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Boosting vaccine efficacy the natural (killer) way

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Abstract

Coordination of the innate and adaptive immune systems is paramount to the development of protective humoral and cellular immunity following vaccination. Natural killer (NK) cells are front-line soldiers of the innate immune system, and recent studies have revealed functions for NK cells in long-lived immune memory and the regulation of adaptive immune responses. These findings suggest that NK cells may play important roles in the development of efficacious vaccines, as well as, in some contexts, failed immunizations. Here we review the current understanding of the immunomodulatory and memory differentiation capabilities of NK cells. We examine the context-dependency of the mechanisms and the nature NK cell-mediated modulation of the immune response, and discuss how these insights may impact immunization strategies and the development of next-generation vaccines.

Keywords

Natural killer cells; vaccination; immune regulation

Introduction

Vaccines have enjoyed unparalleled success as stalwart defenders of human health, including the eradication of smallpox and the imminent demise of polio. These successes largely reflect the ability of vaccines to induce durable production of protective pathogen-specific antibodies by plasma and memory B cells [1]. Despite concerted international efforts, licensed vaccines still do not exist to prevent infections with a number of major microbial threats, including malaria, tuberculosis, hepatitis C virus (HCV), and HIV. In the case of many chronic infections, this lack of success reflects an incomplete understanding of the necessary correlates of protection. Current vaccine strategies must evolve to be able to generate specific types of antibodies capable of antibody-dependent cellular cytotoxicity (ADCC) or broadly specific neutralization in order to combat highly mutable viruses like

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HIV [2, 3]. Furthermore, it is likely that a vaccine must concomitantly generate functional memory T cells and stimulate long-lasting protective *innate* immune memory [4, 5]. In addition to regimens that can prevent infection, there is a need to develop therapeutic vaccines that can stimulate or reinvigorate these types of immune responses against pathogens that have already infected a host. Therefore, next generation vaccines should encompass strategies to overcome natural immunoregulatory roadblocks that restrict development of these types of adaptive immune responses, and that also incorporate novel means of triggering innate immune memory to promote life-long protection against infection.

Natural killer (NK) cells are innate lymphoid cells (ILCs) widely renowned for their role in eliminating transformed and virus-infected cells [6]. This classical view has recently evolved to reflect evidence that NK cells display features of adaptive immune cells [7, 8], including the ability to specifically recognize microbial antigens and the potential to develop into long-lived memory cells that protect against subsequent infections [9, 10]. These findings imply that new vaccine strategies should be developed in order facilitate the induction of long-lived, pathogen-specific memory NK cells that could contribute to prevention or control of infection. Moreover, there is growing appreciation for the ability of NK cells to regulate adaptive immune responses [11, 12]. NK cells inhibit the development of long-lived memory T and B cells as well as the generation of protective neutralizing antibodies after infection [13, 14]. In contrast, NK cells appear to support the development of memory T cells and humoral immunity following immunization with less inflammatory apoptotic tumor cells [15, 16]. Thus, NK cells may be a critical linchpin in the success or failure of vaccination, but their contributions appear to be entirely dependent on the specific circumstances associated with either the immunization milieu or the nature of the pathogen the vaccine is meant to eliminate. Herein we provide a discussion on the means by which NK cells promote, suppress, and participate in adaptive immune responses. Our goal is to provide a framework for further debate and future experimentation concerning the questions of whether and how these new functions of NK cells should be modulated during immunization. In other words, can innovative strategies be developed to harness the beneficial activities of helper or memory NK cells while safely subverting the functions of suppressive regulatory NK cells in order to enhance the efficacy of next-generation vaccines?

Activation of NK cells during vaccination

Unlike antigen naïve T and B cells that must proliferate and differentiate from relatively rare precursors before becoming fully functional, resting NK cells are readily poised to exert effector functions immediately after stimulation [8]. The activation of NK cells is predominately determined by the net input of activating and inhibitory signals from germline encoded NK-cell receptors [17, 18]. A number of these NK-cell receptors recognize class 1 major histocompatibility complex (MHC) molecules and protect host cells from NK-cell attack by delivering an inhibitory signal through mouse Ly49 receptors, human killer immunoglobulin-like receptors (KIRs), or the NKG2A receptor in both species. Thus, NK cells are activated in the absence of self when infection or other stimuli trigger downregulation of MHC, a phenomenon termed missing self [19]. This missing self

recognition can be exploited during immunization by delivering tumor cells that lack class I MHC molecules. Remarkably, injection of MHC deficient or allogeneic NK cell-susceptible target cells into mice triggered an NK cell-mediated enhancement of memory T-cell and humoral immune responses against antigens expressed by the target cells [15, 16]. This is one example of a potential beneficial regulatory role for NK cells during immunization.

NK cells also possess germline-encoded activating receptors that recognize pathogen-encoded molecules or stress-induced proteins expressed on infected and transformed cells [17, 18]. For example, ligands of the NK-cell receptor NKG2D present on tumor cells stimulate potent NK-cell effector functionality [20]. In fact, forced expression of NKG2D ligands in the context of tumor cell lines or a murine cytomegalovirus (MCMV) vaccine vector, augmented the development of memory T cells in mice in an NK cell-dependent manner [21, 22]. In a similar fashion, the MCMV m157 protein is recognized by the activating NK-cell receptor, Ly49H [23–25]. The interaction between Ly49H and m157 is central to the discovery of memory NK cells induced by virus infection [10]. Mouse NK cells also appear capable of responding to specific antigens of influenza virus and HIV, although no antigen-specific receptor of NK cells has been linked to this phenomenon [9]. These results suggest that manipulation of the expression of stimulatory or inhibitory ligands for NK-cell receptors is one means of controlling the activity of NK cells during immunization.

In addition to ligands of conventional NK-cell receptors, cytokines [26], microbial products [27], and inflammatory molecules [28] present during infection or vaccination can also regulate the activation of NK cells. In order to control the contribution of NK cells to vaccine efficacy, it will be important to discriminate the ability of distinct stimuli to differentially trigger the inflammatory, suppressive, and memory-differentiation activities of NK cells. Aluminum salts (alum) are a primary adjuvant in many human vaccine regimens because it stimulates a type 2 cytokine response that facilitates development of humoral immunity [29]. While there is little evidence that alum stimulates NK-cell activation, newer, more inflammatory adjuvants (e.g. MF59 and CpG-DNA) are being introduced that promote type 1 cytokines and induction of memory T-cell responses [30]. Type 1 cytokines, like interleukin-12 (IL-12), IL-15, IL-18, and type 1 interferons (IFN), are capable of potently stimulating NK-cell activation [17]. In fact, type 1 IFN is implicated in the induction of NK-cell suppression of T cells during lymphocytic choriomeningitis virus (LCMV) infection of mice [31]. Conversely, IL-12 and IL-18 are critical in the induction of long-lived memory NK cells induced following MCMV infection [32, 33]. NK cells can also be activated by direct stimulation of receptors on NK cells, including toll-like receptor 2 (TLR2), which bind microbial products present in live or attenuated whole-pathogen vaccines [34]. Thus, consideration should be given to the effects that inflammatory molecules in vaccines may have on NK-cell activity.

Localization of NK cells

Vaccination requires the mobilization of immune cells to different sites within the body, including the injection site and draining lymphoid tissues. Under steady state conditions, human NK cells comprise roughly 10 to 40% of leukocytes in lung and liver, up to 6% in

blood, 1 to 5% in spleen and lymph nodes, and a smaller fraction in the skin and gut [35]. Thus, NK cells are well-placed to respond to vaccine antigens. It is noteworthy that NK cells are far less numerous than T cells in most tissues, since T cells represent more than 50% of the leukocytes present in blood and lymph nodes of humans and mice [36]. Furthermore, inflammation promotes re-distribution of NK cells among and within tissues, including re-localization to zones of lymphoid tissues that are rich in T and B cells, thereby permitting direct interactions of NK cells with adaptive immune cells [37–42].

In humans, most NK cells present in the blood feature dim expression of the adhesion molecule CD56 (CD56^{dim}) but contain high levels of cytolytic proteins, enabling them to rapidly kill target cells [43]. In contrast, NK cells in human lymph nodes are predominately CD56^{bright} and produce large amounts of cytokines following stimulation. Similarly, mouse NK cells demonstrate divergent cytotoxic and cytokine-producing capacity that are related to the tissue in which they reside [44]. For example, while most NK cells are strongly cytolytic and produce IFN- γ , a unique subset of NK cells present in the uterine mucosa is characterized by a weak capacity for both cytotoxicity and IFN- γ production [45]. These cells are thought to promote healthy pregnancy in part by supporting placental development, which involves allogeneic fetal placental cells that might normally be targeted by classical NK cells. Thus, modulation of NK cell function in vaccination during pregnancy presents a unique challenge regarding the additional beneficial contributions of uterine NK cells. In a similar fashion, NK cell-rich tissues like the liver contain a molecularly distinct population of resident NK cells that is weakly cytotoxic but strongly implicated in mediating antigen-specific memory NK-cell responses against haptens [9, 46]. Notably, liver NK cells share a number of molecular features with NK cells present in the skin, a tissue frequently exposed to microbial antigens during vaccination and infection [44]. Thus, the liver and skin may hold clues for how to prime and maintain populations of memory NK cells. In mucosal tissues, many NK-like cells have been reclassified as distinct subsets of ILCs with a variety of functions reviewed elsewhere [47, 48]. Nevertheless, classical NK cells in the gut mucosa have also been attributed with unique functions that include the production of cytokines like IL-22 [49, 50]. This has implications for the development of immune responses against oral vaccines or in immunization against mucosal pathogens. In fact, IL-22-producing NK cells in the lungs of mice were shown to promote protective memory T-cell responses by suppressing regulatory T cells after immunization with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), a vaccine against the major human pathogen *Mycobacterium tuberculosis* [51]. Notably, IL-22-producing NK cells are also present in tonsils and the uterine mucosa in humans [52, 53].

This diversity within the NK cell lineage and in different tissues highlights the potential for functionally distinct subsets of NK cells to be selectively stimulated following immunization via oral, intramuscular, subcutaneous, and intranasal routes. Challenges remain in understanding the environmental factors that contribute to the differentiation of these subsets and whether tissue-restricted subtypes of NK cells can in fact re-localize to vaccine-relevant sites following immunization. Furthermore, it remains unclear whether the regulatory and memory functions of NK cells reside in distinct or overlapping subpopulations of these cells, and to what extent these activities of NK cells are restricted by tissue-specific factors (Box

1). For example, while some types of memory NK cells reside in the liver, other long-lived populations of NK cells have been observed in lymphoid tissues [54]. Due to the tremendous heterogeneity of NK cells in humans [55], further studies are required to ascertain the phenotypic or molecular characteristics that demarcate distinct functional (i.e. regulatory or memory) subsets of NK cells. Identification of such discrete subsets would facilitate specific targeting of beneficial or detrimental NK cells in order to enhance efficacy of a particular vaccine regimen.

NK cell influence on antigen presentation and inflammation

The cytotoxic functions of NK cells are important in the resolution of infection and inflammation. Thus, NK cell-mediated elimination of infectious microbes can be beneficial to the host by preventing exaggerated adaptive immune responses that could lead to immunopathology. However, NK-cell constraint of the magnitude and duration of antigen exposure after infection can also be detrimental to the adaptive response. In other words, NK-cell killing of an infected cell prevents the pathogen (i.e. virus) and its subsequent progeny from continuing to provide antigenic and inflammatory stimulation to adaptive immune cells. For example, depletion of NK cells and loss of NK cell-mediated control of virus replication has both positive and negative consequences for virus-specific T cell responses during MCMV infection (reviewed in [11]). We speculate that in some instances NK-cell elimination of virus results in a low level of antigen that poorly stimulates virus-specific T cells, which translates to higher viral load and stronger stimulation of T cells when NK cells are depleted [56–58]. Alternatively, loss of NK cell control of MCMV can convert a moderate level of virus into a high viral load that is immunosuppressive, which presents as a depressed T-cell response following NK cell depletion [59, 60]. Rather than total levels of antigen and inflammation, NK cells can affect the quality of antigen presentation by killing infected professional antigen-presenting cells (APCs), including dendritic cells (DCs). In the case of MCMV infection of mice, NK-cell killing of infected DCs proved counter-effective to strong T-cell immunity by reducing the number of antigen-bearing DCs available to prime a response [57].

In addition to classical cytotoxic antiviral functions, NK cells also mediate non-canonical interactions with DCs and macrophages that potentiate or diminish subsequent stimulation of adaptive immunity (Figure 1). NK cells can shape adaptive immune responses via a process called DC editing. Immature DCs are implicated in the induction of immune tolerance, whereas mature DCs upregulate co-stimulatory receptors, express pro-inflammatory cytokines, and stimulate T-cell responses. Human NK cells discriminatorily target immature DCs while sparing mature DCs [61]. This dichotomy is rooted in the expression of ligands of Nkp30 and DNAM-1 on susceptible immature DCs as well as the up-regulated expression of inhibitory MHC I ligands that suppress NK-cell killing on resistant mature DCs [62–65]. Notably, DC ‘editing’ by NK cells was reduced by IL-10 in HIV-infected individuals, which contributed to accumulation of poorly immunogenic DCs and immune dysfunction [66, 67]. NK cells can also secrete immunosuppressive or immunomodulatory cytokines that limit antigen presentation or inflammation and thereby diminish adaptive immune responses. Although only one report suggests NK cells secrete transforming growth factor-beta (TGF- β) [68], there is ample evidence that these cells can

produce IL-10 in both humans and mice [69–73]. IL-10-expressing NK cells in mice were triggered during disseminated infections and contributed to the prevention of fatal immune pathology by inhibiting T-cell responses.

NK cells are also able to stimulate survival, function, and maturation of APCs through the release of pro-inflammatory cytokines like IFN- γ and tumor necrosis factor (TNF). During influenza virus infection of mice, NK cell-derived IFN- γ drove recruitment of DCs and T cells to lymph nodes for priming of an adaptive antiviral response [74]. Likewise, immunization of mice with immunogenic DCs promoted NK-cell migration into lymph nodes, where these NK cells produced IFN- γ to stimulate protective Th1 responses [75, 76]. In a contact-dependent manner, activated human NK cells promoted maturation of immature DCs [61, 64, 77–80]. Mouse NK cells promote the survival of splenic CD8 α + DCs, which correspondingly produce IL-18 and IL-12 to support NK-cell proliferation and IFN- γ production [41, 81–83]. Additionally, NK cells limited the levels of type I interferons produced by plasmacytoid DCs (pDCs) early after MCMV infection, thereby preserving the T-cell stimulatory myeloid DC compartment [60]. Likewise, virus-specific Ly49H-expressing NK cells can engage in crosstalk with DCs to enhance the priming of MCMV-specific T cells [84]. The impact of these interactions is reciprocal, as DCs also stimulate NK-cell activation and function. Release of type I IFNs by pDCs stimulated NK-cell effector functions [78, 79, 85–87]. Furthermore, IL-12, IL-15 and IL-18 produced by DCs promoted the maturation, survival and proliferation of NK cells [41, 79]. The mutually beneficial nature of DC-NK cell cross-talk (Figure 1) can act in a cyclical fashion to amplify the overall immune response and support the induction of T-cell responses necessary for pathogen clearance.

The preceding discussion highlights the complex role of NK cells in the regulation of antigen presentation and inflammation. On the one hand, NK cells can function by killing infected cells and microbes, thus limiting the spread of infection and contributing to the resolution of inflammation. The production of IL-10 by NK cells also appears to constrain inflammation by suppressing APC function and cytokine production. These effects are beneficial by preventing immunopathology during disseminated infections [70, 71]. On the other hand, NK cell-mediated restriction of antigen availability and inflammation can undermine effective activation of an adaptive immune response during vaccination. This mechanism is likely to be most relevant in the context of live (whole pathogen) vaccines where NK cells can curtail the persistence of replicating antigens and associated inflammation. Thus, ablation of NK cells may be an effective strategy to enhance immune responses against complex, highly inflammatory vaccine regimens. This fits with data that reveal stronger adaptive responses in the absence of NK cells after virus infection or immunization using potent vaccine adjuvants like poly(I:C) [13, 14, 56, 57, 88, 89]. In contrast, DC-NK cell crosstalk can sometimes augment the inflammatory response and thereby enhance stimulation of adaptive responses. Given that NK cells tended to enhance adaptive responses against mildly inflammatory cell-based vaccines [15, 16, 76], the contribution of NK cell-derived inflammatory cytokines or signals may be most desirable in the context of less inflammatory protein subunit vaccine regimens. More research must be done to refine our understanding of when regulatory effects of NK cells on antigen

presentation and inflammation are desirable (Box 1) in order to guide improved vaccine design.

NK cell interaction with and regulation of T cells

Whereas interactions between NK cells and APCs can indirectly shape the T-cell response that develops following immunization (Figure 1), NK cells can also have direct effects on T cells themselves [13, 31, 88, 90–98]. NK cell-derived IFN- γ can drive the polarization of CD4 T cells to a Th1 phenotype [76, 99, 100]. Moreover, NK cell-derived IFN- γ can also promote CD8 T-cell activation in the absence of CD4 T cell help [101, 102]. In contrast, NK cell production of immunosuppressive cytokines such as IL-10 have been shown to limit Th1 responses and the expansion of CD8 T cells in the context of parasite and MCMV infections [70, 71]. Thus, NK cell-derived cytokine production can modify T cell responses in both detrimental and beneficial ways.

Evidence also supports the notion that NK cells can regulate T-cell responses via direct interactions (reviewed in [11, 12]). Murine experiments suggested that NK cells could kill activated T cells *in vitro* and *in vivo* via engagement of NKG2D by ligands expressed on activated T cells [13, 31, 95, 98]. This phenomenon has also been observed *in vitro* with human NK cells, although differential requirements for NKG2D, NKG2A, TNF-related apoptosis-inducing ligand (TRAIL), DNAX Accessory Molecule-1 (DNAM-1), and lymphocyte function-associated antigen 1 (LFA-1) on NK cells have been noted in this process [90, 91, 98, 103]. Notably, priming of T cells in the absence of NK cells led to larger and more functional pools of memory T cells following immunization of mice with Pichinde virus, LCMV, an ovalbumin (OVA)-expressing adenovirus, or OVA protein admixed with lipopolysaccharide [13, 14]. The greater number of memory T cells may be a function of the enhanced magnitude of the primary T cell response in NK cell-depleted animals, although evidence from our group [14] reveals a concomitant skewing towards IL-7 receptor alpha expressing (IL-7R α^+) and killer-cell lectin like receptor G1 negative (KLRG1^{neg}) memory precursor effector cells (MPECs) rather than IL-7R α^{neg} KLRG1⁺ short-lived effect cells (SLECs) [104] in the absence of NK cells, which may indicate qualitative differences in responding T cells that favor enhanced differentiation into a memory phenotype. To date, studies concerning NK cell influence on memory T cells have focused on the induction of these cells in lymphoid tissues. Given the current interest in tissue-resident memory T cells [105], it will be important to evaluate whether priming of these front-line sentinels is also modulated by NK cells.

NK cells can also influence T cell responses indirectly by targeting accessory suppressor cells, including regulatory T cells. During *M. tuberculosis* infection or BCG immunization of mice, NK cells have even been found to kill regulatory T cells and thereby enhance the responses of effector T cells against the pathogen or vaccine [51, 106]. Conversely, decidual human NK cell crosstalk with myeloid cells promotes the development of regulatory T cells *in vitro*, which appears important for suppressing maternal immune responses against the fetus during pregnancy [107]. It is not currently known whether and how NK cells may crosstalk with other ILC subpopulations, which have also been shown to regulate adaptive immunity [108–110], or how this might impact vaccine responses. Thus, effects of NK cells

on regulatory populations of lymphocytes that subsequently modulate adaptive responses need to be considered as well.

It is interesting to speculate on why this mechanism of NK-cell inhibition of T cells exists and what vital function it conveys to the host. While this may reflect impromptu friendly fire when highly activated NK cells and T cells co-exist in an inflammatory niche at an early stage of the immune response, the stringent control of NK-cell killing of T cells by paired NK cell receptors and their ligands on T cells suggest an element of design. In fact, inhibitory receptors like NKG2A [96, 111] and 2B4/CD244 [112] preclude NK-cell killing of T cells in certain contexts, suggesting that T cells can up-regulate ligands of these receptors in order to protect themselves. Moreover, type I IFN signaling could protect thymocytes [113] and peripheral virus-specific T cells from NK cell-mediated killing during virus infection of mice [93, 94]. This suggests that adjuvants that trigger high levels of type I IFN expression may be ideal in order to protect developing T cells from NK cell-mediated attack during vaccination. However, type I IFN is also a potent activator of NK cells and it is unknown whether type I IFN provides signals that protect other cells, including B cells and APCs, from NK-cell killing. Perhaps NK cell-mediated suppression is meant to prevent immunopathology caused by over exuberant T-cell responses during disseminated infection [70, 88, 114], or to exclude potentially dangerous autoreactive T cells from joining the immune response [115]. In fact, NK cells prolonged MCMV infection in the salivary gland by inhibiting virus-reactive CD4 T cells, such that depletion of NK cells resulted in an autoimmune disease of this tissue in mice that resembles Sjogren's syndrome in humans [116]. Therefore, NK-cell regulation of adaptive responses might be important to protect the host from autoreactive or aberrant T-cell responses. Future studies aimed at revealing enhanced adaptive immune responses against vaccine antigens following subversion of NK-cell regulatory functions should examine in parallel the possible enhancement of autoimmune responses of T and B cells (Box 1), which could limit the safety of this type of vaccination strategy.

Finally, it is worth noting the potential role of NK cells during therapeutic vaccination of chronically-infected individuals or cancer patients. In humans, NK cells were found to delete HCV-specific T cells expressing ligands for TRAIL in the blood and liver of infected patients [90]. This supports data in mouse models of chronic LCMV infection that reveal NK cell-depletion to be an effective means of reinvigorating dysfunctional virus-specific T cells to promote viral control [117]. However, during therapeutic vaccination of hepatitis B virus-infected patients, an increase in the proportion of CD56^{bright} NK cells was associated with an enhanced HBV-specific T cell response [118]. Furthermore, conventional wisdom holds that NK cells contribute to the immune response against HIV, HCV, and cancer [119]. NK cells are also likely to be involved in controlling herpesvirus infections within these patients as well [120]. Therefore, the relative contributions of regulatory and antiviral roles of NK cells need to be understood in these different disease conditions before due consideration can be given to developing means to target NK cells in the context of therapeutic vaccinations aimed at enhancing T or B cell elimination of infected or transformed cells.

Interplay between NK cells and B cells

The ability of B cells to produce antigen-specific antibodies is at the crux of any effective vaccine. Therefore, the identification of immunoregulatory factors that govern key events in the development of long-lived protective humoral immunity is crucial to developing means of enhancing these responses. Of note, the formation of germinal centers in secondary lymphoid tissues permits sustained cognate interactions between helper CD4 T cells and B cells that facilitate isotype class-switching, somatic hypermutation and affinity maturation of immunoglobulin, and differentiation of B cells into long-lived memory or plasma cells [121]. While NK cell inhibition of CD4 T cells is likely to have effects on T cell-dependent humoral responses including the germinal center, there is evidence that NK cells also directly regulate B-cell function. Early studies showed that NK cells could promote IgM and IgG production by B cells during *in vitro* co-culture. In particular, mouse NK cells promoted isotype class switching via both IFN- γ -dependent and -independent mechanisms [122–125]. Human NK cells could also enhance antibody production by B cells *in vitro* via both cytokine production and CD40-CD40 ligand interactions [126, 127]. Moreover, NK cells supported B-cell activation and antibody production following immunization of mice with heat-killed *Brucella abortus* [128], antigen-expressing tumor cells [15], or proteinaceous antigens (e.g. OVA) [129].

However, NK cells also function as suppressors of B-cell responses in various contexts. Injection of the TLR3 agonist, polyI:C, resulted in NK-cell activation and premature collapse of the primary IgM response in immunized mice [89]. Likewise, IL-2 activated NK cells restricted B-cell immunoglobulin production following pokeweed mitogen [130–133] or Epstein-barr virus [134] stimulation *in vitro*. More recently, our lab has shown that NK cells suppress B-cell responses (Figure 1) by impairing the development of CD4⁺ follicular helper T (T_{fh}) cells in a perforin-dependent manner [14]. This, in turn, led to decreased expansion of germinal center B cells, diminished generation of LCMV-specific plasma cells, and weak induction of neutralizing antibodies [14]. A similar effect has been observed in chronic LCMV infection as well [135]. Our results are consistent with past reports of a role for perforin (i.e. cytolytic granules) in NK cell suppression of B cell responses [136], although accounts differ with regards to whether B cells or the helper T cells are the primary targets of this inhibition [132, 137, 138]. The receptors involved in NK-cell targeting of T_{fh} cells and B cells remain to be determined (Box 1).

NK cell interference with germinal center reactions during immunization could have important consequences for the efficacy of vaccine regimens. Within germinal centers, B cells undergo somatic hypermutation and affinity maturation of immunoglobulin sequences [121]. This process is crucial for generation of high affinity antibodies that are capable of neutralizing viruses. For example, development of broadly neutralizing antibodies against a highly mutable virus like HIV requires extensive rounds of somatic hypermutation over the course of years of infection [2]. To date, HIV vaccines have failed to induce this level of mutation or stimulate generation of broadly neutralizing antibodies in uninfected individuals. Understanding how and when NK cells regulate B cells and whether this affects the rate at which GC B cells acquire productive affinity-increasing mutations would enable the design of strategies to target this activity of NK cells to enhance induction of protective

humoral immune responses following vaccination (Box 1). Moreover, work remains to be done to determine when NK cell function is beneficial or detrimental to the developing B cell response during vaccination, and how it may relate to the nature of the adjuvant or inflammatory response that is induced [139, 140].

Memory NK cells

Effective vaccines elicit long-lived memory T and B cells that can be rapidly recalled and expanded following a secondary challenge. A central dogma of the innate immune system is the inability to form long-lived memory pools capable of enhanced recall responses. However, the discovery of long-lived ‘memory’ NK cells capable of responding specifically and more robustly than naïve NK cells following re-exposure to antigen, has blurred the lines between innate and adaptive immunity [7, 8]. In mice, memory NK cells were defined by their ability to expand upon antigen encounter, undergo contraction, and persist indefinitely as memory cells capable of enhanced antigen-specific responses (i.e. IFN- γ secretion) upon secondary challenge [9, 10]. A subset of these cells have been further characterized as being liver-resident and demarcated by expression of CXCR6 and CD49a [9, 141]. Yet another distinct subset of Fc receptor gamma-negative (FcR γ -) memory NK cells capable of preferential expansion in response to human cytomegalovirus- or influenza A virus-infected cells has also been observed in the spleens of mice and in human blood [54, 142, 143].

The first report of memory NK cells occurred in a hapten-induced contact delayed hypersensitivity (DTH) model where NK cells from sensitized animals were transferred into naïve T- and B-cell-deficient mice where these innate immune cells were found to elicit robust recall responses to the original hapten up to four weeks after the first encounter. This effect was mediated by a subset of liver-resident NK cells expressing the chemokine receptor CXCR6 [144]. Memory-like NK cells elicited following cytokine stimulation were also observed following adoptive transfer and homeostatic proliferation in immunodeficient hosts [145]. In addition, MCMV-specific Ly49H⁺ NK cells have been shown to expand, contract, persist at low levels for long periods of time and rapidly re-expand following subsequent re-infection, reminiscent of T-cell memory responses [10]. MCMV-specific [8] and FcR γ - [54] memory NK cell responses, contrary to DTH studies, do not belong to a subpopulation of NK cells residing in the liver, but have been shown to persist in various tissues.

Murine memory NK cells have also been elicited by immunization with influenza virus [146], vaccinia virus [147], inactivated vesicular stomatitis virus, or virus-like particles containing antigens of influenza A virus and HIV [9]. In some cases, these memory NK cells could partially protect against lethal challenge with live virus in the absence of T and B cells [9]. In humans, a long-lived population of ‘memory’ NK cells emerged following infection with hantavirus that persisted for greater than 60 days post-infection and were marked by high expression of the activating NK-cell receptor, NKG2C [148]. Similar populations of ‘memory’ NK cells are expanded in cytomegalovirus infected individuals [149–151] and may contribute to control of virus replication. Overall, it is clear that experienced NK cells can persist in the periphery for great lengths of time, possibly indefinitely, and that these NK

cells maintain some measure of specificity for the original antigen with which they were stimulated. Yet, aside from direct recognition of MCMV infected cells by the Ly49H receptor on NK cells, little is known about the molecular nature of NK cell memory, including the identity of the receptor(s) required for this specific antigen recognition that is inconsistent with the germline-encoded rather than somatically rearranged nature of classical NK-cell receptors (Box 1). Nevertheless, it is clear that memory NK cells are now a novel goal of innovative vaccine efforts that aim to harness the enhanced functions of these cells to prevent infection (Figure 1). Additional information is required concerning how and if these cells can be induced and sustained following vaccination (Box 1).

Recall responses during boosting and infection

It is unclear what role 'memory' NK cells would play when they are present in an intact immune environment along with memory T and B cells that can also mediate specific, rapid elimination of the offending pathogen (Figure 2). Certainly these cells can mediate rapid cytolytic attack and production of IFN- γ , but only marginally more so than antigen-naïve NK cells, with antiviral effects only measureable following transfer of memory NK cells into neonatal mice lacking an endogenous NK cell repertoire [10]. Moreover, in an immunized and immunologically intact mouse or human, there would be enhanced numbers of memory T cells that can perform the same functions as NK cells rapidly and specifically, without the onus for expansion and differentiation of naïve T cells. The most intriguing possibility lies in the ability of NK cells to participate with pathogen-specific antibodies derived from memory B cells and plasma cells in the process of ADCC (Figure 2), thereby removing infected cells or microbes from the host. An enhanced ability of memory NK cells relative to their inexperienced counterparts to mediate ADCC would be a clear advantage in a recall response. In fact, memory NK cells can display altered expression of the adaptor proteins, including FcR γ , involved in ADCC [54]. Notably, these cells have reduced expression of FcR γ and SYK but normal expression of CD3 ζ , a phenotype associated with greater antibody-dependent effector functions of these memory NK cells [142, 143]. Thus, memory NK cells may have enhanced functionality that operates in concert with memory B cells in protection.

A second intriguing aspect of the presence of both inexperienced and memory NK cells during recall infection or vaccine boosting is the possibility that these two types of NK cells may similarly or differentially contribute to regulation of the adaptive immune response in these settings (Figure 2). Although antigen would be more rapidly eliminated in a recall challenge, it is possible that there would be sufficient inflammation to trigger NK cell suppression of memory T and B cells as they respond to re-exposure to their cognate antigen. Future studies should assess whether memory T and B cells remain amenable to NK-cell regulation (Box 1). The kinetics of adaptive immune activation are more rapid in memory recall, migration may be biased more towards the site of infection or immunization rather than to secondary lymphoid tissues, and the expression of ligands of NK-cell receptors may differ following antigen-receptor ligation on memory versus naïve lymphocytes. In addition, memory NK cells present under these conditions may be more adept at regulating adaptive immune responses. This would be particularly exciting in light of the data suggesting that memory NK cells remember specific antigens [9] and could

therefore potently attenuate or potentiate antigen-recall responses at the level of interaction with APCs. Finally, cytokines produced by memory T cells are potent triggers for NK cells [152], which in turn represent a sizeable fraction of the responding cells in human recipients of BCG or a malaria vaccine candidate [153, 154]. This suggests that the presence of responding memory T cells could heavily influence the activation of either memory or regulatory NK cells (Figure 2).

Concluding Remarks

Natural killer cells have demonstrated a powerful role in molding adaptive immune responses to a diverse array of infections and inflammatory conditions, an exciting attribute that could hold immense promise for enhancing or modifying vaccine responses. Ongoing efforts will help elucidate the exact mechanisms NK cells employ to regulate adaptive responses, and identify specifically which NK-cell receptors and corresponding ligands on target cells are mediating interactions that shape adaptive immunity (Box 1). There is a pressing need to dissect the conflicting nature of regulatory functions of NK cells in order to understand when or under what circumstances this activity of NK cells is beneficial versus detrimental. Unraveling the nuances of NK cell receptor specificity will allow us to pair that knowledge with NK cell subset localization within the body to determine the most appropriate adjuvant to pair with a vaccine antigen to elicit either: 1) NK cell recruitment and activation to promote beneficial regulation of adaptive immunity, or 2) deletion or inhibition of NK cells to prevent suppression of adaptive immunity and instead favor strong induction of protective immune responses. There is a parallel need to increase our understanding of how memory NK cells mediate specific recognition of antigen, and whether strategies can be developed to stimulate the generation of these cells during vaccination (Box 1). In summary, there is ample exciting evidence that NK cells are key players in determining and contributing to life-long protection from infection after vaccination. Greater attention to understanding the mechanism and context of these specific activities of NK cells is certain to enhance the development of efficacious new vaccine strategies for major human diseases.

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Box 1**Outstanding Questions**

- Can vaccine adjuvants be optimized to promote activation and recruitment of NK cells to target tissues?
- What environmental factors contribute to the phenotypic differentiation of NK cells in tissues and can tissue-resident cells re-localize during immunization?
- What factors determine beneficial versus detrimental effects of NK cells on adaptive immune responses?
- Is a distinct subset of NK cells responsible for immunomodulatory effects on the adaptive immune system?
- Are different NK cell subsets involved in the positive and negative regulatory effects?
- What NK cell receptors are involved in conferring specificity to memory NK cells and to promoting NK-cell regulation of adaptive immunity?
- Can regulatory human NK cells be effectively targeted to yield enhanced adaptive responses at the time of vaccination?
- Can memory NK cells be induced and sustained following vaccination?
- Do memory NK cells contribute to host defense in immunologically intact hosts?
- Does NK cell regulation of T and B cells serve to prevent development of autoimmunity?
- How do memory NK cells interact with APCs, T and B cells during a recall response following antigen boost or infection?
- Are memory T and memory B similarly amenable to regulatory NK cells during a secondary response?

Highlights

- Vaccination should induce long-lived protective B, T, and NK-cell immunity.
- NK cells can positively and negatively regulate adaptive immunity.
- The mechanism and nature of NK-cell immunomodulation is context-dependent.
- Memory and regulatory NK-cell activities should be targeted in immunization.

Figure 1. NK cells in the priming of an immune response

During a primary response, NK cells can directly interact with and positively or negatively regulate antigen presenting cells (APCs) and downstream T cell responses. NK cells can also interact directly with T cells and, at least indirectly, with B cells to modulate the magnitude of T- and B-cell responses. All of these interactions can shape the memory precursor pool of T and B cells that form following contraction of the primary response. More work needs to be done to decipher the NK-cell receptors and mechanisms involved in NK-cell regulation of adaptive cell populations. In some contexts, memory NK cells have been shown to develop, although here as well, the NK receptors delineating memory NK from other NK cells are largely unknown.

Figure 2. NK cells in recall and re-infection

In a recall response, memory T and B cell populations respond more quickly and expand more rapidly than in a primary response. Thus, memory T cell-derived cytokines can contribute to activation of NK cells. Whether memory T and B cells remain as amenable to NK cell regulation as their naïve counterparts has not been determined. Memory NK cells may also contribute through rapid effector functions or enhanced ADCC function with antibodies from memory B cells. Additionally, there is essentially nothing known about the regulatory capacities of memory NK cell populations and whether they represent another, more specialized subset capable of modulating adaptive immunity or possess another distinct function.