trial of relapsed and refractory cHL showed that the drug was safe and tolerable, with minimal toxicities (101). Patients receiving AFM13 showed an increase in the NK cell activation marker, CD69, after each dose with preliminary evidence of efficacy. A phase II trial with AFM13 is currently underway (GHSG-AFM13 and NCT02321592).

Numerous clinical trials have employed allogeneic NK cells derived from healthy donors with variable outcomes (102). An alternative approach is to use an allogeneic permanent, malignant NK cell line, NK-92, derived from a patient with an NK lymphoma. The parental NK-92 line lacks CD16 expression and hence cannot engage in antibody-dependent cytotoxicity, and its cytolytic activity, lower than for primary NK cells, relies heavily on the activation of the activating receptors NKP30, NKP46, and NKG2D, and the absence of most inhibitory KIRs (103). In addition, given the malignant origin of NK-92, irradiation prior to infusion is required, reducing its cytotoxicity further, including IFN-γ secretion, compared with primary NK cells (28, 104, 105). NK-92 cells nonetheless can kill a variety of cancer cell types (104), and the line is a potentially universal, off-the-shelf source of readily expanded NK cells with uniform cytotoxicity and a high safety profile (106). We recently reported a phase I trial of NK-92 in patients with hematological malignancies relapsing after hematopoietic cell transplantation and found the treatment to be well tolerated and documented several responses, including a patient with refractory cHL who has remained in unmaintained remission for 11 years after NK-92 infusion (107) by an uncertain mechanism.

FUTURE DIRECTIONS
Not only do 15% of cHL patients fail to achieve long-term remission (1, 2), but accumulating evidence indicates that significant long-term toxicities result from aggressive management with chemotherapy and radiotherapy, including an increased risk of mortality from solid tumors and cardiovascular disease, as
well as increased risks in the development of second cancer and diabetes mellitus (108–112). Consequently, studies that explore the immunotherapy of NK cell reactivation to improve long-term disease-free survival and promote harm reduction warrant further investigation.

CONCLUSION

Classic Hodgkin lymphoma is characterized by a potent immunosuppressive TME that inhibits NK cells. It is worth noting that the quantitative and qualitative NK cell deficiencies exhibited by patients with cHL are reversible. Strategies to reactivate NK cell function or block the evasive mechanisms displayed by the TME need further investigation and are likely to identify new immunotherapeutic targets.

AUTHOR CONTRIBUTIONS

JC and DE wrote and revised the final manuscript; JC provided the figure; and AK contributed to manuscript editing and final revision.

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