ROLE OF IL-15 IN NATURAL KILLER CELL RESPONSES TO INFLUENZA

by

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(Under the Direction of KIM KLONOWSKI)

ABSTRACT

Influenza remains a public health priority in the U.S. particularly with new, rapidly emerging strains of the virus. Natural Killer (NK) cells are activated after contacting influenza infected epithelial cell and are crucial for viral clearance. Understanding the mechanisms of the NK cell response to influenza could illuminate ways to augment the non-specific immunity, which could provide rapid, short-term protection. We hypothesize that NK cells are dependent on direct IL-15/IL-15Rα signaling for recruitment to the lung airways after an influenza infection. Here, we examined the relationship between NK cell recruitment and Interleukin-15 (IL-15) at the site of influenza infection. We show that NK cell recruitment is dependent on the local expression of IL-15. In IL-15-ablated models, fewer NK cells migrated to the site of infection, whereas treatment with soluble IL-15 led to increased recruitment of NK cells to the lung airways. Altogether, these data suggest an important role for IL-15 in the recruitment of NK cell responses to influenza, while also revealing a potential therapeutic use of for IL-15.

INDEX WORDS: *Influenza virus A*, Natural Killer Cell, IL-15, IL-15Rα, Trafficking, Innate Immunity

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B.S., University of Georgia Athens, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in

Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2011

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December 2011

ACKNOWLEDGEMENTS

I wish to express appreciation to Dr. Kim Klonowski, my major professor, for her encouragement, suggestions, guidance, and patience. Special thanks to my committee members, Dr. Rick Tarleton and Dr. S. Mark Tompkins. I am also indebted to Kat Verbist, fellow student, for her support and reflections, and to Mary Field for all her time caring for our mice and to Dr. James Murray for all his reading and reflecting on the issues. Finally, special thanks to Laurie Jones (Mom) for a lifetime of believing and supporting.

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INTRODUCTION AND LITERATURE REVIEW

INFLUENZA A VIRUS

1.1 INTRODUCTION TO INFLUENZA

Infection with influenza virus is a major cause of disease worldwide. During seasonal influenza outbreaks, it is estimated that 5-15% of the world population is infected. According to the World Health Organization, 250,000 to 500,000 people die annually as a consequence of infection by seasonal influenza virus [1]. Despite our extensive knowledge of the influenza virus itself and the type of immunity resulting from influenza infection, the virus still eludes global eradication and is a serious detriment to human health and national economies. Moreover, at unpredictable intervals, a novel influenza A virus subtype can appear in humans and give rise to a worldwide influenza pandemic. This occurred three times during the 20th century, namely in 1918, 1957 and 1968 [1]. The 1918 pandemic had the highest mortality, causing approximately 40 million deaths worldwide, with an unprecedented number of deaths of young adults in their 20's or early 30's [2]. It was predicted that if a pandemic with similar associated mortality rates occurred today, the number of deaths could in a worst-case scenario exceed 350 million people [3].

In 1997, the transmission to humans of a highly pathogenic avian H5N1 influenza virus, which had a lethality rate of 33% [4,5], resulted in a renewed interest in influenza research and the development of new pandemic vaccine candidates. Subsequently, several avian influenza subtypes that infect humans have been documented, with a large outbreak in South East Asia [6].

The WHO declared that the recent H5N1 outbreaks in Asia met all but one requirement for pandemic classification, underlining the possible threat that the world is facing [1,7].

1.2 CLINICAL MANIFESTATIONS

Airborne-transmission of influenza virus results in the primary infection of the epithelial cells of the upper respiratory tract. The virus is transmitted via droplets expelled upon sneezing and coughing [8]. The incubation period is usually 2-3 days before onset of illness, but can be as long as 7 days. The patient is generally contagious after the onset of illness, but cases of viral spread have also been observed prior to symptoms. In general, the illness lasts approximately one week and is accompanied by high fever, headache, myalgia, sore throat and rhinitis. The severity of infection is correlated with the level of viral shedding; high levels of viral shedding are often found in people with more severe illness and higher-grade fever, whereas people with low levels of viral shedding have less clinical symptoms or are asymptomatic [9, 10]. Healthy adults usually recover within one week of bed rest without requiring any medical intervention. However, in the very young, the elderly and people with underlying medical problems (e.g. diabetes, cancer, neurological diseases, kidney, cardio or respiratory diseases) influenza poses a serious risk and infection may lead to hospitalization and in some cases death [11]. The cause of death can be the virus itself (viral pneumonia) or secondary infection (often bacterial pneumonia) as the cells of the epithelia are damaged by virus replication. In the United States, the estimated excess mortality during an annual influenza outbreak is up to 35,000 deaths per year [12].

During an influenza pandemic, infection spreads across the planet, increasing the chances that a greater number of deaths and a higher frequency of medical complications occur than in the yearly influenza outbreaks. While influenza virus replication is normally confined to the respiratory tract, viral isolates from the H5N1 outbreaks in 1997 [13] and 2003 [14] were shown to have

an unusually broad cellular tropism with virus detected in lungs, spleen, heart, brain and colon of diseased individuals [15].

1.3 THE INFLUENZA VIRUS

Influenza is a negative sense, RNA stranded virus belonging to the family *Orthomyxoviridae* [17]. The first virus was isolated in 1933 [18] and is referred to as A/Puerto Rico/33/8 or PR/8. There are three influenza genera, *Influenzavirus A*, *Influenzavirus B* and *Influenzavirus C*, which are divided on the basis of antigenic differences in the matrix (M) and nucleoprotein (NP) internal proteins. The three genera differ in epidemiology, host range and pathogenicity. Influenza A and B viruses are important human pathogens, although Influenza A is also found in a wide range of hosts, with waterfowl serving as the reservoir [20]. Influenza C infection typically results in a mild respiratory infection in humans. Influenza C viruses are only rarely isolated from individuals within a population, but by early adulthood 96% of the human population have antibodies directed against influenza C, indicating that infection with influenza C is common [19].

There are two integral viral proteins located in the viral envelope of Influenza A viruses: Haemagglutinin (HA), the most abundant protein in the viral envelope, and Neuraminidase (NA). Currently, there are 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes recognized by the WHO [21]. HA is a type I membrane protein consisting of 566 amino acids, with the C terminus acting as the anchor domain. The protein has two major functions in the viral life cycle. It binds to the receptor on the host cell surface, bringing the virion in close proximity to the cell membrane; it is also responsible for the fusion of the endocytosed virion with the endosomal membrane, allowing release of the viral genome into the host cell cytoplasm. HA is the major antigenic determinant and target of the humoral response, but it has a high mutation rate that helps the virus evade the humoral response. The receptor binding site, however, is highly conserved. For the receptor binding site, the amino acid identity is as high as 39% between influenza A and B and as high as 79% between influenza A subtypes [22].

The neuraminidase is the second most important antigenic determinant in the viral envelope. The protein consists of 453 residues; four identical monomers form the functional NA in the viral envelope. The enzymatic site is located directly over the β-sheets in the globular head, one site on each of the four monomers, and is specific for N-acetyl-neuraminic acid. NA is a class II membrane protein with the N terminus acting as a combined signal and anchor domain spanning the lipid bilayer. NA does not undergo proteolytic cleavage, but can be post-translationally modified by addition of carbohydrates [23]. The protein has four known antigenic sites [24]. The function of NA is to enzymatically remove sialic acid from the cell surface, thereby promoting release of the virion from the cell by preventing the virion from re-binding to the same cell [25]. It is also important in creating a pathway through the mucus layer in the respiratory tract, allowing the virus to gain access to the surface of the epithelial cell [25]. There is large variation in the sequence identity of NA between genera; the globular head of NA has only 30% sequence identity between influenza A and B viruses, whereas within a subtype the identity can be as high as 97% [26]. Currently, nine subtypes of influenza A NA have been identified based on seroreactivity of post infection sera.

1.4 ECONOMIC IMPACT

In the northern hemisphere the annual influenza outbreaks usually start during the winter months. In the tropics and subtropics influenza virus is isolated all year around. The number of suspected influenza cases in periods of known influenza spread, designated by the WHO as Influenza like illness (ILI), is a frequently used measure of epidemiological activity by international and national authorities [27]. The number of ILI cases reported by general practitioners (GP) with patients suffering from typical influenza symptoms and the number of ILI are a good estimate of the magnitude of circulating influenza [28]. The definition of ILI that corresponds best with laboratory confirmed influenza is a sudden onset of fever, cough and fatigue [29], but other case definitions are also used [30, 31].

Additionally, physicians may take nasal/throat samples from ILI patients, and in many countries centralized influenza centers isolate virus in order to identify the type, subtype (if appropriate), and strain of influenza virus circulating in the community. Based on the number of ILI and/or laboratory confirmed influenza infections, a designation of “No activity”, “Sporadic activity”, “Local outbreaks”, “Regional outbreaks” or “Widespread outbreaks” is used [1]. The term pandemic is only used when an antigenic shift occurs, creating a new influenza A subtype which then infects humans, leading to global widespread outbreaks which result in substantial morbidity and mortality [1].

Influenza related deaths are under-reported during an outbreak as influenza often exacerbates underlying disease [8]. The number of influenza related deaths is therefore often reported as the number of excess deaths compared to a period without known influenza spread. During a pandemic, as well as during the annual influenza outbreaks, an excess of morbidity and mortality is reported. People that die from influenza infection are usually from the “at risk” groups, primarily children, the elderly, and people with certain chronic medical conditions. The estimate for influenza related deaths worldwide is about 1 million people per year [12, 32]; in the United States about 60-70% of deaths occur in people above the age of sixty-five [33]. However, the total number of influenza related deaths worldwide is difficult to estimate, due to a lack of knowledge about influenza epidemics in developing countries [21]. The HIV epidemic in developing countries further obscures the situation. Thus the total impact of influenza deaths in developed countries is not known with great accuracy, as updated mortality rates are often not readily available.

During an influenza outbreak there is substantial morbidity; conservative estimates suggest an economic loss of 12 to 17 billion dollars in the United States alone during a single influenza season [9, 21]. Furthermore, influenza morbidity results in significant strain on health care systems. Morbidity after influenza infection has rarely been investigated in population studies, but an outbreak of influenza in Boston, Massachusetts in 1976 and 1977 resulted in an

estimated 37% absenteeism in school children during a 5-week period [34, 35]. Large retrospective cohort studies investigating hospitalization rates in the United States over twenty years found that otherwise healthy children under the age of five had a hospitalization rate similar to adults classified with a high risk of influenza complications [36, 33, 37], demonstrating the substantial impact of influenza infection in children.

1.5 PREVENTION AND PROPHYLAXSIS

The burden of annual influenza infection is substantial, both in terms of illness, loss of life, and economic impact on society [12, 32]. Additionally, threats of emerging illness in both developed and developing countries loom large. Therefore, improving immunity to current influenza strains through vaccination, as well as developing new antiviral drugs, are of vital importance. Continued and focused research efforts are needed in order to understand the immunology, epidemiology, ecology and the etiology of influenza viruses. Despite many years of studies, we still lack a complete understanding about influenza, the infection it causes, and the subsequent immune response.

The two main types of influenza vaccine used today are an inactivated subunit vaccine and a live, attenuated virus. The attenuated viral vaccine (trademarked as FluMist) is administered intranasally, and the virus is attenuated to only produce an upper respiratory tract infection, which does not cause any overt clinical illness. The viral strain used in the current vaccine needs to be of significant epidemiological importance, serologically matching circulating strains of virus that are present in the potential areas of vaccine administration. The vaccine currently produced provides satisfactory protection, with a protective effectiveness in healthy adults of 70-90 percent against laboratory confirmed influenza [38]; this vaccine is also cost effective [39]. However, there are still areas for improvement, especially in increasing the effectiveness in elderly populations where the current efficacy is not optimal.

The resulting immunity following vaccination varies depending on the type of vaccine used. With vaccines that utilize inactivated viruses, immunization of individuals that are immunological naïve, meaning that their immune system has not experienced that particular influenza subtype before, will mainly induce low titers of serum antibody [40] with little or no T-cell response. The resulting antibodies will be largely IgG, with some IgM; little IgA will result. Inactivated vaccines are efficacious in man, likely because most individuals have been exposed to several influenza viruses prior to vaccination, meaning that most individuals have immunological memory.

However, if a vaccine contains inactivated virus that is antigenically similar to the strains that generated the immunological memory during previous exposure, vaccination will activate memory B cell, resulting in a boost of antibody production [41, 42, 43, 44]. The systemic response will be faster after vaccination of primed individuals, with the cellular response peaking after a week; the serum antibody levels continue to increase for as long as twenty-one days after vaccination [45]. Because the main antigenic determinates are located on HA, most neutralizating serum antibodies will be directed against HA [46, 47]. Vaccination with inactivated virus prevents laboratory confirmed influenza in up to 70-90% of those who receive the vaccine, although in those under the age of five or over the age of sixty-five the vaccine provides lower rates of protection [38]. In years with a sub-optimal match between the vaccine strains, the vaccine is less effective in preventing illness, both in children [48] and young adults [49].

If there is no pre-existing immunity, like in a pandemic of a novel influenza subtype, one standard dose of inactivated vaccine is unlikely to confer adequate protection. Repeated vaccination with vaccines containing an adjuvant is often necessary to elicit a satisfactory immune response to a novel subtype [40, 50]. Rubens *et al.* demonstrated in 1973 that whole virus vaccines elicited rates of protection similar to split virus vaccines in protection against influenza illness, with a 69% reduced infection rate among vaccinated individuals compared to unvaccinated individuals [51]; numerous subsequent studies have shown that

whole virus is more immunogenic than split virus vaccine, especially in unprimed populations [52-54]. Annual immunization has been shown to not compromise the immune response, meaning there has been no demonstrated reason not to vaccinate annually, especially if multiple strains of virus are prevalent in the population [55].

1.6 THE IMMUNE RESPONSE TO INFLUENZA

The immune system can be divided into two interconnected parts: the innate and the adaptive system. The innate system responds generically to a broad range of pathogens and does not generate immunological memory. An important secondary function of the innate immune system is to signal the activation of the adaptive response and to slow pathogen replication until the adaptive immune system is activated and fully functional. The adaptive response of the immune system has the capacity to selectively identify a particular antigen and to produce a long-lived pool of memory cells.

Several mechanisms are in place to recognize pathogens at mucosal surfaces. The first line of defense against all respiratory pathogens is an intact mucosal membrane with cilia and mucosal secretions in the lungs removing foreign particles. Another potent defense system against influenza infection is the induction of type I interferons. Type I interferons are known for their strong antiviral effects. During an influenza infection, single-stranded viral RNA are present in infected cells [133]. Multiple pattern recognition receptors (PRRs) are reported to play an important role in sensing the single-stranded viral RNA [134, 135]. Following the detection of pathogen, PRRs initiate the transcription factors responsible for the production of soluble type I interferon [137]. Secreted IFNα/β can then bind to its ubiquitously expressed receptor, IFNα/β receptor (IFNAR), that, in turn, initiate signal transduction via phosphorylation of Janus kinases (JAKs) which in turn phosphorylate signal transducers and activators of transcription (STATs) [74]. Upon phosphorylation, STAT molecules, along with interferon response factors (IRFs), bind to each other, translocate to the nucleus

and begin to encode proteins that mediate the antiviral response. These defensive proteins include the cellular enzymes, protein kinase R (PKR), which combats viral infections by destroying RNA and reducing protein synthesis in the cell [161]. Also, IFN signaling limits viral spread by increasing p53 activity, which kills virus-infected cells by promoting apoptosis [162]. Another function of interferon is upregulation MHC molecules. Higher MHC expression increases presentation of viral peptides to T cells thereby increasing the recognition and killing of infection cells. It is this IFNα/β-induced anti-viral state that initiates the early response of the innate immune system to control viral spread by slowing viral replication, initiating the presentation of pathogenic peptide to and activating the innate immune response, and initiating programmed cell death of infected cells.

The importance of IFNα/β signaling for controlling influenza infection has been demonstrated in knockout mouse models. Mice lacking genes important for IFNα/β signal transduction are more susceptible to infection [137]. Also, this important role is demonstrated by the fact that many pathogenic viruses including influenza virus possess antagonistic proteins that hinder the protective effect of IFN and allow establishment of infection [163]. The viral nonstructural protein 1 (NS1) is a potent virulence factor for influenza A viruses. It is implicated in "masking" viral presence and inhibition of immunity via multiple mechanisms [164]. NS1 binds to RIG-I/IPS-1 complexes and blocks downstream signaling [165,166], resulting in attenuation of type I IFN expression. The activities of NS1 protein result in diminished activation of DCs, blocked cytokine expression, and diminished T-cell activation, thus counteracting both innate and adaptive immune responses [167].

NATURAL KILLER CELLS

Natural killer (NK) cells are involved in the early antiviral response. NK cells are a subtype of lymphocytes that can be distinguished by their large size and the presence of granules in their cytoplasm [61]. They are widely distributed

throughout the body and can be found in peripheral blood, spleen, lung, liver, bone marrow and the lymph nodes. The recognition strategies used by NK cells are diverse; NK cells recognize both tightly regulated self-proteins on the surface of all cell types and viral proteins [60].

One of the earliest recognized functions of NK cells is their ability to kill tumor or virus-infected cells. In contrast to other killer lymphocytes such as the cytotoxic T-lymphocytes (CTLs), NK cells do not need previous encounter and antigen-specific priming in order to develop their cytotoxic response [62]. Shortly after activation, NK cells deploy their cytotoxic machinery, mostly made of combinations of perforin, a pore forming protein, and granzymes, a serine protease [63] stored in the intracellular granules, in order to destroy target cells. Moreover, NK cells also modulate other immune functions by releasing various cytokines and chemokines, including IFN-γ, and other molecules like tumor necrosis factor-alpha (TNF-α), macrophage inflammatory protein 1a (MIP-1a), macrophage inflammatory protein 1b (MIP-1b), and RANTES [64]. The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly, and most importantly from its immunostimulatory and immunomodulatory effects. IFN-γ production by NK cells augments antigen presentation by macrophages to enhance the downstream adaptive immunity to viral infection [65].

In 1986, Karre observed that NK cells were able to kill tumor cells [66]. This observed cytotoxicity was correlated to the level of MHC-class I expressed by the target cells. The less MHC-class I expression, the more the cells were targeted for NK-mediated destruction. This new phenomenon, which differed from previously observed MHC-class I-dependent destruction [67], led to a new concept of “missing-self” in which NK cells play a leading role. Because many viruses have evolved strategies to interfere with the host’s MHC-class I and II expression, allowing the viruses to evade adaptive T-cells immune responses [68], NK cells are an important player in the anti-viral immune responses as they can provide a complementary defense strategy by specifically targeting those

cells that evade adaptive T-cell responses. Importantly, NK cells are vital in limiting viral replication as depletion of NK cells dramatically increases morbidity and mortality in hamsters and mice [183].

The recognition or sensing of pathogen presence is more complex than that described by the missing self hypothesis. A culmination of studies over the last several years have determined that the ability of NK cells to sense and trigger downstream responses is finely orchestrated by the integration of opposing signals coming from activating and inhibitory receptors [69]. The summation of the activating and inhibitory signals determines whether NK cell will trigger the killing of target cells or enhance cytokine secretion. However, there are multiple pathways that can activate NK cells in response to infection both directly and indirectly. As discussed, NK killing can be inhibited by MHC class I molecules expressed on the cell surface of normal, uninfected cells and by inhibitory cytokines such as TGFβ.

NK cells can be directly stimulated by positive signals through activating receptors that either recognize specific self or pathogen-specific proteins. NKG2D is a C-type lectin-like receptor and is the best characterized [52]. A number of NKG2D target ligands have been identified. The most intriguing of these are a pair of closely related proteins called MICA and MICB (major histocompatibility complex (MHC) class I chain-related) [181,182]. In addition, NKG2D is required for NK cell-mediated cytolysis on virus-infected targets. Efficient NK cell activation and function in response to viral infection is critically dependent on the NKG2D pathway.

Alternatively, NK cells express activating receptors that are unique to NK cells and recognize non-MHC I molecules. These receptors are known as the natural cytotoxicity receptors (NCRs) [71]. NK cell activation requires recognition of influenza-induced ligands on the infected cells by both NKG2D and the NCR, NKp46. Unlike the NKG2D receptor, which recognizes to self-proteins, the NKp46 receptor's specific ligand is specific HA molecules of distinct influenza strains [171,172]. The first evidence of NCR-influenza virus interaction was

published in 2001 in experiments demonstrating that when HA was blocked using monoclonal antibodies, lysis of target NK cells was significantly reduced [74]. The interaction of NCRs with their targets were further demonstrated using a human Ig-recombinant NCR fusion protein (NKp46) [76]. Since many viral families express HA, this mechanism may explain how NK cell can kill a broad range of viruses using a limited repertoire of lysis receptors. NKp46 knockout mice have been generated and 60% of the WT homozygous and heterozygous mice survived the influenza virus infection, but none of the homozygous NKp46 -/- littermates were able to survive [77].

In addition to the important role of NK cells in murine models of influenza infection, NK cells are also an important component in the human response to influenza infection [185]. It has been shown that acute influenza infection readily activates NK cells in humans. Also, severe infection with the 2009 pandemic H1N1 virus positively correlated with reduced numbers of NK cells in the lungs [184].

T CELLS

The adaptive immune response, which induces long-lived memory, consists of two interlinked parts, the humoral and the cell-mediated immune system. The cell-mediated immune response consists of two main cell lineages, CD4 and CD8 positive T-cells. The main function CD4+ T-cells or T-helper (Th) cells is regulation and control of the immune system. After recognizing an antigen, Th cells begin to divide and give rise to effector cells, whose main task is to secrete cytokines. Based on the cytokines they secrete, Th cells are divided into two subsets responsible for the effector functions, Th1 and Th2 [80], and several subsets responsible for regulation [81]. The major Th2 cytokines in mice are IL-4, IL-5, IL-6 and IL-10, which stimulate B-cells to produce antibodies, inducing a humoral immune response [80, 81]. A Th1 response induces a different cytokine profile with the most important cytokines being IL-2 and IFN-γ [80, 81]. An important function of INF-γ is to increase the expression of FcR [82].

It also up-regulates the poly immunoglobulin receptor (pIgR) expression [83] and activates cytotoxic T lymphocytes [81]. After Th cells have been activated by antigen, long-lived Th memory cells are produced and maintained [84, 85].

Addition of adjuvants that shift the Th response after immunization have been reported, but have not yet reached clinical use in humans [86, 87]. CD8+ T-cells and macrophages are activated and supported by Th1 cells [88]. After an antigen is presented by MHC I and recognized by the TcR, the activated CD8+ cell differentiates into a CTL. The mechanism of cell lysis has been described as two-fold [89]. The first is the perforin/granzyme mediated pathway, which is similar to but not identical to that of NK cells; in this pathway, CTLs release perforin which forms a pore in the target membrane, allowing the granzyme pass into the target cell and causing cell death. Perforin activity has been shown to be important in protection from influenza infection as knockout mice for perforin show an increased susceptibility to influenza infection coupled with prolonged viral shedding [90]. The second mechanism of cell lysis involves the Fas pathway, which leads to the recruitment of Fas associated death domain (FADD) and the subsequent induction of apoptosis [7]. Following activation, some of the effector CTLs differentiate into memory CD8+ [84, 13]. These memory cells can be reactivated if the same antigen is re-encountered. This reactivation will give rise to a faster immune response with a subsequent faster elimination of the antigen.

B CELLS

The humoral immune system consists of B cells that produce and secrete antibodies. After encountering an antigen, B cells differentiate into plasma cells and memory cells, a process that is aided by cytokines produced by Th cells. Activated B cells secrete antibodies, even before they fully differentiate into plasma cells [91]. While fully differentiated plasma cells are normally short-lived and secrete large quantities of antibody, up to several thousand antibodies per

second [92], a subset of long-lived plasma cell migrates to the bone marrow and contributes to the serum antibody pool [93].

Influenza virus undergoes substantial antigenic drift allowing the virus to escape the host's immune response. The host's immunological defenses against viral pathogens are multifaceted and involve a range of antiviral mechanisms. The primary target for influenza virus is the respiratory tract, which is also the site of the initiation of immune response against influenza, with secretory IgA, CTL, and non-specific innate immunity responses.

Mucosal immunity is not an independent part of the immune system but a function of the innate, with humoral and cell-mediated responses acting in concert. The mucosa is very important in viral defense; many major viruses, including the influenza virus, rotavirus, corona virus, human immunodeficiency virus (HIV) and measles virus, enter the body via the mucosal route. Immunity to mucosal viruses is complex, with a range of complementary and compensatory functions. It is believed that because of the difficulty of maintaining a very high level of mucosal immunity over long periods of time, the major function of the mucosal immune system may be to reduce the severity of infection by aiding the clearance of virus [100]. Th1 cells will secrete cytokines in a process called delayed type hypersensitivity (DTH); this includes IFN-γ, which induces an antiviral state in the epithelial cell layer and recruits the cells of the immune system.

The mucosal immune response will include local mucosal IgA and CTLs, as well as systemic IgG and IgM [101]. The resulting immunological memory will consequently be largely memory IgA B cells, with some memory IgG B cells; these memory cells are directed against the main antigenic epitopes on HA and NA. The cytotoxic T cell response, which is important in viral clearance and recovery from infection, is mainly directed against the internal viral proteins, NP and M1 protein [102, 103]. The internal influenza proteins are more conserved than the surface proteins, thus the memory T cells may be more cross-reactive against viruses which have undergone antigenic drift and possibly against

multiple influenza A subtypes. However, in the murine model, there is still conflicting data concerning what provides immunity against lethal challenge [104]. In humans, viral infection usually results in long-lived immunity [84]. This was seen after the re-appearance of H1N1 in 1977. H1N1 had not been circulating for 20 years [105], but people 30-35 years old had antibodies that reacted against the 1977 H1N1 influenza virus [106], likely as the result of an early childhood infection with an antigenically similar virus; the 1977 H1N1 was found to be antigenically similar to a 1950 isolate [107]. People under the age of twenty were almost exclusively infected [108], and young adults also had a markedly lower immune response to inactivated vaccines containing H1N1, suggesting that they did not have any immunological memory against the virus [40].

1.7 INTERLEUKIN 15 AND INTERLEUKIN-15 RECEPTOR

Interleukin-15 is a 14-15 kDa membrane-associated cytokine from the common gamma chain family of cytokines. Many cell types, including epithelial cells, activated dendritic cells, and stromal cells, produce IL-15. Although IL-15 mRNA is constitutively expressed in many tissues, IL-15 protein is not easily detected in normal mice and humans [111]. IL-15 binds and signals via a trimeric receptor composed of the γc chain, the IL-2/15Rβ chain (CD122), and a high-affinity α chain that binds to IL-15 [112]. In the absence of IL-15Rα, IL-15 can bind to the γc and IL-2/15Rβ chains with intermediate affinity and transduce signals through these subunits [113]. It is important to note that recent studies have indicated that cells do not require IL-15Rα expression to receive IL-15-mediated signals [114]. A transpresentation model for IL-15 was proposed that demonstrated that IL-15Rα can bind IL-15 alone and subsequently transpresent this cytokine to adjacent cells [115]. Thus, cells expressing the γc and β chains but lacking the IL-15Rα chain are still able to receive IL-15 signals [114, 116].

IL-15 plays a major role in the development and maintenance of NK cells. IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice have profound defects in NK cell numbers [117, 118]. In fact, NK cells are nearly absent in IL-15$^{-/-}$ animals [138]. Alternatively, IL-15 transgenic mice have increased numbers of NK cells [119]. The underlying mechanisms involved in the effect of IL-15 during NK cell ontogeny entail transpresentation of IL-15 by bone marrow stromal cells to NK cell precursors [120]. Transpresentation of IL-15 promotes NK cell survival and induces the developmentally regulated expression of NK cell receptors in mice [121] and humans [122]. The cytokine supports survival of NK cells through the induction of Bcl-2 [123 -125] and stimulates NK cell proliferation [126,127–129,130].

Additionally, IL-15 has also been shown to be important for NK cell activation. Following infection, dendritic cells produce IL-15, which results in the activation and proliferation of NK cells [139]. Furthermore, transient systemic stimulation of NK cells with soluble IL-15/IL-15Rα complexes also results in an accumulation of phenotypically and functionally mature NK cells [141,142]. IL-15 can stimulate the cytotoxicity of NK cells against tumor cells [131] and upregulate the expression of receptors such as NKG2D, which might facilitate target cell recognition and subsequent activation of effector functions [132]. Conversely, IL-15 alone is a poor inducer of cytokine production by NK cells [133].

IL-15 can also stimulate the migration of NK cells *in vitro* [143]. Allavena et al. demonstrated that treating NK cells with IL-15 primarily stimulated LFA-1-dependent adhesion to endothelial cells. Furthermore, utilizing a computer-assisted chemotaxis assay, this group showed that NK cells chemoattract to a positive IL-15 gradient [180]. By increasing NK cell adhesion to vascular endothelium and migratory response, IL-15 may be an important determinant of NK cell recruitment into tissues. Since IL-15 has been shown to regulate aspects of NK cell migration and our recent data demonstrates the chemotactic potential of infection-induced IL-15 on the trafficking of other IL-15-sensitive populations of lymphocytes, we hypothesize that NK cells are dependent on IL-15/IL-15Rα signaling for recruitment to the lung airways after influenza infection.

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CHAPTER 2

INTRODUCTION

Influenza spreads around the world in seasonal epidemics, resulting in the deaths of between 250,000 and 500,000 people annually [1], with millions perishing in some pandemic years. Despite this, the current influenza vaccines are not completely protective and are subject to failure due to antigenic shift and drift; modern vaccines are therefore not completely reliable. Thus, alternative strategies of immune intervention might be used to improve current vaccine strategies.

It has been demonstrated that NK cells are important for timely clearance of influenza virus. In NK cell deficient mice, influenza viral titers are higher and the adaptive immune response is delayed compared to WT mice [2] and humans [22]. Since, NK cells are known for their rapid, non-specific response to viral infections in the mucosa. Augmentation of the timely, broad NK cell response could be a viable option for inducing a rapid immune response against newly emerging strains of influenza. Thus, understanding the mechanisms controlling NK cell migration might elucidate methods that could be used to improve the innate immune response through targeted mobilization of NK cells to respiratory tract.

The molecular basis responsible for NK cell recruitment into tissues is not completely known. NK cells migrate in response to classical chemotactic factors [176, 177] and to certain cytokines [e.g. tumor necrosis factor (TNF), IL-2, and IL-12 , and to some chemokines, including MCP-1, -2, and -3, RANTES, MIP-I and IL-8. In addition, NK cells express many known adhesion molecules and are able to actively transmigrate across endothelial monolayers [178]. The adhesive capacity of NK cells to endothelial cells can be modulated by various cytokines. For instance, IL-2 and IL-12 increases NK cell adhesion to endothelial cells, whereas IL-4 has inhibitory activity [179].

Important to the work presented in this thesis, IL-15 can also stimulate the migration of NK cells *in vitro* [143]. Allavena et al. demonstrated that treating NK cells with IL-15 primarily stimulated LFA-1-dependent adhesion to endothelial cells. Furthermore, utilizing a computer-assisted chemotaxis assay, this group showed that NK cells chemoattract to a positive IL-15 gradient [180]. By increasing NK cell adhesion to vascular endothelium and migratory response, IL-15 may be an important determinant of NK cell recruitment into tissues. Thus, while many previous studies have shown that IL-15 is chemotactic for NK cells in vitro [3], studies demonstrating the chemotactic functions of IL-15 in disease models are lacking. Since IL-15 could play a role in NK cell recruitment to the lung airways to promote the inhibition of early viral replication, it holds great promise as an adjuvant not only in anti-influenza responses but also in tumor immunotherapy. We hypothesize that NK cells are dependent on direct IL-15/IL-15Rα signaling for recruitment to the lung airways after influenza infection and that modulation of this interaction could be used to enhance immunity at mucosal surfaces.

MATERIALS AND METHODS

2.1 MICE, VIRUS, AND INFECTION

C57BL/6 CD45.1 wild type (WT) mice were obtained from Charles River through the NCI program (Bethesda, MD) or Taconic Farms (Germantown, NY), and IL-15$^{-/-}$ mice were generously provided by Dr. Leo Lefrancois (University of Connecticut, Farmington, CT) or obtained from Taconic Farms. Influenza viruses A/HongKong(HK)-x31(x31, H3N2) and A/Puerto Rico 8 (PR8, H1N1) were generously provided by Dr. S. Mark Tompkins (University of Georgia, Athens, GA). Age and sex-matched groups of animals were infected with either 10^3 PFU x31 or 5 x 10^3 PFU PR8 intranasally (i.n.) in 50 µl of PBS.

2.2 TISSUE PREPARATION AND FLOW CYTOMETRY

Single-cell suspensions were obtained from spleens and lymph nodes by passing homogenized organs through cell strainers. Spleens were depleted of erythrocytes using Tris-buffered ammonium chloride. Lung airway-resident cells were harvested after intratracheal introduction and recovery of 1 ml PBS three to five times. After bronchoalveolar lavage (BAL) recovery, lymphocytes were isolated from the perfused lung parenchyma. After perfusion with ~25 ml PBS/heparin, lungs were excised, minced, and incubated at 37˚C with 1.25 mM EDTA for 30 min followed by 6 mg/ml collagenase for 60 min at 37˚C. After passage through cell strainers, cells were resuspended in 44% Percoll underlaid with 67%, centrifuged, and lymphocytes at the interface were collected.

For flow cytometry, antibody staining was conducted at 4°C for 1 h using mAbs from eBioscience (PerCP/Cy5.5-conjugated αCD4 or αCD8, and PE/ Cy7-conjugated αCD44 or αCD45.1, αNK1.1), BD Pharmingen (San Diego, CA; PE/ Cy7-conjugated αCD11a and allophycocyanin/Cy7-conjugated αCD8), or Caltag Laboratories (Burlingame, CA; FITC-conjugated αCD122). Stained cells were analyzed using a BD LSR II digital flow cytometer (BD Biosciences, San Jose, CA) and BD FACSDiva software.

2.3 QUANTITATIVE RT-PCR

Total RNA was purified from tissues of naive or 10^3 PFU x31-infected C57BL/6 mice using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Reverse transcriptions were primed with random primers and performed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Quantitative PCR assays were done using the ABI TaqMan Gene Expression Master Mix from ABI 7500 Real Time PCR System (Applied Biosystems). Only ratios with an SE < 0.2 log (95% confidence limits) were considered for determination of induction levels. Quantitative real-time RT-PCR was performed using IL-15–FAM (#mm00434210_m1) and 18s-VIC (#4319413E) assays (Applied Biosystems). Thermal cycling conditions were 30 min at 48˚C,

10 min at 95˚C, and 40 cycles of denaturation (95˚C for 15 s) and annealing (60˚C for 60 s). Samples were analyzed in triplicate, normalized against 18s, and expressed relative to mock-infected animals. The results are expressed as fold induction of gene expression (relative quantity of template) using the Dcycle threshold method (5).

2.4 ENRICHMENT OF NATURAL KILLER CELLS AND ADOPTIVE TRANSFER

Single-cell suspensions from spleens and all visible lymph nodes (cervical, axillary, mesenteric, and inguinal) were depleted of erythrocytes, washed, and stained with anti-PE-CD3, anti–CD8-PerCP, anti-NK1.1 and Fc block for 1 h at room temperature in 1 ml FACS buffer. Cells were subsequently washed, resuspended in 500 ml MACS buffer (PBS with 0.1% NaN3, 0.5% BSA, and 2 mM EDTA, degassed), and labeled with 50 ml anti-PE microbeads (Miltenyi Biotec, Auburn, CA) for 20 min at 4˚C. Cells were again washed and passed over a magnetized LS column (Miltenyi Biotech). Bound cells were eluted from the columns and discarded. The remaining population of cells was stained with a PE/Cy7-labeled dump gate (including anti-CD4, CD19, and CD11c). The frequency and number of NK cells in each cohort of animals was determined by gating on CD3-, NK1.1+, dump$^-$ lymphocytes.

NK cells were enriched to 90% purity. NK cells were enriched from only the spleen C57BL/6 CD45.1$^+$ WT mice. After enrichment, 9×10^4 purified NK cells were injected intravenously, via the lateral tail vein in 200 µl of PBS, into each CD45.2 recipient.

2.5 MIGRATION ASSAYS

For in vitro migration assays, cells from the indicated tissues were placed in the top insert of a 0.4-mm chemotaxis Transwell (Fisher Scientific, Waltham, MA). The bottom chamber contained either warm media alone or supplemented with 100 ng/ml recombinant murine IL-15–IL-15Rα Fc–chimeric complexes (IL-15c) [6] (R&D Systems, Minneapolis, MN). Percent migration was calculated as

number of NK1.1+ NK cells in the bottom chamber divided by number of NK 1.1 cells in the input sample. For in vivo migration assays, IL-15c consisting of 1.5 mg recombinant murine IL-15 and 7 mg IL-15Rα Fc-chimera (R&D Systems) were generated by incubation at 37˚C for 20 min followed by incubation at 4˚C for 10 min and delivered i.n. in 72.5 ml PBS.

2.6 PLAQUE ASSAYS

Plaque assays were performed as previously described (7). In brief, lungs from x31-immune WT and IL-15-/- mice challenged with PR8 were lysed with a TissueLyser at the indicated times in 1 ml MEM+ 1 mg/ml TPCK- treated trypsin. Confluent monolayers of Madin-Darby canine kidney cells were incubated with 10-fold serially diluted 10% homogenate for 1 h at 37˚C. The inocula were removed and cells were washed with PBS. Monolayers were then overlaid with MEM containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA), 0.04 M HEPES, 0.02 mM L-glutamine, 0.15% NaHCO3 (w/v), and 1 µg/ml TPCK-trypsin. Seventy-two hours p.i., monolayers were fixed with cold methanol/acetone (60:40%) and stained with crystal violet.

2.7 STATISTICS

Where appropriate, an unpaired two-tailed Student t test was applied using Prism GraphPad software (GraphPad, San Diego, CA). The p values are indicated in the figure legends where statistical significance was found. For multiple comparisons, an ANOVA was applied with Tukey's post hoc analysis using Prism GraphPad software. The p values (indicated with a *) less than 0.05 were considered significant.

RESULTS

To first examine the relationship between NK cells and IL-15, we asked whether influenza infection in the lung airways correlates with a modulation in expression of IL-15 in the local mucosa. IL-15 mRNA is constitutively expressed throughout the entire body [8] and IL-15 protein expression has been shown to be produced by DCs, macrophages and mucosal epithelial cells [9,10,11]. Although IL-15 expression regulation is complex and broad, cellular expression of IL-15 has been shown to be regulated by multiple anti-viral factors [12]; for example, the IL-15 promoter contains a type I IFN upstream regulatory element [13, 12] and IL-15 expression can increase in response to type I IFNs [14], which are abundant after influenza infection [15]. In order to determine whether respiratory infection with influenza virus modulates IL-15 expression in the lung and lung airways, we infected C57BL/6 WT mice with influenza A/Hong Kong x31 (HKx31) and monitored IL-15 mRNA expression in the lung airways via bronchial-alveolar lavage (BAL) fluid, lung parenchyma, lung-draining mediastinal lymph nodes (MdLNs), and spleens. Beginning at day three post-infection, there was a 4-fold increase in the relative expression of IL-15 in the lung airways of infected animals when compared with mock-infected animals and IL-15 mRNA levels remained increased until day 7 post-infection. (Fig.1). Although IL-15 transcription in the airways began to decline by day 10 p.i., the levels remained three times higher than the levels in naive mice. Expression of IL-15 also increased to a lesser degree in the lung parenchyma and spleen after infection. Subsequent to these studies, our group has also demonstrated that IL-15c levels in lung serum drastically increase upon influenza infection [16]. Together, these data suggest the inflammatory response initiated by influenza infection causes a local increase in the expression of IL-15, therefore suggesting that this cytokine may regulate early immune responses to influenza infection.

NK cells are one of the first populations of cells recruited to the lung airways following influenza infection. The early response of NK cells plays a crucial role in

the initial control of viral spread by killing infected cells and directly signaling and activating the adaptive immune response. The majority of biological effects of IL-15 on NK cells are mediated through the expression of two receptor subunits, CD122 and CD132 [17,18,19], while IL-15Rα is only required by accessory cells which transpresent IL-15. It has been suggested that IL-15Rα expression alone contains some signaling moieties, and therefore, may participate in distinct biological functions. Therefore, in order to evaluate the IL-15 signaling potential in our model, it is important to determine whether responding NK cells express each of the three IL-15 receptor components. To demonstrate that NK cells migrating into the lung airways express components of IL-15R and are thus sensitive to IL-15 mediated signals in situ, lymphocytes were collected from the lung airways of x31-infected WT mice at the various times post infection. NK cells were identified as $CD3^-$ $NK1.1^+$ lymphocytes and the cells were analyzed via flow cytometry for each of IL-15 receptor's three subunits: IL-15Rα, CD132, and CD122 (Fig. 2). At day two p.i., around 20% of these cells expressed IL-15Rα, and 30-40% of them expressed CD122 and CD132. By day 3 p.i., greater than 90% of these cells expressed CD122 and CD132. Expression of IL-15Rα however, was variable but consistently much lower that the expression levels of CD122 and CD132. Therefore, responding NK cells are capable of responding to locally produced IL-15 through their expression of CD122 and CD132 IL-15R subunits. This low level of IL-15Rα coupled with an increase in CD122 and CD132 expression suggests that IL-15-mediated effects on the NK cell response to influenza are via the IL-15 transpresentation route.

In other IL-15 sensitive cells, specifically $CD8^+$ T effector cells, IL-15 has been shown to recruit the cells to the lungs after influenza infection [6]. Thus, it is quite possible that NK cells might also be recruited to the lung airways following influenza infection in an IL-15-dependent fashion. Indeed, IL-15-mediated NK cell chemotaxis has been demonstrated in vitro [17]. Moreover, the transient augmentation of IL-15 mRNA expression immediately preceding the documented influx of NK cells into the lung airways supports our theory that IL-15 plays a role

in NK cell recruitment. To test our hypothesis that IL-15 could also participate in the recruitment of the NK cells to the site of infection, IL-15$^{-/-}$ and WT animals were infected i.n. with x31 and the kinetics of the NK cell response were assessed. Because IL-15$^{-/-}$ animals have a severe developmental deficiency in NK cells compared to WT animals, we also treated WT mice with IL-15 blocking antibody throughout the infection to assess any recruitment deficiencies in IL-15 ablated models. The lung airways of IL-15$^{-/-}$ or IL-15-blocked mice had a significantly reduced population of responding NK cells by day 4 p.i. (Fig. 3A, B); a 20-fold reduction in the frequency of NK cells in the lung airways was observed as early as day 3 (Fig. 3A). Additionally, the total number of NK cells recovered from the airways of IL-15$^{-/-}$ or IL-15-blocked at day 4 p.i. was assessed. The initial NK cell numbers in IL-15$^{-/-}$ mice were below a reliable limit of detection. The loss of NK cells in the airways of IL-15-blocked mice was greater than a 50-fold numerical reduction compared to the total number of NK cells in IL-15 competent mice. The frequency of NK cells was also reduced in the lung parenchyma of IL-15-blocked animals at day 4 p.i., though not to significant levels. Interestingly, fewer NK migrated to the lung parenchyma in the IL-15$^{-/-}$ mice compared to the IL-15-blocked mice. This could be due to a differential requirement for IL-15 in the development of specific NK cell subsets [21], which may be both absent in the IL-15 knockout mice and particularly dependent on IL-15 for migration to the lung. Thus, an IL-15 blockade resulted in a specific reduction in the number of influenza-responding NK cells at the site of infection.

Because NK cells are known to be important for efficient influenza clearance, plaque assays were used to determine how IL-15 deficiency and IL-15 antibody blockade affected viral loads. To test the quality of this response, we challenged WT and IL-15$^{-/-}$ animals with HKx-31; the animals then received intranasal treatments of either PBS or IL-15 blocking antibody every day for three days. On indicated days p.i., lung viral titers were determined by plaque assay (n = 3 mice/group). At the earliest time (day 3) p.i., the lungs of WT mice contained around four times less virus than those of IL-15$^{-/-}$ mice, with the lungs of IL-15-

blocked animals containing the highest viral load (Fig. 4). IL-15$^{-/-}$ and IL-15 blocked mice did not begin to reduce the viral load until day 4 p.i. The IL-15 deficiency or blockade decreased the levels NK cells isolated from the site of infection which inversely correlated with lung viral titers, indicating that in the absence of IL-15 control of the virus is severely impaired.

It has been previously shown that recombinant IL-15 recruited NK cells in vitro [20]; however, it is unclear how NK cells from different tissue reservoirs are affected by soluble IL-15c. To distinguish between these possibilities, the direct effects of IL-15c on NK cell migration were monitored in an in vitro chemotaxis assay. Single-cell suspensions from the lung parenchyma, BAL fluid, and spleen of day 2 x31-infected animals were placed in the top chamber of a transwell chemotaxis chamber, with media or media supplemented with IL-15c placed in the bottom (Fig. 5A). After 90-min incubation, cells in the bottom chamber were collected, counted, and the percentage of NK cells that had migrated toward the IL-15c was determined. Although NK cells isolated from both draining lymph nodes migrated fairly efficiently toward the IL-15c (data not shown), the NK cells isolated from the lung parenchyma and spleen exhibited a marked increase in migration to IL-15c (Fig. 5B). However, the NK cells isolated from the BAL fluid migrated poorly toward IL-15c; exhibiting only a slight increase in migration over those stimulated with PBS. These data demonstrate that exogenous IL-15 results in increased numbers of NK cells from the spleen and lung parenchyma to the lung airways. Therefore, we hypothesized that IL-15 may be responsible for a substantial amount of migration of NK cells into the lung airways following influenza infection.

IL-15 deficient models show reduced the numbers of NK cells in the lung airways resulting in increased viral titers. Conversely, exogenous IL-15c promoted the migration of NK cells and resulted in increased numbers of responding NK cells. We therefore hypothesized that IL-15c treatment could be used to enhance the early innate immune response to influenza and augment viral control. Because IL-15$^{-/-}$ mice do not have adequate populations of NK cells

to respond to an influenza challenge, we moved to an adoptive transfer system to assess whether IL-15c treatment could reverse the blocked NK cell migration in IL-15$^{-/-}$ recipient mice. This experimental design also allowed the detection of responding NK cells from one anatomical location preferentially migrating to IL-15c in vivo. Donor NK cells were enriched from the spleen of x31-infected CD45.1+ WT donor mice 2 days post infection and transferred into WT or IL-15$^{-/-}$ congenic CD45.2+ recipients that were 2 d p.i. with x31 (Fig. 6A). At the time of adoptive transfer, recipient animals were given either IL-15c or PBS i.n.; 12 h later, the BAL fluid from these animals was collected and analyzed for the presence of donor-derived NK cells. Donor NK cells were detected in the BAL fluid of IL-15c–treated animals only, confirming that IL-15 is capable of inducing the migration of NK cells in vivo (Fig. 6B). These data indicate that reservoir NK cells are dependent on IL-15 for their migration into the lung and lung airways and that donor NK cells can be homed to an infected site via exogenous, local IL-15 recruitment. This is not the case for migration to the spleen.

Since exogenous IL-15 recruits NK cells to the site of infection in vivo and IL-15 recruits NK cells to the lung airways, we asked whether adding an IL-15 adjuvant to the respiratory mucosa could numerically boost the NK cell response, which could used to non-specially control an infection and reduce morbidity. To determine if IL-15c as an adjuvant increases the number of responding NK cells, WT mice infected with x31 were given IL-15c or PBS i.n. between day 1 and 2 p.i. (Fig 7A). At day 2 p.i., cells from the lung airways and other indicated tissues were collected, counted, and analyzed for the presence of NK cells. Animals receiving IL-15c retained a greater frequency and number of NK cells in their lung airways compared with animals receiving PBS alone (Fig. 7B). Taken together, these results indicate that by altering the dynamics of the NK cell response, IL-15c enhanced responding NK cell migration to the lung airways. These data reveal a direct role for IL-15 in NK cell recruitment to influenza infected lung mucosa and reveal a potential avenue for improved non-specific control of an ever-evolving virus.

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CHAPTER 3

DISCUSSION

The role of IL-15 on NK cell proliferation and survival has been well documented. It has been shown previously with in vitro migration assays that NK cells can migrate toward IL-15 [1]. However, only recently has work focused on a potential role of IL-15 as a chemoattractant of lymphocytes, particularly in response to infection [2].

In this study we investigated the potential chemotactic properties of IL-15 on NK cells. We have shown that upon influenza infection, IL-15 expression levels are increased early in the lung airways, the site of infection [2]. Our findings indicated that while a quarter of the responding NK cells express IL-15Rα, the majority express CD122 and CD132, demonstrating NK cell receptivity to IL-15 via the transpresentation route. We next sought to further our understanding of IL-15's role on NK cell migration during an influenza infection in a mouse model. In IL-15-ablated mice, we noted a decrease in the immigrating NK cells in the lung airways, with the IL-15$^{-/-}$ mice showing the greatest deficiency in the rate of influxing NK cells. We also examined the effect of IL-15 deficiency on viral loads at day three p.i., which closely coincides with the peak of the NK cell response. Indeed, in WT mice, viral loads were significantly lower than in IL-15$^{-/-}$ mice. Taken together, these results suggest that IL-15 functions as a chemoattractant, signaling the migration of NK cells to the lung airways soon after an influenza infection and the enhanced presence of these lymphocytes improves viral control.

Whether or not IL-15 directly or indirectly is important for NK cell migration is still unclear. The in vitro transwell migration assays suggest that this is a direct effect. Moreover, responding lung airway NK cells were not chemotactic to IL-15 in vitro despite expression of CD122 and CD132. This could possibly suggest that this specific anatomical subset of NK cells is less susceptible to IL-15 signals while emigrating lung and spleen NK cells are IL-15 dependent. Nonetheless,

these assays revealed that blood-borne NK cells can respond directly to IL-15 and migrate toward the lung airways in a chemotactic manner.

It has been shown that IL-15 is constitutively expressed throughout the body being produced by a variety of cell types, including dendritic cells, stromal cells, endothelial cells, and epithelial cells [6]. Although dendritic cells are known to be an important source of IL-15 for control of influenza infection [7], it is not known whether other cell types might be contributing to IL-15 production in response to influenza infection. Lung epithelial cells, for example, are known to constitutively express IL-15 and IL-15Rα [8]. It is still unknown which cell types produce IL-15 during an influenza infection and how respondent NK cells "see" IL-15. Since IL-15 has been shown to be crucial to elicit a proper response to influenza infection virus, further investigation on the identification of these cell types could be beneficial.

Nonetheless, our study assessing the NK cell chemotactic properties of IL-15 on NK cells in the context of influenza infection highlights its potential as a vaccine adjuvant. Our adoptive transfer studies demonstrate that NK cells can be induced to migrate to sites of infection upon IL-15c treatment in IL-15 (and NK cell) deficient animals. Thus, IL-15 plays an important role in NK cell homeostasis, proliferation, and now shown by our group, recruitment. These and our data further shine the light of inquiry on the multiple roles of IL-15 on NK cells and garners support for the need for additional experiments to determine if and how NK cell function can be modulated via IL-15c administration during the innate phase to enhance NK cell proliferation and survival, lower viral titers and/or accelerate viral clearance during a primary infection.

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FIGURE 1.

Infection with influenza virus induces localized IL-15 expression in the respiratory tract. Levels of IL-15 gene expression were quantified from BAL, lung tissue, spleen, and MdLNs by quantitative RT-PCR at the indicated time points p.i. with x31 and expressed as relative expression greater than baseline in naive animals (normalized to 1 using the Δcycle threshold method). Values are represented as mean ± SD compared with mock-infected mice. *$p < 0.05$ by ANOVA with Tukey's post hoc analysis; n = 3 mice repeated 3 times.

FIGURE 1

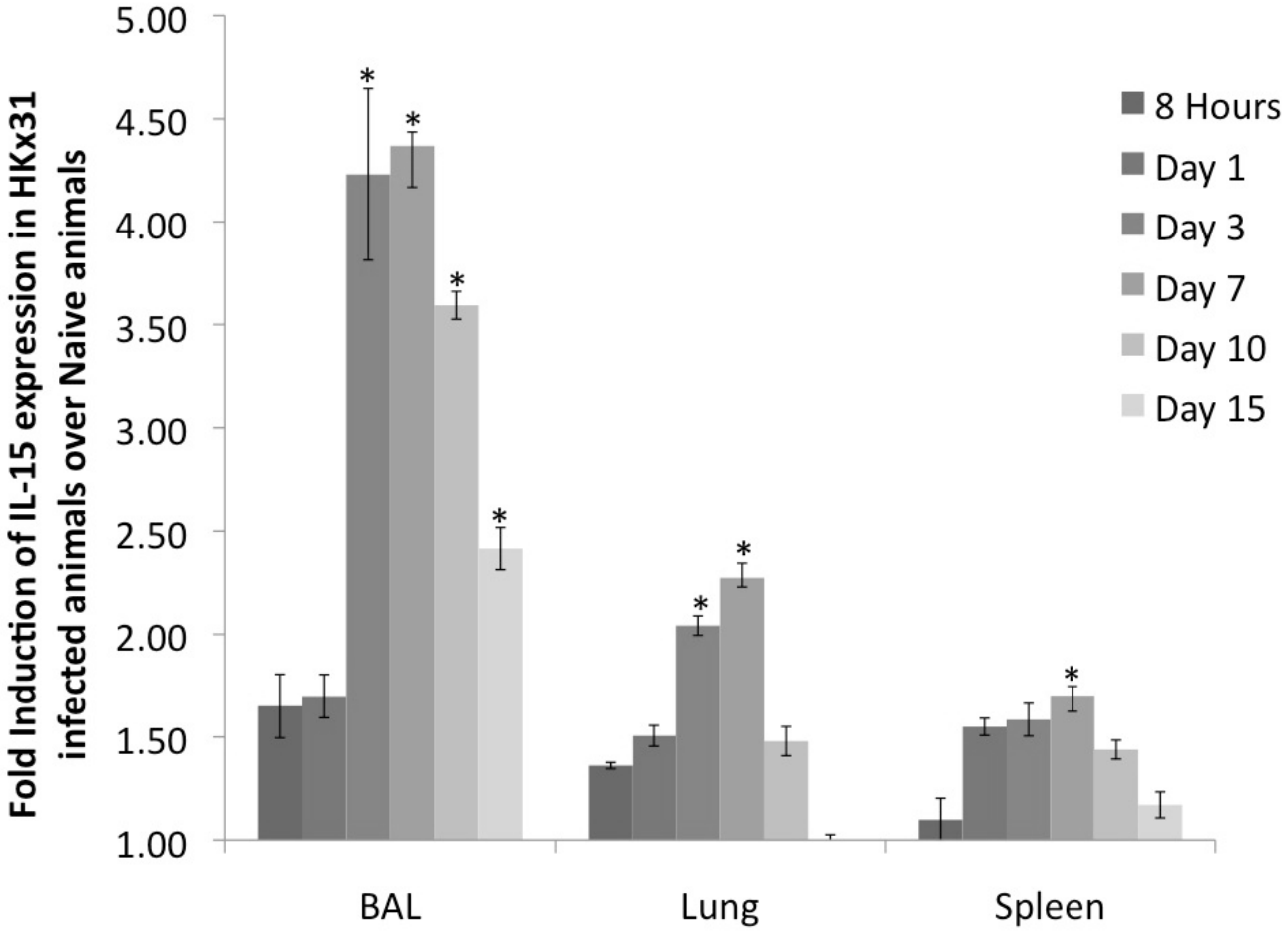

Fold Induction of IL-15 expression in HKx31 infected animals over Naive animals
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
8 Hours
Day 1
Day 3
Day 7
Day 10
Day 15
BAL
Lung
Spleen

FIGURE 2:

NK cells in the lungs airways following influenza infection express IL-15 receptors. Lymphocytes were isolated from mice on days 2, 3, and 4 with Influenza A virus. Lung airway lymphocytes were stained for surface expression of CD3, NK1.1 IL-15Rα, CD122, and CD132. Cells shown are gated on $CD3^-$ $NK1.1^+$. Darker histograms indicate expression of indicated surface proteins while lighter histograms indicate straining with the appropriate isotype controls.. Data are representative of 2 independent experiments. ($p < 0.05$; n = 3-4 mice/exp)

FIGURE 2

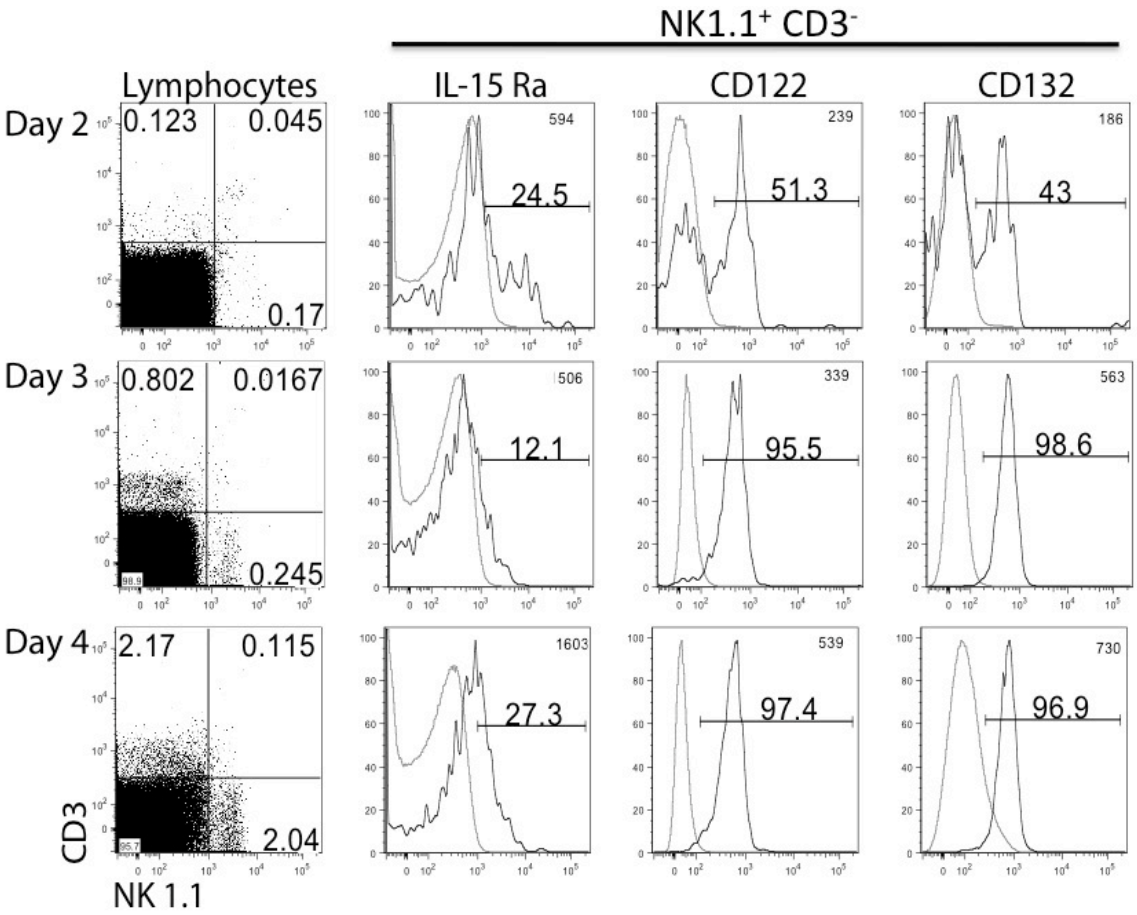

NK1.1+ CD3-
Lymphocytes
IL-15 Ra
CD122
CD132
Day 2
0.123
0.045
0.17
594
24.5
239
51.3
186
43
Day 3
0.802
0.0167
0.245
506
12.1
339
95.5
563
98.6
Day 4
2.17
0.115
2.04
1603
27.3
539
97.4
730
96.9
CD3
NK 1.1

FIGURE 3:

An IL-15 deficiency impairs NK cell accumulation in the lung airways following influenza infection and impacts early viral control. Lymphocytes from the indicated tissues were isolated and analyzed for NK cell numbers at days 1 through 4 p.i. Total cell numbers for WT, IL-15 blocked and IL-$15^{-/-}$ animals are shown for days 1 through 4 p.i. with x31 i.n. Cell numbers for WT (circle), IL-15 blocked (square) and IL-$15^{-/-}$ (triangle) are represented ± SEM (*$p < 0.05$; n = 3 mice/ group). Data are representative of two independent experiments.

FIGURE 3

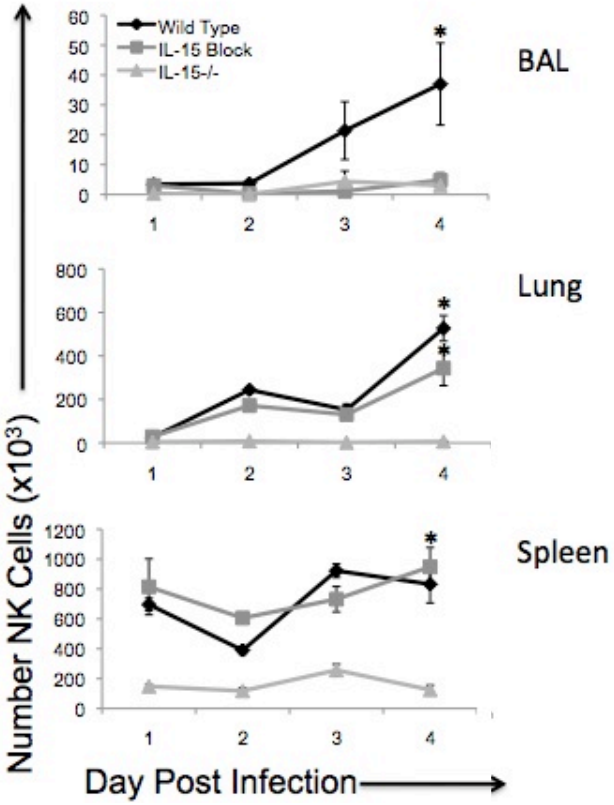

Wild Type
IL-15 Block
IL-15-/-
BAL
Lung
Spleen
Number NK Cells (x10³)
Day Post Infection
60
50
40
30
20
10
0
800
600
400
200
0
1200
1000
800
600
400
200
0
1
2
3
4
*

FIGURE 4:

IL-15 deficiency impairs early control of influenza virus.

Starting 2 d after x31 infection, WT and IL-15$^{-/-}$ animals received either PBS or IL-15 blocking antibody every day for 3 days. On indicated days p.i., lung viral titers, were determined by plaque assay (n = 3 mice/group). PFU/mg are represented ± SEM (*$p < 0.05$; n = 3 mice/ group).

FIGURE 4:

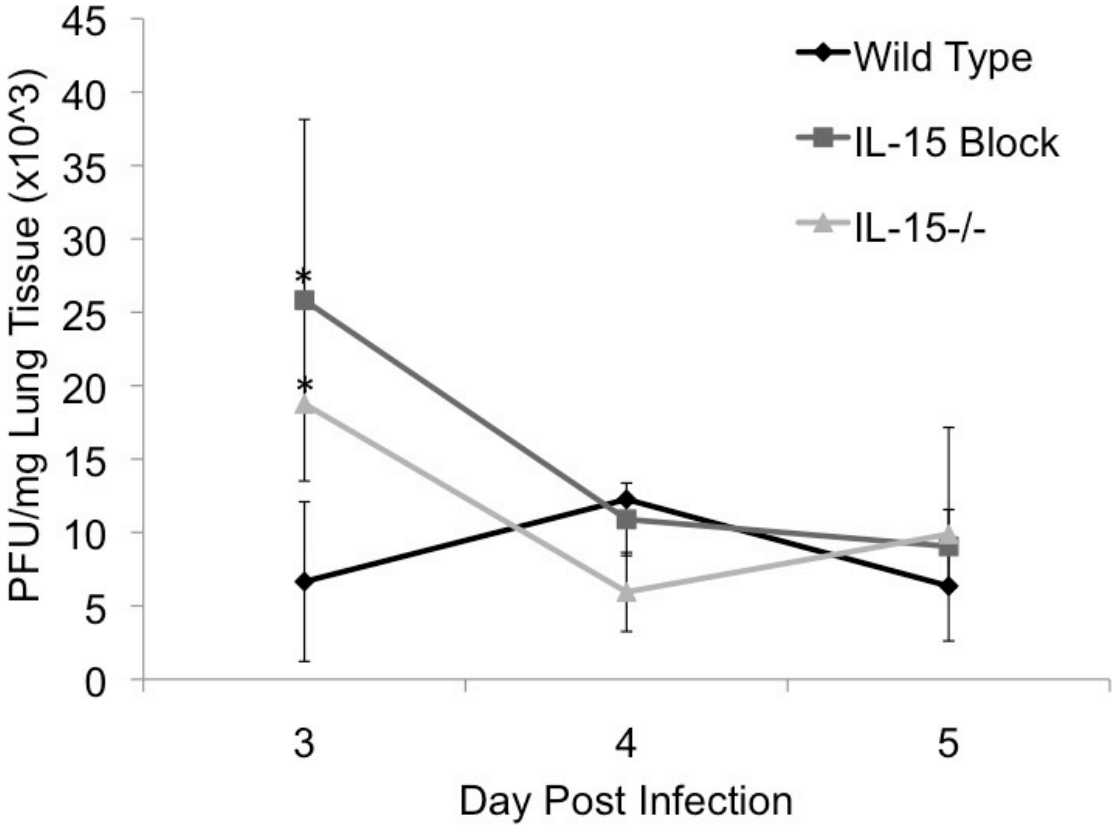
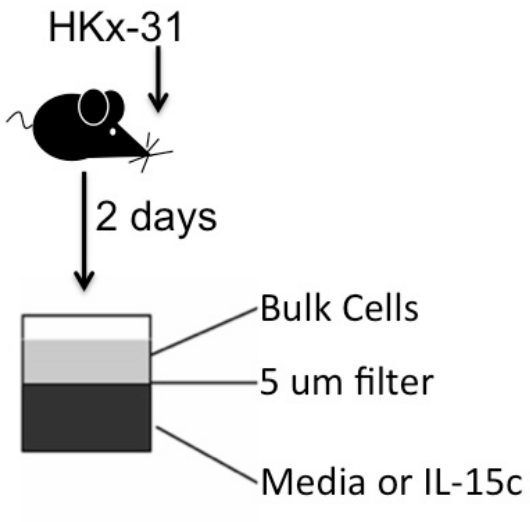

45
40
35
30
25
20
15
10
5
0
PFU/mg Lung Tissue (x10^3)
Wild Type
IL-15 Block
IL-15-/-
*
*
3
4
5
Day Post Infection

FIGURE 5:

NK Cells migrate to IL-15c in vitro. A, Methods Schematic. 1 x 10^6 bulk cells isolated from the indicated tissues of WT animals infected 2 days previously with x31 were placed in the upper chamber of a 0.5-mm transwell with media alone or supplemented with IL-15c in the bottom chamber. Percentage migration was calculated as number of NK cells in the bottom chamber divided by number of NK cells in the input sample. Mean percentage migration is plotted ± SEM among three replicates for each tissue sample. B, The mean percentage of migrated NK cells among day 2 NK was determined and plotted ± SEM (*$p < 0.05$; $n = 3$ mice/group).

FIGURE 5

A.


HKx-31
2 days
Bulk Cells
5 um filter
Media or IL-15c

B.

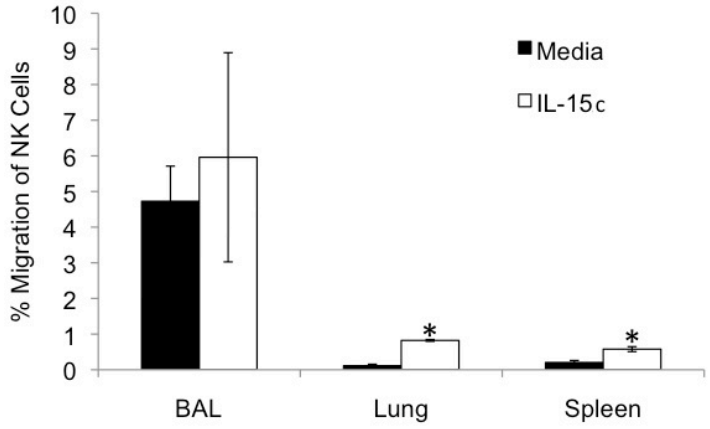

% Migration of NK Cells
10
9
8
7
6
5
4
3
2
1
0
Media
IL-15c
*
*
BAL
Lung
Spleen

FIGURE 6:

Exogenous IL-15c can rescue impaired NK cell trafficking in IL-15-/- mice. A, Methods Schematic for B. B, 9 $X10^4$ CD45.1+ NK cells were adoptively transferred into WT or IL-$15^{-/-}$ CD45.2+ recipients subsequently infected with x31. At 36 hours p.i., WT or IL-15-/- received PBS or IL-15–IL-5Ra complexes (IL-15c). BAL, Lung and Spleen was collected 12h post-treatment, and isolated lymphocytes were analyzed for NK cell numbers (B.) and frequency (C.) Data are representative of two independent experiments. The mean number of donor NK cells isolated from indicated tissues at day 12 p.i. is plotted ± SEM (*$p < 0.5$; n = 4 mice/group) on right. Data are representative of two independent experiments.

FIGURE 6:

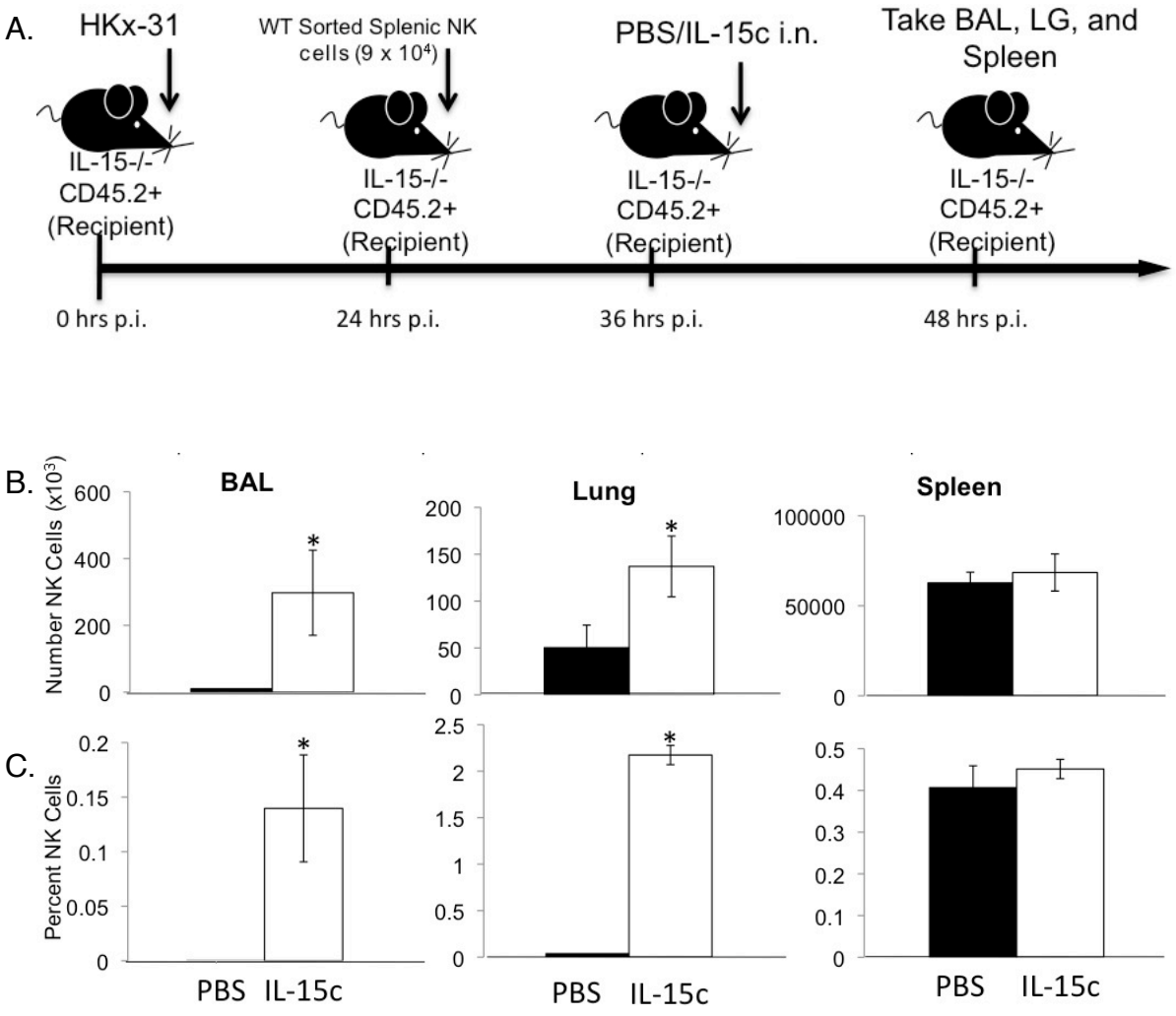
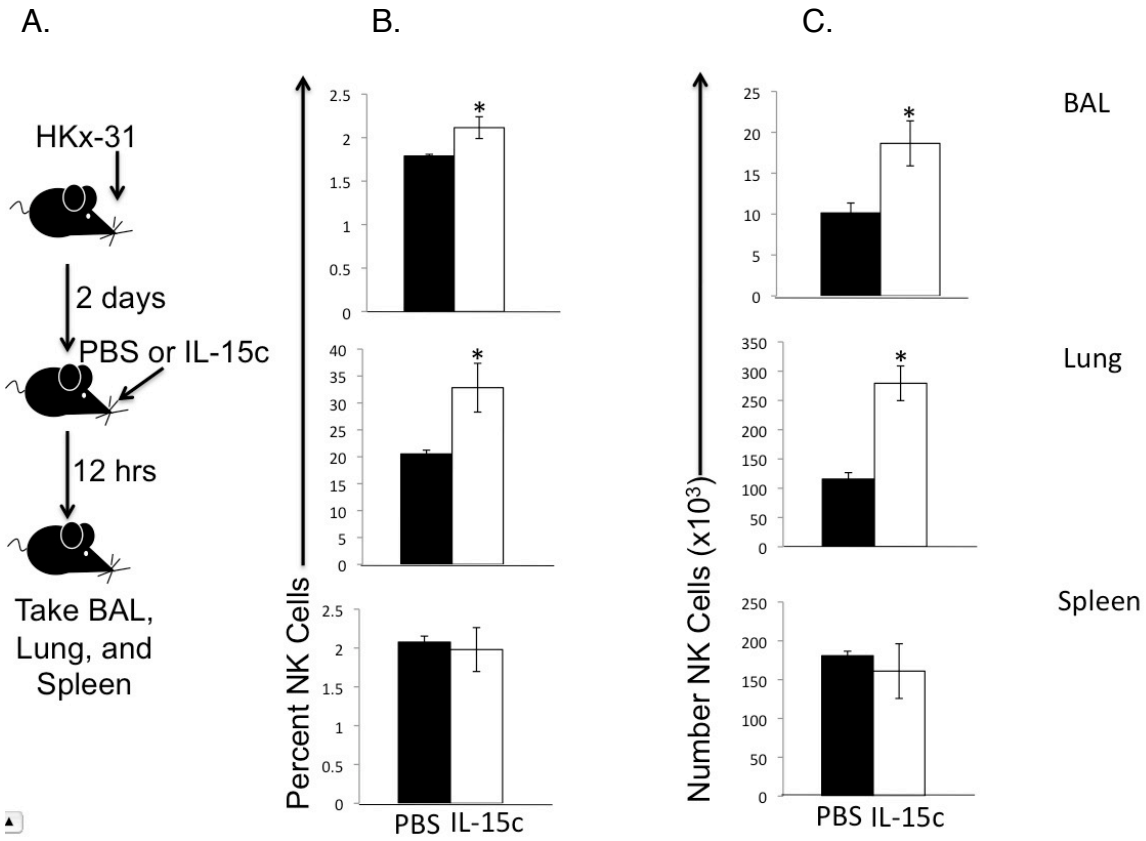

A.
HKx-31
WT Sorted Splenic NK cells (9 x 10⁴)
PBS/IL-15c i.n.
Take BAL, LG, and Spleen
IL-15-/- CD45.2+ (Recipient)
IL-15-/- CD45.2+ (Recipient)
IL-15-/- CD45.2+ (Recipient)
IL-15-/- CD45.2+ (Recipient)
0 hrs p.i.
24 hrs p.i.
36 hrs p.i.
48 hrs p.i.
B.
BAL
Lung
Spleen
Number NK Cells (x10³)
600
400
200
0
200
150
100
50
0
100000
50000
0
C.
Percent NK Cells
0.2
0.15
0.1
0.05
0
2.5
2
1.5
1
0.5
0
0.5
0.4
0.3
0.2
0.1
0
PBS
IL-15c
PBS
IL-15c
PBS
IL-15c

FIGURE 7

NK migrate to IL-15c in vivo. IL-15 induces the migration of NK cells in vivo. A, Methods schematic for B and C. B, 2 d after x31 infection WT mice received either PBS or IL-15c i.n.; 12 h later, BAL, Lung, and spleen were collected and the number of Natural Killer cells migrating into the lung airways was determined by flow cytometry. C, Frequency of NK cells recovered is plotted 6 SEM. $p < 0.05$; $n = 6$ mice.

FIGURE 7


A.
B.
C.
HKx-31
2 days
PBS or IL-15c
12 hrs
Take BAL, Lung, and Spleen
Percent NK Cells
Number NK Cells (x10³)
PBS IL-15c
PBS IL-15c
BAL
Lung
Spleen