IDENTIFICATION OF A ROLE FOR B7-H4 IN NEUTROPHIL HOMEOSTASIS AND INNATE IMMUNITY

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Abstract

B7-H4 is an immunoglobulin superfamily molecule and already been shown to be inhibitory for T cell responses through cell cycle arrest and inhibition of T cell proliferation and cytokine release (Sica et al., 2003). To further explore the physiologic role of B7-H4, we created B7-H4-deficient mice by genetic targeting. B7-H4KO mice are healthy and their T and B cell responses to polyclonal antigens are in normal range. However, B7-H4KO mice are more resistant to infection by Listeria monocytogenes than their littermates. 3 days after infection, bacterial colonies in liver and splenic tissues are significantly lower than the controls, suggesting a role of B7-H4 in enhancing innate immunity. Further studies demonstrate that neutrophils increase in peripheral organs of B7-H4KO mice more so than their littermates but their bactericidal functions remain unchanged. Augmented innate resistance is completely dependent on neutrophils, even in the absence of adaptive immunity. *In vitro*, B7-H4 inhibits the growth of bone marrow-derived neutrophil progenitors, suggesting an inhibitory function of B7-H4 in neutrophil expansion. Our results identify B7-H4 as a negative regulator of the neutrophil response to infection and provide a new target for manipulation of innate immunity.

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Neutrophils and innate immunity

Our bodies are constantly exposed to microorganisms in the environment that are capable of penetrating both epithelial and non-epithelial surfaces to establish infection. Pathogenic microorganisms have evolved sophisticated strategies to invade host organisms at different sites and cause disease by evading host defense mechanisms. Despite the constant exposure to pathogens, the frequency of infection is fortunately quite rare as a result of barrier mechanisms to pathogen invasion and an immune system that has evolved to combat disease at early and late time points. Pathogens that have found a way to escape the host organism's barrier defenses are countered initially by an *innate immune system* that presides in the underlying tissue and can rapidly combat their establishment and progression within minutes of invasion. This innate immune system is composed of cells that utilize germline-encoded, invariant receptors that selectively recognize and activate signaling systems to mobilize and eradicate these disease-causing microorganisms. Phagocytic cells of the innate immune system play a pivotal role in innate immunity as they function to recognize, ingest, and destroy pathogens without the initial aid of an acquired, adaptive immune response.

One major family of phagocytes is the neutrophil or polymorphonuclear leukocyte, which are terminally differentiated hematopoietic precursor stem cells that, under normal conditions, originate within the bone marrow. Paul Ehrlich originally discovered these cells while experimenting with new techniques of cell staining (Sulek, 1967). He described seeing a diverse group of cells that were initially thought to be just one population. One of these newly identified cell types possessed the tendency to retain neutral dyes and exhibited a "polymorphous nucleus". He labeled these cells "neutrophils". The function of these cells

was determined by Elie Metchnikoff, an evolutionary biologist interested in the phagocytic capacity of cells who discovered through experiments on the injured starfish embryo that there was recruitment of phagocytic cells to the site of injury (Sulek, 1967). He theorized that these phagocytic cells ingested microbes that invaded at these locations. He named these cells "polymorphonuclear leukocytes" (a term used interchangeably with neutrophils) based on the appearance of a multilobulated nucleus.

Much has been learned about neutrophils since these seminal observations by Ehrlich and Metchnikoff. Neutrophils are a tightly regulated, but readily mobilized, innate immune cell type that play a critical role as a first line of defense in combating infection by recognizing, ingesting, and destroying foreign materials and organisms (Nathan, 2006). They are the most abundant leukocytes in human blood, produced at a rate of 10^9 cells/kg body weight per day and are kept in a homeostatic balance within the bone marrow, peripheral circulation, and parenchymal tissue (Basu, Hodgson, Katz, & Dunn, 2002; Price, Chatta, & Dale, 1996). Under normal conditions, mature neutrophils, not their precursors, are found in the bloodstream. While they are the most abundant subset of white blood cells in circulation, they are also the shortest-lived. In humans, their half-life is around 7-10 hours, at which point they undergo an organized process of cellular death called *apoptosis* (Cartwright, Athens, & Wintrobe, 1964; Price et al., 1996). While many theories exist, it is unclear why neutrophils are programmed to live so transiently. One postulate is that a short half-life and death through apoptosis may ensure a healthy population of phagocytic cells with the highest functional integrity and efficacy. As a result, this replenished neutrophil

population may be less likely to release toxic mediators indiscriminately into the external environment that could injure tissue and cause unwanted inflammation to develop.

As stated earlier, to maintain neutrophil numbers at a steady state, turnover is rapid with around 10^9cells/kg body weight released from the bone marrow per day (Basu et al., 2002; Price et al., 1996). Maintenance of this cell population requires regulation at various stages including production, bone marrow egress, margination, extravasation, and clearance. It is believed that these processes establish the feedback loops, both positive and negative, that help control neutrophil circulating numbers. Imbalances in this homeostatic system may result in microbial pathogenesis, excess tissue damage in inflammatory disease states, or the development of malignancy.

Granulocyte differentiation

As stated earlier, granulocytes and monocytes are key mediators of the innate immune response, participating as the first line of defense against microbial pathogens. They and their committed progenitors are collectively referred to as myeloid cells. The term *myeloid* is derived from the Greek word for marrow, the niche from where pluripotent hematopoietic stem cells (HSCs) are derived. These hematopoietic stem cells have the potential to differentiate into multiple cell types and to replenish the pluripotent population ("self-renew"). With each progressive step in the linear and hierarchical differentiation of myeloid cells, the cellular fate becomes progressively restricted and defined. Myeloid cells selectively express sets of cell surface proteins that distinguish them from their predecessors and offspring. Through the use of specific antibodies to stain cell surface proteins, these cell

populations can be immunophenotyped, providing a periodic table to identify and characterize them during development (Friedman, 2002, 2007). HSCs do not express many of these lineage markers. They are typically isolated and enriched through the process of negative selection. HSCs are lin-IL7Ra-Kit+Sca-1-CD34+FcgRlo cells that give rise to a common myeloid progenitor (CMP), which in turn gives rise to a lin-IL7Ra-Kit+Sca-1-CD34+FcgRhi granulocyte/macrophage progenitor (GMP) population (Akashi, Traver, Miyamoto, & Weissman, 2000; Traver et al., 2001). These cells, which reside in the medullary cavity of the bone marrow and localize to niches supported by osteoblasts and endothelial cells, are characterized by the expression of both CD34 and Fcγ receptor (II/III) cell surface receptors. These progenitor cells give rise to granulocyte-, monocyte-, and granulocyte/monocyte-colony forming units (CFU). Neutrophil maturation progresses from the myeloblast to the promyelocyte stage- the phase at which the appearance of primary granules occurs. The subsequent differentiation of promyelocytes to myelocytes results in the expression of secondary or specific granules and the cessation of cell division. Successive stages of cellular differentiation (metamyelocyte, bands, and neutrophils) result not only in the presence of the mature three-lobed nucleus and tertiary granules, but also in the selective expression of cell surface molecules that permit analysis of gene expression and cellular events that drive hematopoietic differentiation.

Transcriptional regulation of granulocyte differentiation

Gene transcription plays a critical role in hematopoietic differentiation with the orchestrated expression of select genes and the silencing of others reflecting different subsets

along myeloid cell development. There is no single master myeloid transcription factor that alone governs myeloid lineage commitment. Instead, multiple transcription factors work cooperatively and coordinately to regulate both temporal and lineage-specific gene expression. One of these transcription factors is PU.1. PU.1 is a member of the *ets* family of transcription factors (Klemsz, McKercher, Celada, Van Beveren, & Maki, 1990). PU.1 is expressed by myeloid cells and binds to numerous myeloid promoters, suggesting an important role for this transcription factor in myeloid differentiation. However, PU.1 is not specific to myeloid cells as it is expressed in B cells as well. The role for PU.1 in myeloid differentiation was revealed through two experimental studies showing that loss of this transcription factor resulted in disruption of granulocyte and monocyte progenitor generation (McKercher et al., 1996; Scott, Simon, Anastasi, & Singh, 1994). Disruption of PU.1 was also shown to block B cell differentiation and delay T cell maturation. It would appear that modulation of the level of expression of PU.1 may tip the scale between lineage commitment to B cells or myeloid cells with higher levels of PU.1 expression inducing commitment to the myeloid fate (DeKoter & Singh, 2000). PU.1 expression is maintained at stable levels during differentiation of myeloid cell lines to monocytic and neutrophilic cells. PU.1 is expressed at highest levels in mature human monocytes and human peripheral blood neutrophils (H. M. Chen et al., 1995). PU.1 null embryonic stem cells do not express characteristic myeloid cell surface molecules, including CD11b and CD18 (McKercher et al., 1996). Hematopoietic cells derived from PU.1-null neonates and fetal liver can generate neutrophil colonies (Anderson, Smith, Pio, Torbett, & Maki, 1998; DeKoter, Walsh, & Singh, 1998). Reintroduction of PU.1 into PU.1-null hematopoietic progenitor cells by retrovirus-mediated

expression rescued myeloid and lymphoid development *in vitro* but introduction of G-CSF-receptor or M-CSF-receptor was not sufficient to recapitulate granulocyte or monocyte development (Anderson et al., 1998; DeKoter et al., 1998). Studies of PU.1 expression and its genetic disruption suggest that hematopoietic cells can commit to myeloid lineages in the absence of PU.1, but they fail to mature in a normal manner.

C/EBPα is a member of the family of leucine–zipper transcription factors that influence myeloid differentiation. C/EBP family members regulate multiple, important targets including G-CSF-R, M-CSF-R, and GM-CSF-R as well as mediators of the inflammatory response including CD14 and primary and secondary granule protein genes such as myeloperoxidase, lysozyme, neutrophil elastase, lactoferrin and neutrophil collagenase (Faust, Bonifer, & Sippel, 1999; Gombart et al., 2003; Khanna-Gupta, Zibello, Simkevich, Rosmarin, & Berliner, 2000; Pan, Hetherington, & Zhang, 1999; Verbeek et al., 1999). One member of this family, C/EBPα, is expressed in immature granulocytes (Hohaus et al., 1995; Scott et al., 1994).

C/EBPα is expressed in early myeloid cells where it binds and activates key myeloid target genes. Induction of granulocyte differentiation by retinoic acid results in expression of C/EBPα mRNA and protein in early myeloid cells. Conditional expression of C/EBPα in factor-dependent granulocytic cell lines results in the expression of granulocyte-related genes including G-CSF-R, MPO, lysozyme, and lactoferrin (Wang, Scott, Sawyers, & Friedman, 1999). C/EBPα null neonates lack mature granulocytes, but have normal erythrocytes, megakaryocytes, lymphocytes, and monocytes/macrophages (D. E. Zhang et al., 1997). There is a significant increase in neutrophil precursors in the peripheral blood and a selective

loss of colony forming unit-granulocyte colonies in these mice. Since PU.1 deficiency results in loss of B cell and monocytes lineages, with reduced numbers of neutrophils, while C/EBPα appears to regulate granulocyte development, it would be reasonable to conclude that PU.1 is upstream of C/EBPα in the developmental hierarchical pathway.

It is thought that granulopoiesis progresses under two distinct conditions: during normal steady state conditions or as a response to infection (Bugl, Wirths, Muller, Radsak, & Kopp, 2012). These two states are commonly referred to as *steady-state* and *emergency granulopoiesis* and appear to involve members of the C/EBP transcription family. Under non-infectious conditions, C/EBPα-deficient mice lack granulocytes as a result of a differentiation block at the common myeloid progenitor level, strongly suggesting a master regulator function for C/EBPα in steady state granulopoiesis (D. E. Zhang et al., 1997; P. Zhang et al., 2004). In contrast, emergency granulopoiesis has been described to be dependent on C/EBPβ which appears to promote granulopoiesis under inflammatory conditions (Hirai et al., 2006).

Granulocyte-Colony Stimulating Factor (G-CSF)

The production and maintenance of hematopoietic cells is under the tight control of a set of cytokines. Through their interaction with specific cell-surface receptors, various processes including survival, proliferation, differentiation, commitment, maturation, and functional activation are regulated. Granulocyte colony-stimulating factor (G-CSF) is one of these hematopoietic cytokines, a polypeptide growth factor that plays a critical role in regulating the proliferation and differentiation of neutrophils (Lieschke et al., 1994). G-CSF

was identified and subsequently purified as a unique factor with novel activity that could selectively stimulate the formation of granulocytic colonies from normal hematopoietic progenitor cells *in vitro*. The murine form of G-CSF is a hydrophobic glycoprotein with a molecular weight of 24-25Kd (Nicola, Metcalf, Matsumoto, & Johnson, 1983). The human G-CSF molecule was subsequently discovered and found to have cross-species activity (Nicola, Begley, & Metcalf, 1985). G-CSF was cloned and purified from a cDNA library derived from two tumor cell lines, 5637 (bladder carcinoma cells) and CHU-2 (squamous cell carcinoma) (Nomura et al., 1986; Welte et al., 1985). Southern blot analysis of human genomic DNA showed that a single gene encodes human G-CSF (Souza et al., 1986). This gene, located on chromosome 17q11-22, consists of 5 exons spread over a locus of approximately 2.3kb (Nagata, Tsuchiya, Asano, Kaziro, et al., 1986; Nagata, Tsuchiya, Asano, Yamamoto, et al., 1986). The murine G-CSF gene is located on chromosome 11 in a region that is highly homologous to human chromosome 17. The murine G-CSF gene was cloned by cross-hybridization with a human G-CSF cDNA probe under low stringency conditions (Tsuchiya, Kaziro, & Nagata, 1987). Murine G-CSF is highly homologous with the human gene, with 69% nucleic acid sequence homology in both coding and noncoding regions and a 73% sequence homology in the predicted amino acid sequence of the protein (Tsuchiya et al., 1987). Both the mouse and human proteins contain conserved cysteine residues that are necessary for maintenance of tertiary structure and biologic activity (Nicola et al., 1983). The genomic organization of the murine G-CSF gene is very similar to the human G-CSF gene, consisting of 5 exons spanning 2kb. Of note, the human G-CSF gene is distantly related to the pro-inflammatory IL-6 gene with localized homology. The positions

of the aforementioned cysteine residues have been generally conserved. It is quite possible that the pro-inflammatory cytokines IL-6 and G-CSF arose from a gene duplication event that resulted in subsequent divergence.

Both transcriptional and post-transcriptional mechanisms are involved in the regulation of G-CSF expression. The 300 nucleotides upstream of both the murine and human transcription initiation codon for the G-CSF gene are highly conserved and contain areas that could be important promoter elements. Three *cis* regulatory elements are required for the endotoxin-mediated induction of G-CSF in murine cell lines (Nishizawa & Nagata, 1990; Nishizawa, Tsuchiya, Watanabe-Fukunaga, & Nagata, 1990). Posttranscriptional events have also been implicated in the regulation of G-CSF production. Several cell types constitutively transcribe G-CSF mRNA, including blood monocytes, fibroblasts, and mesothelial cells (Demetri et al., 1990; Ernst, Ritchie, Demetri, & Griffin, 1989; Koeffler, Gasson, & Tobler, 1988). The half-life of the mRNA transcript is less than 15 minutes (Demetri et al., 1990; Ernst et al., 1989; Koeffler et al., 1988). G-CSF mRNA possesses a poly-AUUUA sequence at the 3' untranslated region that has been associated with mRNA stability and has been identified with other growth factor/cytokine genes including GM-CSF, IL-1, IL-6, interferons, and TNF (Shaw & Kamen, 1986). Exposure of fresh human monocytes to lipopolysaccharide, IL-1, TNF, phorbol myristate acetate (PMA), and cycloheximide transiently increases the stability and thereby the half-life of G-CSF mRNA, resulting in an accumulation of G-CSF transcript (Demetri et al., 1990; Ernst et al., 1989; Koeffler et al., 1988). The relative importance of transcriptional versus posttranscriptional mechanisms in the physiologic control of G-CSF gene expression is unclear. G-CSF gene

expression is transient with gene expression diminishing approximately 8 to 12 hours after stimulation of normal cells despite continued cytokine exposure. Tumor cells that constitutively express G-CSF would appear to have disordered regulation of these transcriptional and post-transcriptional processes that result in its prolonged expression.

Various cell types can produce G-CSF under appropriate stimulating conditions. One major source of G-CSF production comes from cells of the monocyte/macrophage lineage. Fibroblasts (Koeffler et al., 1988), vascular endothelial cells (Zsebo et al., 1988), and mesothelial cells (Demetri, Zenzie, Rheinwald, & Griffin, 1989) have also been shown to secrete G-CSF under appropriate stimulatory conditions including LPS, TNF, IL-1, GM-CSF, IL-3, IL-4, and interferon-γ. Production of G-CSF in normal cells is under tight regulation and not constitutive so even under conditions of constitutive expression of the transcript, protein expression is tightly controlled. Conversely, regulation of G-CSF transcription and translation is altered in multiple cell lines including bladder carcinoma (Welte et al., 1985), hepatoma (Gabrilove, Welte, Lu, Castro-Malaspina, & Moore, 1985), oropharyngeal squamous cell carcinoma (Nagata, Tsuchiya, Asano, Kaziro, et al., 1986), melanoma (Lilly, Devlin, Devlin, & Rado, 1987), mesothelioma (Demetri et al., 1989), sarcoma (Sakai, Kubota, Shikita, Yokota, & Ando, 1987), and glioblastoma (Tweardy et al., 1987).

The absence of G-CSF has profound clinical implications. Mice and humans deficient in G-CSF and its receptor suffer from profound neutropenia (F. Dong et al., 1994; Lieschke et al., 1994; Liu, Wu, Wesselschmidt, Kornaga, & Link, 1996). Clinically, G-CSF is well-established as a method to treat or prevent the development of clinical neutropenia. It

appears that G-CSF alone does not support the sustained growth of murine multipotential progenitor cells. Instead, G-CSF stimulates proliferation and differentiation of neutrophil colony-forming cells and alters several functions of mature neutrophils. G-CSF appears to activate colony formation from a more mature population of progenitor cells committed to myeloid differentiation (Ema, Suda, Miura, & Nakauchi, 1990). The presence of G-CSF leads to the down-regulation of CD34+ marrow progenitor cells indicating that G-CSF diverts these cells away from a self-renewing population (Ema et al., 1990). Early blast cells, in contradistinction to promyelocytes and myelocytes, were unable to form clones when exposed to stimulating concentrations of G-CSF (Begley, Nicola, & Metcalf, 1988). As stated above, G-CSF does not support the sustained growth of murine multipotential progenitor cells. However, the addition of other hematopoietic growth factors along with G-CSF appears to stimulate the proliferation of progenitor cell populations. The presence of G-CSF and IL-3 in cultures of non-separated human bone marrow preparations resulted in additive numbers of granulocyte-monocyte colonies. The combination of G-CSF and GM-CSF resulted in synergistic increases in these same colonies in comparison to when these growth factors are added alone (McNiece et al., 1989). Furthermore, the *in vitro* administration of G-CSF to multipotential blast cell colonies failed to demonstrate a direct effect of this growth factor on multipotential progenitors. The rate of appearance of these blast cell progenitors was enhanced by the addition of IL-3 to G-CSF (Ikebuchi, Clark, Ihle, Souza, & Ogawa, 1988). IL-4 in co-stimulation with recombinant G-CSF augmented the proliferation of granulocyte-macrophage progenitor cells (Peschel, Paul, Ohara, & Green, 1987; Vellenga et al., 1990). This stimulatory effect could only be seen when combined with

G-CSF, as IL-4 does not support colony formation by itself. The addition of IL-4 to hematopoietic progenitors 5 days after G-CSF administration failed to increase the number of neutrophil colonies, indicating that IL-4 works within a therapeutic window to potentially sensitize committed cells to the effects of G-CSF (Sonoda et al., 1990). IL-6 appears to selectively augment colony formation in more mature myeloid progenitors in the presence of G-CSF (Rennick et al., 1989). This role of G-CSF in the presence of IL-6 appears dichotomous. One study indicated that high levels of G-CSF were required for IL-6 to augment colony formation. However, at low levels of G-CSF, IL-6 appeared to suppress colony formation (Sonoda et al., 1990). G-CSF is also a potent granulocytic differentiation factor and it can initiate proliferation of multipotential and erythroid progenitor cells but is unable to sustain their continued proliferation to result in colony formation.

G-CSF receptor

Nicola and Metcalf, using radioiodinated G-CSF, were able to identify high-affinity receptors on granulocyte lineage cells (Nicola & Metcalf, 1984, 1985). This expression was seen on select cells as G-CSF receptor was identified on cells from the myeloblast to the mature neutrophil lineage, but not on any erythroid or megakaryocytic lineage cells. Mature neutrophils had the greatest number of G-CSF receptors per cell, with the number of receptors per cell increasing with further hierarchical levels of maturation.

The murine G-CSF receptor has an apparent molecular mass of 150,000 daltons, consisting of 812 amino acids with a single transmembrane domain and a cytoplasmic domain of 187 amino acids (Nicola & Peterson, 1986). In humans, two cDNAs have been

cloned that encode predicted G-CSF receptors of 759 and 812 amino acids. The two human G-CSF receptors are identical except for different carboxyl terminus sequences derived from apparent alternative gene splicing. The purified receptor has been shown to bind to G-CSF with two affinities. An oligomeric form of the receptor binds with high-affinity, while the monomeric form possesses moderate-binding activity.

GCSFR belongs to the cytokine receptor type I superfamily. Signaling progresses through the Janus kinase (Jak)/signal transducer and activator of transcription (STAT), Ras/Raf/MAP kinase, and Protein Kinase B (PKB)/Akt pathways. Different regions of the receptor appear to dictate different activities. It has been shown that a 55 amino acid region adjacent to the transmembrane domain was sufficient to transduce proliferative and survival signals (F. Dong et al., 1993; Fukunaga, Ishizaka-Ikeda, & Nagata, 1993; Ziegler et al., 1993). These signals could be enhanced by a 30 amino acid downstream sequence immediately adjacent to the aforementioned 55 amino acid region (F. Dong et al., 1993; Fukunaga et al., 1993; Ziegler et al., 1993). In contrast, the carboxy-terminal 98 amino acids were shown to inhibit growth signaling (Touw & van de Geijn, 2007). Mutations causing a truncation of the C-terminal cytoplasmic region of the GCSFR had abnormally high proliferative responses but failed to mature when cultured in G-CSF (F. Dong et al., 1995). This hyper-proliferative response to G-CSF is accompanied by prolonged activation of STAT complexes resulting in extended expression of the receptor on the cell surface due to poor internalization. Negative regulators of CSF3 signaling linked to the C-terminal region include the protein tyrosine phosphatase SHP-1 and suppressor of cytokine signaling (SOCS3). SOCS signaling is associated with ubiquitin (E3) ligase function and is thought to

contribute to GCSFR ubiquitination, resulting in receptor internalization and routing to lysosomal compartments for degradation (Beekman & Touw, 2010). Mutations in the GCSFR result in development of congenital neutropenia. On the contrary, acute myeloid leukemia or the myelodysplastic syndrome, diseases associated with dysregulated production of white blood cells, may develop as a result of prolonged administration of G-CSF in patients with severe congenital neutropenia as the genotoxic effects of G-CSF compound into malignant transformation.

Neutrophil migration

Neutrophils migrate into the blood stream after passing through the vascular barrier that separates the bone marrow compartment from the circulation. The interaction of stromal derived factor-1 (SDF1) with the chemokine receptor CXCR4 is important for retention in the bone marrow compartment. This was exemplified in CXCR4-deficient mice that exhibited low numbers of granulocyte precursors in the bone marrow but elevated levels in the blood (Ma, Jones, & Springer, 1999). The administration of recombinant G-CSF has been shown to stimulate granulopoiesis and the subsequent release of neutrophils into the blood. This is thought to occur at least in part to down-regulation of cell surface expression of CXCR4 in murine Gr1+ myeloid cells by G-CSF exposure resulting in the poor attachment of these cells to SDF1 (Kim, De La Luz Sierra, Williams, Gulino, & Tosato, 2006). Furthermore, the administration of a CXCR4 antagonist as well as blocking antibodies results in neutrophil mobilization (Iyer et al., 2008; Suratt et al., 2004). WHIM syndrome is an immunodeficiency disease resulting in increased susceptibility to bacterial

and viral infections characterized by peripheral neutropenia but abundant mature myeloid cells (myelokathexis) (Balabanian et al., 2005; Hernandez et al., 2003). Mutations in the CXCR4 gene result in enhanced G-protein-dependent responses that prevent CXCR4 refractoriness and internalization. The receptor remains in an activated state, resulting in retention (kathexis) of neutrophils in the bone marrow.

In mice, the administration of a single injection of G-CSF results in a 5-fold expansion in circulating neutrophils that begins at 30 minutes and peaks at 12 hours (Ulich, del Castillo, & Souza, 1988. This injection leads to a selective boost in circulating mature and immature (band) neutrophils. This augmentation in circulating neutrophils has also been shown in humans {Cohen, 1987 #172). Within the peripheral circulation, neutrophils are informed of tissue injury and stress, requiring their immediate response. The activation of neutrophils results in a highly choreographed process localizing them to sites of infection and mobilizing them to destroy foreign pathogens. Both host and pathogen-derived molecules are produced or elicited at inflammatory or infection sites, resulting in the activation of nearby endothelial cells. Bacterial-derived N-formyl-methionyl-leucyl-phenylalanine (fMLP) and lipopolysaccharide (LPS), as well as host-derived tumor necrosis factor alpha (TNF-a), interleukin 1B (IL-1B), IL-8, and IL-17 are potent mobilizing and chemoattractant agents that induce the expression of adhesion molecules including P-selectins, E-selectins, and members of the ICAM family on the luminal surface of endothelial cells. Neutrophils are in a continual state of surveying the endothelial luminal surface as they are transported through the blood stream. It is at the postcapillary venule where optimal binding to stimulated endothelial cells occurs. Here, the vessel wall is very thin and the luminal

diameter narrow, allowing for the easiest route out of the blood vessel without occluding it. Neutrophils express proteins on their cell surfaces that bind to the endothelial cell surface receptors. Initially, P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin on the surface of the neutrophil microvilli interact with the endothelial P- and E-selectins to mediate the initial binding of neutrophils to the vessel wall (McEver & Cummings, 1997). This initial tethering allows for the "rolling" of neutrophils on the endothelial surface. This engagement leads not only to the recruitment of neutrophils but also results in their activation. Binding of PSGL-1 and L-selectin induces activation of intracellular kinases including Src family kinases, phosphoinositide 3-kinase (PI3K), Syk, and p38 mitogen-activated protein kinase (MAPK) (S. I. Simon, Hu, Vestweber, & Smith, 2000). The activation of these intracellular pathways induces changes that result in the expression of integrins necessary for "firm adhesion" to the vascular endothelial wall.

Selectin-mediated rolling gives way to integrin-mediated firm adhesion mediated by the β2-integrin family of proteins, which include LFA-1 and Mac-1 on neutrophils. These β2-integrins engage members of the immunoglobulin family ICAM-1 and -2 on the endothelial cells (Chesnutt et al., 2006; Salas et al., 2004). Deficiency of β2-integrin causes leukocyte adhesion deficiency type I, characterized by the inability of neutrophils to establish firm adhesion and diapedesis, resulting in an immunodeficiency characterized by the onset of recurrent bacterial infections during the neonatal period (Etzioni, Doerschuk, & Harlan, 1999; Rotrosen & Gallin, 1987). This firm adhesion transitions neutrophils away from rolling, leading to the migratory arrest of these cells on the surface of vascular endothelial cells. Integrin-mediated firm adhesion sets the stage for the transmigration of neutrophils

from the luminal side of the blood vessel to the underlying host tissue where the inflammatory process was first established. It is believed that the interaction of β2-intergrins with ICAM-1 and-2 results in tyrosine kinase activation leading to phosphorylation and destabilization of vascular endothelial-cadherin bonds which form homotypic contacts that stabilize endothelial cell-cell junctions. Neutrophil surface proteins and junctional proteins interact, resulting in the transmigration of these cells through the endothelial junction. ICAM-2 is concentrated at the junctional surfaces and guide neutrophils to enter the endothelial cell junctions. Further transmigration is assisted by junctional adhesion molecule-A (JAM-A). Jama-/- mice show accumulation of neutrophils deeper between the endothelial cells than seen in Icam2-/- mice (Corada et al., 2005; Khandoga et al., 2005). Platelet endothelial cell adhesion molecule-1 (PECAM-1) is thought to be one of the final molecules necessary for the transmigration of neutrophils across the endothelial cell. Pecam1-/- mice show accumulation of neutrophils between the endothelial cells and the basement membrane suggesting that this molecule is necessary for breaching this final threshold to gain access to the host tissue (Schenkel, Chew, & Muller, 2004; R. D. Thompson et al., 2001). Blockade of highly O-glycosylated proteins CD99 and CD99L2, which are both expressed at endothelial cell-cell junctions, also results in the accumulation of neutrophils at the interface between the endothelial cell and the basement membrane (Bixel et al., 2007). Since neutrophils also express these molecules, it is postulated that activated neutrophils may exploit this to gain access to the host tissue. Navigation through the thick basement membrane, composed largely of laminins and collagens, requires the release of

proteases, including elastase, matrix metalloprotease 8 (MMP8), MMP9, and MT6-MMP, from neutrophil granules to digest the protein matrix.

Having crossed the vascular endothelial threshold, neutrophil migration will now rely upon its sensitivity to chemotactic gradients to pursue invading microorganisms. These gradients are derived from both host cytokines and pathogen chemoattractants. These substrates bind to and activate G protein-coupled receptors within neutrophils, resulting in activation of the MAP kinase pathway. Activation of this pathway leads to assembly of the oxidative burst machinery. Neutrophils also express Toll-like receptors (TLR) that are responsible for recognizing specific pathogen motifs. These pathogen-associated molecular patterns (PAMPs), including LPS, lipopeptides, flagellin, and DNA, bind specific TLRs thereby activating both the oxidative burst apparatus and degranulation.

Neutrophil activation and killing

Once neutrophils have been activated and migrate to sites of inflammation or infection, they possess highly regulated substances to carry out microbial eradication. The four major mechanisms through which neutrophils fulfill this task are degranulation, oxidative burst, release of antimicrobial peptides, and phagocytosis. Neutrophils carry toxic substances from the blood stream into host tissue. These substances are stored in special organelles called the granule. Neutrophils possess three major types of granules: azurophilic, specific, and gelatinase. *Azurophilic granules*, which take up the basic dye azure A due to their content of acid mucopolysaccharide, are the earliest granules to develop during neutrophil maturation. They form during the promyelocytic stage. These granules contain

myeloperoxidase (MPO), an enzyme necessary for oxidative burst. These granules also carry defensins, lysozyme, and serine proteases. *Specific granules* are formed after azurophilic granules and do not possess MPO, but are characterized by their expression of antimicrobial glycoprotein lactoferrin. *Gelatinase granules* are the last subgroup of granules to form during neutrophil maturation. Similar to specific granules, they do not contain MPO, but are a repository for metalloproteases, including gelatinase. It is believed that these granules may be mobilized as the neutrophil transmigrates across the vascular endothelium and into the basement membrane where they release their cargo of metalloproteases. A fourth class, *secretory vesicles*, contain plasma-derived proteins including albumin and their membrane serves as a reservoir for membrane-bound molecules utilized by neutrophils during migration, including β2-integrins, complement proteins, and the fMLP receptor. Fusion of the secretory vesicle with the plasma membrane exposes these substances to the external environment that facilitates firm adhesion. The further activation of neutrophils occurs at sites of inflammation or infection resulting in the initiation of the respiratory burst cycle and the mobilization of both azurophilic granules and specific granules. Specific granules release their antimicrobial peptides into phagosomal compartments or out into the external environment through fusion with the plasma membrane.

Neutrophils carry a diverse cargo of peptides and proteins that are utilized to destroy microbes. It appears that many of these microbicidal substances are redundant and it is this presumed redundancy that makes studying their specific role challenging. As a result, the unique biologic significance of any one of these molecules is unclear. There are three main types of antimicrobials. The first are the cationic antimicrobial peptides and proteins that

include α-defensins and cathelicidins. These antimicrobial substances bind to microbial membranes and permeabilize membrane bilayers by exploiting the negatively charged phospholipids. The defensins have also been shown to inhibit bacterial cell wall synthesis. BPI, a cationic antimicrobial protein binds LPS resulting in hydrolysis of bacterial phospholipids and increased bacterial permeability. The second class of antimicrobials includes a diverse array of proteolytic enzymes. Lysozyme, a glycoside hydrolase breaks down bacterial cell walls by attacking and hydrolyzing the glycosidic bonds found in peptidoglycans. Neutrophils also contain several serine proteases that can cleave bacterial virulence factors and membrane proteins. Lastly, the final class of antimicrobial substances includes metal chelator proteins. These proteins chelate essential metals from microbes thereby inhibiting bacterial cell growth. Lactoferrin alters bacterial growth by binding to iron, depriving bacteria of this essential nutrient. Calprotectin, another member of the metal chelator proteins, sequesters zinc and manganese.

Neutrophils utilize a set of enzymes to perform respiratory burst to produce reactive oxygen species that are directly toxic to bacteria. The NADPH oxidase system is a membrane-associated enzyme complex that is utilized to perform respiratory burst (Weiss, 1989). Dysfunction of the NADPH oxidase system results in chronic granulomatous disease (CGD), an immunodeficiency resulting in chronic bacterial and fungal infections (Clark et al., 1989; Curnutte, Scott, & Mayo, 1989; Leto et al., 1990; Lomax, Leto, Nunoi, Gallin, & Malech, 1989; Rotrosen, Yeung, Leto, Malech, & Kwong, 1992; Segal, 1987; Teahan, Rowe, Parker, Totty, & Segal, 1987). NADPH oxidase is assembled on the phagosomal and plasma membrane surfaces where it is thought to be involved in the generation of three oxygen

metabolites: superoxide anion, hydrogen peroxide (H_20_2), and hydroxyl radical (OH). This is accomplished by the production and transport of electrons across the vacuolar membrane. Superoxide and H_2O_2 can combine to form the highly reactive hydroxyl radical, which is thought to contribute to neutrophil function as a microbicidal agent. NADPH oxidase activity coincides with degranulation. Another enzyme, myeloperoxidase, is found in high concentrations in the cytoplasmic azurophilic granules of neutrophils. It is thought that this enzyme catalyzes H_2O_2-dependent oxidation of halides that react and destroy microbes (Hampton, Kettle, & Winterbourn, 1996; Klebanoff, 1967, 1968, 1975). With the ubiquitous availability of chloride in plasma, myeloperoxidase will use this to generate HOCL. The role of myeloperoxidase in facilitating bacterial eradication has been shown as myeloperoxidase-deficient human neutrophils display poor microbial killing (Lehrer, Hanifin, & Cline, 1969).

Phagocytosis is the major mechanism to remove pathogens and cell debris. It is an active, receptor-mediated process during which a particle is internalized by the cell membrane into a vacuole called a phagosome. The interaction between neutrophil and particle can be either direct or indirect. The direct interaction is exemplified by the physical interaction between pathogen-associated molecular patterns (PAMP) exhibited by the microbe and their cognate pathogen recognition receptor (PRR). Indirect or opsonized-mediated recognition by coating of a particle with proteins that facilitate phagocytosis is typically characterized by FcγR-mediated phagocytosis which results in the engulfment of IgG-opsonized particles or complement receptor-mediated phagocytosis in which complement plasma proteins interact with pathogens to mark them for destruction by phagocytes expressing complement receptors. Ingestion of these particles is just the first step

toward degradation. Initially, the phagosome in which cargo is stored and transported intracellularly has no bactericidal or enzymatic activity. Once this occurs, the phagosome must mature to carry out these functions. In neutrophils, this maturation process coincides with the degranulation of antimicrobial molecules into the phagosomal compartment. At the same time, the NADPH oxidase system is assembled on to the surface of the phagosomal surface resulting in ROS production. Neutrophil phagosomal pH, unlike that in macrophages, is initially acidic and then alkaline providing a permissive environment for the activation of serine proteases, neutrophil elastase, and cathepsin G.

Mechanisms are in place to retard the accumulation and further activation of neutrophils once in the site of tissue injury. Endogenously-derived anti-inflammatory mediators including neutrophil-manufactured lipoxins and macrophage-derived resolvins and protectins are produced in response to ingestion of apoptotic neutrophils resulting in inhibition of neutrophil recruitment (Serhan, 2005). Secretory leukocyte protease inhibitor (SLPI), produced by neutrophils, macrophages, and epithelial cells has been shown to suppress neutrophil respiratory burst and tissue proteolysis by inhibiting neutrophil elastase (Grobmyer et al., 2000). Furthermore, SLPI was shown to bind proepithelin (PEPI), inhibiting elastase conversion of PEPI, thereby preventing pro-inflammatory epithelin (EPI) activation of neutrophils through promotion of epithelial-cell production of IL-8, one of the most potent neutrophil chemoattractants (Zhu et al., 2002). PEPI signals epithelial cells to promote wound healing, closing a portal for further bacterial entry and migration (He, Ong, Halper, & Bateman, 2003).

In the absence of external stimuli that promote their localization, migration, and microbicidal activity, neutrophils are thought to undergo apoptotic cell death. It appears that apoptotic neutrophil death has an anti-inflammatory role. The pro-inflammatory cytokine IL-23 is thought to play an important role in neutrophil homeostasis. Transgenic overexpression of IL-23 subunit p19 induces neutrophilia (Wiekowski et al., 2001) while IL-23 deficiency or blockade with an antibody decreased neutrophil counts in normal and neutrophilic mice (Smith et al., 2007). IL-23 is a potent inducer of IL-17 (Steinman, 2007). It is found at elevated levels in all strains of neutrophilic, adhesion molecule-deficient mice. IL-17 blockade by soluble IL-17 receptor severely reduced neutrophil counts, indicating that neutrophilia is in part caused by IL-17 (Forlow et al., 2001). Mice deficient in the IL-17 receptor have decreased neutrophil numbers (Kelly et al., 2005; Smith et al., 2008). IL-17 stimulates G-CSF secretion and G-CSF levels were elevated in all neutrophilic mouse strains (Fossiez et al., 1996). IL-23 levels are decreased by apoptotic neutrophils (Stark et al., 2005). It is thought that under normal conditions, transmigrated apoptotic neutrophils are phagocytosed by macrophages and DCs, resulting in the decreased production of IL-23. Macrophages and DC are the major source of IL-23, which induces IL-17 in γδ and αβ T cells as well as NK and NKT cells.

B7-H4

T cells require at least three signals to transition from a state of naïve incompetency to differentiated functionality. T cells recognize peptide antigens that are displayed on cell surfaces. Cell surface expression requires antigen processing through one of two major pathways depending on whether the antigen was degraded in the cytosol or through endocytic vesicle formation. These antigens are displayed on the surface for evaluation by T cells through association with the major histocompatibility complex (MHC) molecules. MHC: peptide complexes are assessed by the T cell through its T cell receptor (TCR). This has been classically defined as "signal one". This crucial signal is essential in determining specificity and initiating T cell signaling. However, this coupling of the MHC and TCR is not sufficient to determine the fate of T cell activation. What is required is a "second signal" provided by costimulatory or co-inhibitory ligands. These are glycoproteins expressed on the cell surface of antigen presenting cells (APC), providing contextual signals that guide the fate of T cells maturations and differentiation through interaction with costimulatory or co-inhibitory receptors. The end product is broadly defined into positive and negative signaling. Positive signals typically result in T cell proliferation, expansion, survival, specific cytokine release, and may deliver cytotoxic effector responses. Negative signaling attenuates T cell responses, resulting in T cell death, reduced memory responses, suppressor T cell induction, and immunosuppressive cytokine secretion. This second signal is most likely the integrated output of multiple costimulatory and co-inhibitory interactions with their respective ligand.

The best-characterized costimulatory molecules are members of the B7 family. B7-like molecules belong to the immunoglobulin (Ig) superfamily. The best understood costimulatory receptor is CD28. CD28 is present on the surface of all naïve T cells and binds to the costimulatory ligands B7.1 (CD80) and B7.2 (CD86), expressed on specialized antigen presenting cells such as dendritic cells. It is this interaction that facilitates naïve T cell activation, proliferation, differentiation, and survival. Mice lacking either CD28 or B7.1 and B7.2 demonstrate impaired T cell responses *in vivo* and *in vitro* (Lenschow et al., 1996). CTLA-4 is an inducible co-inhibitory molecule expressed by T cells that engages B7.1/B7.2 with higher affinity than CD28, resulting in an inhibitory signal to activated T cells (Chambers, Kuhns, Egen, & Allison, 2001). CTLA-4 knockout mice develop profound autoimmune diseases (C. B. Thompson & Allison, 1997). CD28 and CTLA-4, through their interactions with B7.1/B7.2, regulate the fine-tuning of early T cell activation.

B7-H4 (B7S1/B7x) is a member of the B7 immunoglobulin superfamily and was isolated and identified in three separate labs. (Prasad, Richards, Mai, & Dong, 2003; Sica et al., 2003; Zang et al., 2003) B7-like molecules contain single IgV and IgC domains and share approximately 20-40% amino acid identity. Sica et al. identified human B7-H4 by using sequences of the IgV and IgC domains of the B7 family molecules and queried them against an expressed sequence tagged (EST) database. (Sica et al., 2003) This allowed the group to piece together the nucleotide sequence that encoded the full-length B7-H4. A BLAST search of the NCI database with the human B7-H4 sequence identified the mouse ortholog. Zang et al. searched a mouse EST database, identifying three overlapping ESTs with homology (Zang et al., 2003). Using primers along with DNA from dendritic,

macrophage, and fibrosarcoma cell lines the group was able to obtain cDNAs of the full-length transcript. Prasad et al. identified B7-H4 by performing a homology search using a mouse and human EST database using the sequences for B7h and B7-H3 (Prasad et al., 2003).

The mouse B7-H4 gene is located on chromosome 3 consisting of six exons and five introns. Its human counterpart is located on chromosome 1p11.1. It consists of six exons and five introns. The coding region spans 849bp and encodes a protein consisting of 282 amino acids with an N-terminal hydrophobic region that most likely serves as the leader peptide, four conserved cysteine residues that help form the IgV and IgC-like domains, seven sites for N-linked glycosylation, a hydrophobic transmembrane domain, as well as a short intracellular domain consisting of only 2 amino acids. From the amino acid sequence, B7-H4 most closely resembles a type I transmembrane protein. The mouse and human B7-H4 sequences have approximately 87% amino acid identity, suggesting high evolutionary conservation. The IgV domains of human and mouse B7-H4 share 91% sequence identity and only 23% identity with B7.1/B7.2.

The extracellular domain of B7-H4 has approximately 25% amino acid homology with other B7 family members. However, the intrachain disulfide bonds formed by cysteine residues are all conserved between B7-H4 and other B7 family members. From our understanding of other B7 family members, the IgV domain is usually involved in counter-receptor binding (Schwartz, Zhang, Fedorov, Nathenson, & Almo, 2001; Stamper et al., 2001). For example, CD80 and CD86 IgV domains bind CD28/CTAL-4. The IgV domains of human and mouse B7-H4 exhibit high homology (91%). However, when compared to

CD80 and CD86, B7-H4 shares only 23% sequence identity. (Sica et al., 2003) Many core or Ig superfamily consensus residue positions are conserved, or conservatively replaced, between CD80/CD86 and B7-H4 indicating that the general structure of these molecules is similar. However, there is a lack of residue conservation in the binding face of the CD80/CD86 and B7-H4, strongly suggesting that B7-H4 does not bind CTLA-4 or CD28. The intracellular portion of B7-H4 comprises a glycosyl phosphatidylinositol(GPI)-linked structure that is distinctly different from other members of the B7 family (Prasad et al., 2003; Zang et al., 2003). However, there is a lack of published uniformity regarding this structure (Choi et al., 2003; Salceda et al., 2005).

Northern blot analysis revealed human B7-H4 mRNA expression in spleen, lung, and thymus (Sica et al., 2003). RT-PCR analysis demonstrated human B7-H4 in the placenta, ovary, testis, prostate, skeletal muscle, kidney, small intestine, liver, and pancreas (Sica et al., 2003). Mouse B7-H4 transcript was detected in the heart, lung, liver, skeletal muscle, kidneys, and testis (Sica et al., 2003). RT-PCR analysis identified mRNA in all these organs as well as the brain and liver (Sica et al., 2003). Zang et al. showed that mouse B7-H4 mRNA was expressed at high levels in splenic dendritic cells and at low levels in splenic B cells, T cells, and thioglycolate-induced peritoneal macrophages (Zang et al., 2003). Despite the wide transcript expression profile of B7-H4, immunohistochemical staining of these same tissues could not reveal its expression. B7-H4 expression could be demonstrated upon stimulation of T cells, B cells, monocytes, and dendritic cells (Sica et al., 2003). Treg cells, but not conventional T cells, were shown to induce B7-H4 expression on CD14+ monocytes (Kryczek, Wei, et al., 2006). This capacity for B7-H4 expression on monocytes by Tregs

was dependent on IL-10 as neutralizing antibodies to IL-10 reduced B7-H4 expression (Kryczek, Wei, et al., 2006). Blockage of B7-H4 by blocking oligonucleotides significantly reduced T cell suppression by Treg-conditioned monocytes. These results indicate that B7-H4 expression on antigen presenting cells plays an important participatory role in immunosuppression along with Treg cells. B7-H4 is expressed intracellularly as well (Choi et al., 2003; Tringler et al., 2005). Intracellular B7-H4 was identified in primary fresh ovarian tumor cells from ovarian cancer ascites fluid and from the tumor mass (Tringler et al., 2006). This expression was exclusively intracellular and could not be identified on the tumor cell surface. In this same study, cell surface B7-H4 expression was identified on ovarian tumor-associated macrophages and was found to be up-regulated upon exposure to IL-6 and IL-10.

One explanation for the exclusive identification of intracellular B7-H4 is that it is stored for release in a soluble form. High levels of the soluble form of B7-H4 are found in the sera of patients with rheumatoid arthritis when compared to healthy controls (65% vs. 13%) (Azuma et al., 2009). Patients with elevated soluble B7-H4 were found to have increased disease severity scores using the DAS28 scoring system. In a collagen-induced arthritis (CIA) mouse model, soluble B7-H4 was able to exacerbate disease. This result was complemented by the exacerbation of CIA in B7-H4KO mice and was abrogated with the depletion of neutrophils using an antibody against B7-H4. Serum B7-H4 has also been detected in patients with clear cell renal cell cancer. Patients with RCC more often had elevated levels of soluble B7-H4 and had higher median concentrations in their sera. The levels of soluble B7-H4 correlated with higher grade tumors, tumors with positive nodes, and

distant metastases (R. H. Thompson et al., 2008). It is yet to be determined where the source of soluble B7-H4 originates. One theory regarding the possible role of soluble B7-H4 is that it acts as a decoy for surface-bound B7-H4 preventing the binding of this molecule to its receptor, thereby preventing inhibitory responses. The discrepancies between protein and mRNA expression as well as intracellular, cell surface, and soluble expression may be related to differences in posttranscriptional regulation in various cell types.

The receptor for B7-H4 has not been conclusively identified. Early studies utilizing a B7-H4 fusion protein suggested a possible receptor on anti-CD3 activated T cells (Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003). B and T lymphocyte attenuator (BTLA), a glycoprotein with two immunoreceptor tyrosine-based inhibitory motifs, is induced on activated T cells. B7-H4 was found to be a ligand of BTLA through the differential binding of B7-H4-Ig fusion protein with wild-type and BTLA-deficient TCR transgenic cells (Watanabe et al., 2003). This fusion protein was found to inhibit T-cell activation and interleukin-2 production (Carreno & Collins, 2003; Watanabe et al., 2003). Unfortunately, no direct binding of B7-H4 to BTLA has been established. A follow-up study indicated that herpesvirus entry mediator (HVEM), and not B7-H4, was the ligand for BTLA (Sedy et al., 2005).

B7-H4 was shown to inhibit T cell responses. Immobilized B7-H4 could inhibit stimulation of anti-CD3-activated D011.10 TCR transgenic mice and purified CD4+ T cells from B6 mice (Sica et al., 2003). These results were confirmed by *in vivo* studies showing the inhibition of OT-1 expansion with soluble mouse B7-H4Ig. Furthermore, allogenic cytotoxic T cell activity was inhibited by soluble B7-H4Ig. The mechanism of B7-H4

suppression involves cell cycle arrest. Secretion of IL-2, IL-4, IL-10, and IFN-g was inhibited by B7-1 costimulated T cells. Further studies performed around the same time supported the role of B7-H4 in suppression of T cell proliferation and cytokine production (Prasad et al., 2003; Zang et al., 2003).

B7-H4 has been identified in a variety of cancer types. B7-H4 expression was detected in the cytoplasm and on the membrane of primary serous, endometrioid, and clear cell carcinomas (I. Simon et al., 2007; Tringler et al., 2006). Hyperplastic and malignant endometrial epithelium showed overexpression of B7-H4 and was correlated with tumor-associated T cells (Miyatake et al., 2007). B7-H4 was found in invasive ductal carcinomas and associated with decreased number of tumor infiltrating lymphocytes (Mugler et al., 2007). It was detected in 92% of pancreatic ductal adenocarcinomas (Awadallah et al., 2008). B7-H4, along with B7-H3 are highly expressed in human prostate cancer and associated with disease spread and poor outcome (Zang et al., 2007). 60% of renal cell carcinoma tumor specimens exhibited B7-H4 expression and the presence of this molecule correlated with adverse clinical and pathologic features. Patients with tumors expressing B7-H4 were three times more likely to die from RCC compared to tumors lacking B7-H4 (Krambeck et al., 2006). Cultured tumor cells from human gliomas and medulloblastomas were found to express B7-H4 (Yao et al., 2008). B7-H4 expression was found in 45% of gastric cancer specimens and was positively correlated with cancer invasiveness and lymph node metastasis (Jiang et al., 2010). The median overall survival time of patients with lower B7-H4 expression was 13 months longer than that of patients with higher expression levels (Jiang et al., 2010).

B7-H4 was transfected into an insulinoma cell line, NIT-1, and transplanted into diabetic C57BL/6 mice by intraperitoneal injection. Utilizing splenocyte proliferation assays, B7-H4-NIT exhibited suppressed alloreactive T cell activation with lower proportion of IFN-g secreting cells and higher Treg cells compared to control. mRNA and secreted cytokine analysis revealed less IFN-g and higher IL-4 in B7-H4_NIT transplanted animals than control animals. Mice with B7-H4 reached normoglycemia faster and gained weight after transplantation and exhibited longer survival times (Yuan et al., 2009). A separate study showed that prediabetic NOD mice that received intraperitoneal injections of B7-H4-Ig in comparison to control mouse IgG reduced the incidence of autoimmune diabetes. B7-H4 treatment was shown to reduce T cell proliferation in response to GAD65 stimulation *ex vivo* (Wang et al., 2011). Expression of B7-H4 in islet allograft was shown to induce a beneficial effect on controlling alloreactive CD8+ T cell responses, reduce immune infiltrating cells, up-regulate Tregs, and protect allograft B-cell function (Wang et al., 2009).

Listeria monocytogenes

Listeria monocytogenes is a facultative intracellular Gram-positive bacterium that is capable of causing sepsis, brain infection, abortion, and prenatal infection (Drevets & Bronze, 2008). It is a highly studied bacterium that has been used as a model for understanding the function of both the innate and adaptive immune system as well as the relationship of microbes to their host. Both the innate and adaptive immune systems are required for the effective recognition and eradication of these bacteria.

Listeriosis is caused by the ingestion of food contaminated with *Listeria monocytogenes* resulting in a spectrum of disease from gastroenteritis to meningo-encephalitis, sepsis, and possible death (Drevets & Bronze, 2008). While we are exposed to *Listeria* on a frequent basis, the development of fulminant bacterial infection is rare. However, there is an increased risk of infection and death in immunocompromised hosts, including neonates and fetuses, immunodeficient patients, and patients taking immunosuppressant medications. As a facultative bacterium, *Listeria* can exist in the external environment. After ingestion, *Listeria* is able to cross the human intestinal epithelial barrier and spread through the blood stream and lymphatics to distant organs. The liver and spleen are two primary sites of bacterial residence and it is here that the bacteria can divide and replicate, resulting in possible septicemia and abscess formation. Listeria can also migrate across the blood-brain barrier and the materno-fetal membrane resulting in meningo-encephalitis or fetal infection, stillbirth, or abortion.

As an invasive intracellular microbe, *Listeria* utilizes a variety of mechanisms to enter host cells, evade intracellular killing, and infect adjacent cells. This is accomplished through the hijacking of host cellular proteins and machinery. Nearly all of the gene products that contribute to bacterial invasion, cytosolic entry, growth, intracellular motility, and spread to adjacent cells are regulated by a transcriptional regulator PrfA. To first gain entry into cells, *Listeria* utilizes two cell surface proteins, internalins A and B (InlA and InlB), to bind to cell surface proteins on host non-phagocytic cells (Seveau, Pizarro-Cerda, & Cossart, 2007). InlA binds the adherens junction protein E-cadherin while InlB utilizes the receptor tyrosine kinase Met. The endogenous ligands of E-cadherin and Met are E-cadherin and hepatocyte growth factor, respectively. Binding of InlA and InlB to their receptors does not occur at the same sites as the endogenous ligands E-cadherin and HGF but leads to the recruitment of a similar set of downstream signaling molecules. The binding of InlA and InlB to their respective cell surface receptors results in receptor ubiquitination, recruitment of clathrin, rearrangement of the cortical cytoskeleton, and pathogen uptake. Met is broadly expressed on human cells, including intestinal epithelial cells, and as a result, infection can be accomplished through oral ingestion of bacterial inoculum. However, in mice, a single amino acid change at position 16 of mouse E-cadherin results in weak binding between InlA and E-cadherin, preventing *Listeria* infection by the enteral route. InlB is also species-specific.

After adhesion to the cell surface, *Listeria* induces its own uptake via cytoskeletal and membrane rearrangements involving the use of the host endocytic protein clathrin and receptor-mediated entry. Clathrin recruitment leads to subsequent local cortical actin

rearrangement required for internalization. Once inside the cell, *Listeria* is confined to a vacuole that has a mildly acidic pH. Through the use of two phospholipases and the pore-forming toxin listerolysis O (LLO), the bacterium is capable of escaping the vacuolar organelle prior to its fusion with lysosomes and the subsequent formation of the phagolysosome (Camilli, Goldfine, & Portnoy, 1991; Kathariou, Metz, Hof, & Goebel, 1987; Mengaud, Braun-Breton, & Cossart, 1991; Schnupf & Portnoy, 2007; Scortti, Monzo, Lacharme-Lora, Lewis, & Vazquez-Boland, 2007; Vazquez-Boland et al., 1992). It is within the phagolysosome that enzymes, proteins, and peptides are released that mediate an intracellular antimicrobial response.

In the cytosol, *Listeria* expresses several virulence factors, including the surface protein ActA (Pizarro-Cerda & Cossart, 2006). ActA are critical for the intracellular and intercellular movement of the bacterium and allow bacterial dissemination in the host. ActA is able to recruit host cell actin-related protein 2/3 (Arp2/3) complex and the actin polymerization machinery to form an actin structure at the posterior pole of the bacterium. Actin polymerizes exclusively at one end of the bacterium, because ActA accumulates in a polar fashion on the cell surface after bacterial division. This allows for unidirectional propelling of the bacterium through the cytoplasm. Eventually, motile *L. monocytogenes* form protrusions on the host cell membrane and thereby spread to neighboring cells without inducing cell lysis, allowing bacterium to multiply without being accessible to antibodies produced by the humoral host immune response. In secondarily infected cells, *L. monocytogenes* again escapes from the double membrane vacuole to start a new cycle of

actin polymeriztion and cell-to-cell spread. Lysis of the double membrane secondary vacuole occurs via the conjugated action of LLO and phospholipases.

Mice are not a naturally susceptible host for *L. monocytogenes*. InlA does not recognize mouse and rat E-cadherin, preventing *L. monocytogenes* entry in rodent epithelial cells. Therefore, in mouse experiments, the intravenous and intraperitoneal routes are frequently used. 10 minutes after intravenous inoculation of mice with sub-lethal doses of L. monocytogenes, 90% of the inoculum is taken up by the liver and 5 to 10% by the spleen. Early after *L. monocytogenes* infection, neutrophils act as the major cell of defense. Neutrophils migrate to sites of infection through the bloodstream, transmigrate between endothelial cells and into interstitial tissues, and utilize the chemotactic gradient induced by infected hepatocytes to advance toward the site of infection. Neutrophils phagocytose the bacterium and release reactive oxygen species to kill intracellular bacteria. Mice depleted of neutrophils display increased susceptibility to *L. monocytogenes* and have increased bacterial burden in both the liver and spleen (Conlan & North, 1994; Czuprynski, Brown, Maroushek, Wagner, & Steinberg, 1994; Rakhmilevich, 1995; Rogers & Unanue, 1993). In addition to neutrophils, NK cells are thought to provide immediate immune support as a critical source of IFN-γ. It is this source of IFN-γ, as well as neutrophil-derived IFN-γ, that stimulates the activation of macrophages to become bactericidal through the production of nitrogen and oxygen species. During the first 6 hours, the number of viable *Listeria* in the liver decreases 10-fold, predominated by a rapid destruction of most bacteria. Surviving *Listeria* then multiply within permissive macrophages and grow exponentially in the spleen and liver for the next 48 hours. This proliferation peaks 2-3 days following infection before starting to

disappear from the organs as a result of immune response induction. Innate immunity plays a crucial role upon *L. monocytogenes* infection, and T cells are required for the final clearance of *L. monocytogenes*, while humoral immunity is believed to provide only has a small contribution in protection. Within 1-2 weeks the infection is cleared and any subsequent re-infection will result in an expedited clearance within 4-5 days.

Purpose

B7-H4 is an immunoglobulin superfamily molecule and already been shown to be inhibitory for T cell responses through cell cycle arrest and inhibition of T cell proliferation and cytokine release (Sica et al., 2003). To further explore the physiologic role of B7-H4, we created B7-H4-deficient mice by genetic targeting. B7-H4KO mice are healthy and their T and B cell responses to polyclonal antigens are in normal range. However, B7-H4KO mice are more resistant to infection by *Listeria monocytogenes* than their littermates. 3 days after infection, bacterial colonies in liver and splenic tissues are significantly lower than the controls, suggesting a role of B7-H4 in enhancing innate immunity. Further studies demonstrate that neutrophils increase in peripheral organs of B7-H4KO mice more so than their littermates but their bactericidal functions remain unchanged. Augmented innate resistance is completely dependent on neutrophils, even in the absence of adaptive immunity. *In vitro*, B7-H4 inhibits the growth of bone marrow-derived neutrophil progenitors, suggesting an inhibitory function of B7-H4 in neutrophil expansion. Our results identify B7-H4 as a negative regulator of the neutrophil response to infection and provide a new target for manipulation of innate immunity.

Introduction

Neutrophils are an indispensible component of the host innate immune defense mechanism against infection and also contribute to autoimmune pathogenesis and chronic inflammation. Neutrophils launch the immune response during infection. While multiple theories are posited regarding how neutrophils are able to discriminate benign inflammation from pathogenic inflammation, it is generally believed that the release of so-called "danger signals" released at the behest of host cells activate antigen presenting cells, initiating neutrophil migration to sites of inflammation. Once there, neutrophils become activated and initiate a cascade of defense mechanisms including phagocytosis, killing, and degradation of microorganisms by antimicrobial and proteolytic proteins, along with the generation of reactive oxygen species. Neutrophils also participate in tissue breakdown, remodeling, wound healing, and modulation of other inflammatory and adaptive immune components (Nathan, 2006). Due to their short life span, neutrophils are continuously resupplied during infection and inflammation by expansion from myeloid progenitor cells in the bone marrow (Ley, Laudanna, Cybulsky, & Nourshargh, 2007).

B7-H4 (B7x and B7S1) is a member of the B7-CD28 co-signaling molecule family/immunoglobulin superfamily with signature IgV and IgC regions in the extracellular domain, a single transmembrane domain and an intracellular domain (L. Chen, 2004). Although the mRNA for B7-H4 is broadly detectable in the majority of normal tissues and cell types, its cell surface expression is relatively rare (Choi et al., 2003; Prasad et al., 2003; Sica et al., 2003; Tringler et al., 2006; Zang et al., 2003). Expression of B7-H4 protein, however, can be induced *in vitro* on hematopoietic cells (Kryczek, Zou, et al., 2006; Sica et

al., 2003). In contrast to its limited distribution in normal tissues, constitutive cell surface and cytoplasmic expression of B7-H4 is found on various cultured cancer lines and freshly isolated human carcinomas of breast, lung, ovarian and kidney (Choi et al., 2003; Krambeck et al., 2006; Kryczek, Zou, et al., 2006; Salceda et al., 2005; I. Simon et al., 2007; Sun et al., 2006; Tringler et al., 2006; Tringler et al., 2005). Previous *in vitro* studies demonstrate that B7-H4 fusion protein could inhibit polyclonal T cell responses including growth and cytokine secretion, (Coussens, Tinkle, Hanahan, & Werb, 2000; Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003) as well as antigen-induced stimulation of transgenic T cells *in vivo* (Coussens et al., 2000; Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003). Blockade of B7-H4 by monoclonal antibody (mAb) was shown to partially inhibit antigen-specific T cell responses (Sica et al., 2003) and accelerate autoimmune disease progression *in vivo* (Prasad et al., 2003). The receptor for B7-H4, which mediates inhibitory functions on T cells, remains unknown at the present time. Indeed, the inhibitory activity of B7-H4 on T cells combined with its upregulation on many human cancers (Choi et al., 2003; Krambeck et al., 2006; Kryczek, Zou, et al., 2006; Salceda et al., 2005; I. Simon et al., 2007; Sun et al., 2006; Tringler et al., 2006; Tringler et al., 2005) and tumor-infiltrating macrophages from the ascites of ovarian cancer patients, has implicated this molecule in tumor immune evasion (Kryczek, Zou, et al., 2006). Interestingly, upregulation of B7-H4 on antigen-presenting cells (APCs) has been previously proposed as a potential inhibitory mechanism of regulatory T cells (Kryczek, Wei, et al., 2006). These studies provide direct evidence that B7-H4 may contribute to the suppression of adaptive immunity in the tumor microenvironment.

Interestingly, B7-H4 has also been shown to be expressed at high levels in a soluble form in the sera of patients with rheumatoid arthritis, an inflammatory disease state closely associated with the presence of neutrophils (Azuma et al., 2009). The synovial fluid of patients with rheumatoid arthritis contains significant quantities of neutrophils, indicating an important role for these cells in tissue destruction (Pillinger & Abramson, 1995). The depletion of circulating neutrophils was shown to prevent joint inflammation and synovial leukocyte influx (Brown, Blaho, & Loiacono, 2004; Eyles et al., 2008; Santos, Morand, Hutchinson, Boyce, & Holdsworth, 1997; Wipke & Allen, 2001). Serum G-CSF levels correlate strongly with clinical indicators of rheumatoid arthritis (Nakamura et al., 2000) and the administration of G-CSF in mice exacerbates collagen-induced arthritis (Campbell, Rich, Bischof, & Hamilton, 2000). The use of a neutrophil elastase inhibitor reduced the incidence and severity of collagen-induced arthritis in an animal model (Kakimoto, Matsukawa, Yoshinaga, & Nakamura, 1995). We considered the possibility that the presence of B7-H4 and neutrophils may be linked in cases of inflammation and immunoregulation.

The current study reveals a novel activity of B7-H4 as an endogenous negative regulator for neutrophil responsiveness to *Listeria* infection, done so by directly inhibiting expansion of neutrophil progenitors from bone marrow (BM). As a result, B7-H4-deficient mice are highly resistant to *Listeria* infection resulting from enhanced neutrophil-mediated innate immunity.

Results

Generation of B7-H4KO mice

We generated B7-H4KO mice by homologous recombination in 129 ES cells by deleting the entire IgV and IgC regions of the B7-H4 gene to completely eliminate their interaction with any potential receptor. Exons encoding both the IgV and IgC domains of the B7-H4 gene were replaced with a *Neo* gene cassette **(Fig. 1A).** Targeted recombination of ES cells was confirmed by Southern blot analysis and the data from 4 independent ES clones are shown. The B7-H4 allele is predicted to have a 12.25 kb SpeI fragment and the B7-H4- allele has an 8.9 kb SpeI fragment. The clones 2 and 3 with both fragments indicate a recombination (**Fig. 1B**). Chimeric male mice were derived from these ES clones by standard procedures. They were backcrossed to C57BL/6 (B6) females and heterozygous mutant mice were established from two independently targeted ES clones. Heterozygous or homozygous B7-H4 mutant mice were then identified by PCR analysis of genomic DNA isolated from tail biopsies. Southern blot analysis confirmed the replacement of genomic DNA. RT-PCR analysis demonstrated that B7-H4 mRNA was not expressed in livers of B7-H4-deficient mice **(Fig. 1C).** B7-H4KO mice develop normally and give normal litter numbers. These mice were backcrossed to the B6 background for 10 generations before they were used in the studies described below.

B7-H4KO mice have enhanced neutrophil-mediated resistance to Listeria infection

B7-H4KO mice display normal numbers and ratios of T, B, NK, NKT cells, and macrophages using specific monoclonal antibodies (mAb) in flow cytometric analysis. In addition, T cells from B7-H4KO mice do not have obvious alterations than littermates in responding to polyclonal stimuli, judged by *in vitro* proliferation of purified T cells by CD3

cross-linking, allogeneic antigen stimulation, or cytolytic T cell response to alloantigens. These results indicate that the lack of B7-H4 does not globally affect T cell responses to antigens. These results are consistent with studies showing normal T-cell proliferation in B7-H4 knockout mice (Suh et al., 2006). In these studies, single cell suspensions of total lymph nodes from wild-type and B7-H4KO mice were stimulated with soluble anti-CD3 or T-cell mitogen concavalin A (ConA) and pulsed with tritium-labelled thymidine. Consistent with these *in vitro* findings, we also found that B7-H4KO mice have normal responses to ConA-induced hepatitis (H. Dong et al., 2004), hapten-induced hypersensitivity (Tsushima et al., 2003), and OVA-induced airway inflammation (Kamata et al., 2003). B7-H4KO mice were also found to be comparable to littermate control in OT-I and OT-II cell expansion to OVA proteins upon transfer (Sica et al., 2003), CD4-V8.1/8.2 T cell expansion to superantigens (Tamada et al., 2002), and CTL responses to allogeneic antigens *in vivo* (Tamada et al., 2000). Normal B cell responses in B7-H4KO mice were also observed after immunization by TNP-KLH (Tamura et al., 2001). Finally, we were unable to identify the development of spontaneous autoimmune diseases up to 1.5 years in B7-H4KO mice housed under specific pathogen-free environment.

While our data indicates that B7-H4 plays a minimal role in antigen-driven T and B cell responses, especially polyclonal responses, in our assays, these responses were conducted in the absence of active infection, which usually requires a much more sophisticated coordination between innate and adaptive immunity. To test this possibility, we next evaluated the effect of B7-H4 ablation in mice infected with *Listeria monocytogenes* (LM) to examine whether B7-H4 contributes to immune responses against infection. Since

mice do not acquire infection through the enteral route, we challenged B6 mice with an intra-peritoneal dose (i.p.) of Listeria (2 x 10^6 CFU). We used this dose based on our observation that it was sufficient to induce lethality when tested using dose escalation in our mouse facility. The survival of these mice was then subsequently evaluated. B7-H4KO mice were significantly more resistant to LM infection than their WT counterparts, and as a result, B7-H4KO mice survived much longer than their B7-H4 wild-type (Wt) littermates. Up to 40% of mice were able to clear the bacteria and lived indefinitely. In contradistinction the B7-H4KO mice, littermate controls died around day 9 (**Fig. 2A**). This effect correlated with decreased Listeria in the spleen (**Fig. 2B**) as well as the liver of B7-H4KO mice. Interestingly, the majority of mice succumbed to infection within 3-4 days, time points at which adaptive immunity is usually not yet developed and were indicative of a role of innate immunity. Our results suggested a potential role for B7-H4 in altering the context of the innate immune response.

To address mechanisms for this resistance at potentially lethal doses of inoculation, we examined the cell composition of both the innate and adaptive immune response. Mice were infected with Listeria and T, B, NK, macrophages, and neutrophils in peripheral blood and in lymphoid organs were examined by specific mAb. Although there were no significant differences in the numbers of NK, macrophages, T cells, and B cells of spleens within the first 3 days after LM infection, significantly more neutrophils in composition (**Fig. 2C**) and in absolute numbers (**Supplemental Table 1**) were found from LM-infected B7-H4KO mice than identically infected WT littermates. Similar results were also obtained in neutrophils isolated from livers and in peripheral blood after infection.

To exclude the possibility that the increase in neutrophil levels were due to other genetic modifications in B7-H4KO mice, and were not limited to LM infection, we first attempted to validate this finding by blocking B7-H4 in normal B6 mice. Injection of previously described anti-murine B7-H4 mAb (Sica et al., 2003) showed a small or minimal effect, consistent with the known weak neutralizing ability of this mAb. We then took an alternative approach to express a soluble decoy B7-H4 in B6 mice as a means of blocking endogenous B7-H4. The rationale was that soluble decoy B7-H4 would be able to bind to its unknown receptor thereby recapitulating the blocked state. We first constructed two plasmids encoding only an extracellular portion of murine B7-H4 with deletion of both the transmembrane and intracellular domains (B7-H4VC). In addition, we also constructed another plasmid with only the IgV domain (B7-H4V). Hydrodynamic injection uses controlled hydrodynamic pressure in capillaries to enhance the permeability of endothelial cells and permeabilize parenchymal cells to a large volume of DNA solution for gene delivery was used to provide soluble B7-H4 ((Anand et al., 2006), also see figure legend of Supplemental Fig. 1). The delivery of these plasmids in B6 mice led to the rapid appearance of soluble B7-H4 in sera as detected by ELISA. Soluble B7-H4 could be detected at up to 2ug/ml sera at day 1 and dropped to basal levels at day 7 after injection (Azuma et al, Unpublished data). We subsequently tested the effect of soluble B7-H4 blockade in the neutrophil response to LPS in a pouch assay, in which neutrophils in a skin air pouch could be quantified after LPS challenge. Expression of soluble B7-H4 (either B7-H4VC or B7-H4V) significantly increased the number of neutrophils in the pouches (**Supplemental Fig. 1**) and inhibited the growth of Listeria in spleen (**Supplemental Fig. 2**). These results also

indicated that the V region of B7-H4 was sufficient to compete for binding of endogenous B7-H4. We then performed these experiments in B7-H4KO mice and WT controls. Consistent with these findings, pouches created in B7-H4KO mice had significantly increased neutrophils relative to littermate controls (**Supplemental Fig. 3**). Thus, our results indicated that B7-H4 was capable of modulating the innate immune response by regulating the number of peripheral neutrophils in response to bacterial infection. Our results also further supported the role of soluble B7-H4 as a decoy receptor capable of blocking endogenous cell surface B7-H4.

Removal of neutrophils by the use of depleting antibody has been shown to enhance Listeria infection and implicates neutrophils in early protection against this microorganism (Conlan & North, 1994; Czuprynski et al., 1994; Rakhmilevich, 1995; Rogers & Unanue, 1993). To determine if neutrophils are required for the resistance of Listeria infection in B7-H4KO mice, we depleted neutrophils by *in vivo* injection of Gr-1 mAb. Injection of Gr-1 mAb led to the rapid decline of neutrophils to undetectable levels at day 2 in spleens (unpublished). Depletion of Gr-1+ neutrophils led to a significant increase of LM load in livers from B7-H4KO mice, in comparison with those treated with either PBS or isotype-matched control mAb (**Fig. 3A**). Plasmacytoid dendritic cells (pDC) are shown to express Gr-1 and also have anti-bacterial function (Gilliet, Cao, & Liu, 2008). Elimination of pDC by specific mAb against PDCA1, however, did not increase LM colonies. Depletion of NK cells by NK1.1 mAb also did not affect LM colony formation in liver, while depletion of macrophages by carrageenan increased LM colonies to a moderate but less significant level as compared to Gr-1+ cell depletion **(Fig. 3A)**. Our experiments also demonstrate that

treatment by these methods eliminated specific subsets for up to two weeks. Our results support the notion that Gr-1+ neutrophils play a critical role in the resistance to LM infection in the absence of B7-H4.

We next determined whether the augmented clearance of Listeria by B7-H4KO mice was caused by alterations in neutrophil function. B7-H4 deficient neutrophils displayed normal growth inhibition of LM colony formation in culture, which is comparable to littermate neutrophils (**Fig. 3B**), suggesting that B7-H4 deficient neutrophils have normal bactericidal functions. To confirm this, we evaluated two aspects of neutrophil bactericidal function; phagocytosis and oxygen burst response of B7-H4KO neutrophils to LM infection. To do so, neutrophils from WT or B7-H4KO mice were incubated with dichlorodihydrofluorescein diacetate (H2DCFDA) and PE-labeled latex beads. H2DCFDA is a cell-permeable green fluorogenic probe that is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species. Upon acetate group cleavage by esterases and oxidation, H2DCFDA molecules are converted from non-fluorescence to green fluorescent dichlorofluorescein (DCF) in the cytosol thereby measuring oxidative burst in neutrophils and macrophages. PE-labeled latex beads measure phagocytosis capacity. As shown, both respiratory burst and phagocytosis by B7-H4KO neutrophils are within the normal range (**Fig. 3C**), supporting that neutrophils from B7-H4KO mice are functionally comparable to WT neutrophils. Therefore, increased resistance to LM infection in B7-H4KO mice could not be attributed to functional modification of neutrophils including phagocytosis and reactive oxygen species production that enhance bactericidal activity, but likely mediated by an increased number of neutrophils.

Neutrophil-mediated innate resistance in B7-H4KO mice is independent of adaptive immunity

While our data supports that resistance of B7-H4KO mice to LM infection requires neutrophils and that B7-H4KO mice have enhanced clearance due to elevated numbers of neutrophils, it is still possible that adaptive immunity also contributes to neutrophil growth. To exclude this possibility, we backcrossed B7-H4KO mice to the recombination activating gene-1 knockout (RAG-1KO) background. RAG-1 encodes one of two enzymes that lead to V(D)J rearrangement and recombination which are essential to the generation of mature B and T cells. Our rationale was that in crossing B7-H4KO to RAG-1KO, we could eliminate T and B cells as confounders of the enhanced immunity. Unlike RAG-1KO (RKO) mice, which possess small spleens, double knockout mice (DKO) display enlarged spleens (**Fig. 4A**) accompanied with increased spleen weight (**Fig. 4B**). Further analysis of cell components in the spleen, peripheral blood, liver, and bone marrow revealed an increased percentage and absolute number of Gr-1+CD11b+ neutrophils (**Fig. 4C**) (**Supplemental Table 1**) of DKO mice compared to those of RKO mice. Our results indicate that the lack of T and B cells further revealed an influence of B7-H4 on neutrophil homeostasis under unstimulated conditions.

RKO and DKO mice were then challenged by the administration of a lethal dose of Listeria to examine the efficacy of their respective innate immune resistance. Infection of RKO mice by Listeria induced massive growth of the bacteria within the liver (**Fig. 5A, 5B**) and spleen (**Fig. 5C**). We observed 100% mortality by day 4 as well under these conditions

(**Fig. 5D**). In sharp contrast, DKO mice had significantly less bacterial load in their livers **(Fig. 5A, 5B)** and spleens **(Fig. 5C)**. Unlike their counterparts, the majority of DKO mice survived more than 10 days post-LM challenge **(Fig. 5D)**. In contrast to the long-term survival of a significant fraction of infected B7-H4KO mice on the B6 background, **(Fig. 2A)** all DKO mice eventually succumbed to infection on day 15 indicating that some Listeria under these conditions escape innate immune surveillance. These results confirm an important role of adaptive immunity in providing long-term anti-Listeria immunity and demonstrate that the acute enhancement of anti-Listerial immunity in B7-H4KO mice occurs in the absence of an adaptive immune system.

To confirm that increased innate resistance against LM in DKO mice is related to the neutrophil population, but not other factors, we compared the effect of neutrophil depletion in DKO vs. RKO mice. Injection of Gr-1 mAb led to comparable formation of LM colonies in the livers (**Fig. 5E**) and spleens (**Fig. 5F**) of both RKO and DKO mice. Our results suggest that the lack of B7-H4 confers enhanced innate immunity against LM infection, which is largely mediated through the presence of increased neutrophils.

Absence of B7-H4 in bone marrow-derived cells promotes proliferation of Gr-1+CD11b+ neutrophil progenitors

We next examined growth potential of neutrophil progenitors in the presence and absence of B7-H4. To do so, total bone marrow cells which contain large numbers of neutrophil precursors from WT, B7-H4KO, RKO, and DKO mice were labeled with the fluorescent dye CFSE and cultured *in vitro*. CFSE is retained within the cell and is used to

monitor lymphocyte proliferation due to the sequential halving of CFSE fluorescence within daughter cells following each cell division. Flow cytometric analysis of cultured bone marrow cells at day 3 showed that the majority of live cells in the culture (>95%) were Gr-1+CD11b+ (data not shown). Importantly, 70% of Gr-1+CD11b+ cells from B7-H4KO mice had diluted CFSE, while only 56% Gr-1+CD11b+ cells from WT B6 mice exhibited evidence of cellular division. Mice possessing the RAG-1KO background exhibit similar changes: 86% Gr-1+CD11b+ cells from DKO mice divided whereas only 64.8% Gr-1+CD11b+ cells from RKO mice had diluted CSFE (**Fig. 6A**). Similar results were also obtained by comparing proliferative potential of purified lineage negative (Lin-) bone marrow progenitor cells from RKO vs. DKO mice in the presence of G-CSF (data not shown). These results suggest that B7-H4 is a potent inhibitor for the growth of neutrophils that may be responsible, at least in part, for the decreased response of innate immunity against bacterial infection in wild type mice.

To further evaluate the role of B7-H4 in the inhibition of neutrophil progenitors, we examined the effect of exogenous B7-H4. We first purified Lin- bone marrow cells to enrich the hematopoietic stem cell population via the process of negative cell selection. In the presence of G-CSF, these stem cells differentiated into Gr-1+CD11b+ neutrophils. Recombinant B7-H4Ig fusion protein was then supplemented at the beginning of stem cell culture. The cultures treated with B7-H4Ig had significantly lower cell numbers than those treated with control media or isotype-matched control immunoglobulin at day 2 and day 3 (**Fig. 6B**). This inhibition was evident in a dose-dependent fashion (data not shown). Cell death did not appear to be a contributing mechanism as there was no significant difference

between controls and cells exposed to B7-H4Ig treatment (data not shown). As shown in **Fig. 6C**, normal bone marrow cells contain approximately 50% Gr-1+CD11b+ myeloid cells (46.5%) and depletion of Lin+ cells by negative mAb cell selection efficiently removed the majority of these cells (from 46.5% to 1.3%, left upper panel). Inclusion of G-CSF in culture media resulted in a significant increase in Gr-1+CD11b+ cells; up to 83.1% of cells in the culture at day 3 were Gr-1+CD11b+ cells **(upper panel)**. Addition of B7-H4Ig in the culture significantly inhibited this effect **(middle panel)**. This inhibition is associated with decreased cell division as indicated by dilution of CFSE **(lower panel)**. Our results provide direct evidence that B7-H4 inhibits expansion of neutrophils from their progenitors.

Discussion

During analysis of the phenotypes in a newly generated B7-H4KO mouse strain, we observed that these mice are resistant to the challenge of an otherwise lethal dose of *Listeria monocytogenes* in comparison to wild-type mice. This resistance reveals itself as early as 2-3 days after infection, indicating that in innate but not adaptive immunity plays a pivotal role. This is further supported by the enhanced resistance observed in B7-H4KO mice in the absence of recombination-associated gene-1 in which lack of RAG-1 leads to developmental deficiencies resulting in abrogated T, B, and NKT cell production. Enhanced innate immunity in B7-H4KO mice requires neutrophils because depletion of Gr-1+ cells completely abolished the resistance to the infection. Therefore, our data supports B7-H4 as a checkpoint molecule in negative control of innate immunity through growth inhibition of neutrophil progenitors.

In B7-H4KO mice, the gene encoding the majority of the extracellular portion of B7-H4 protein, including entire IgV and IgC domains, is deleted to ensure complete elimination of interactions between endogenous B7-H4 and its putative receptor. As predicted from our knockout construct, the recombinant murine B7-H4 gene could encode only 47 amino acids in the N-terminus, including the 20 amino acid signal peptide (Fig. 1). Therefore, it is unlikely that residual 27 amino acid B7-H4 polypeptide could signal any B7-H4 receptor. To our surprise, ablation of B7-H4 does not have a profound effect on T cell responses to either polyclonal or allogeneic antigen stimulation *in vitro* or *in vivo*. Similar observations have been made in a recent study (Suh et al., 2006). These findings appear to be different from previous studies showing that neutralizing mAb to murine B7-H4 could enhance T cell

responses to several antigens (Prasad et al., 2003; Sica et al., 2003). A potential interpretation for this seemingly contradicting data is that B7-H4 does not influence global T cell responses but it affects specific effector functions downstream of T cell responses. To support this notion, while B7-H4KO mice responded normally to several types of airway inflammatory responses, as well as LCMV and influenza infection, they have slightly enhanced T-cell immune responses to *Leishmania major* infection. This was attributed to a mildly enhanced Th1 response (Suh et al., 2006). However, innate immune responses in this knock-out system were not examined, and importantly, the modulation of T effector cell functions by innate immunity was not conducted. Our experiments indicate that a dominant role of B7-H4 in *Listeria* infection is to suppress neutrophil-mediated innate immunity and this effect could also be more profoundly observed in the absence of T, NKT, and B cells. Our findings thus reveal a new function of B7-H4 as a negative regulator for innate immunity against bacterial infection.

Interestingly, bactericidal functions of neutrophils from B7-H4KO mice are normal while the percentage of neutrophils increased in spleens and other organs after infection, indicating that B7-H4 influences the quantity of available neutrophils available for bacterial eradication and does not appear to significantly modulate the effector function of neutrophils as determined by selective, available functional assay systems. The results from our experiments indicate that B7-H4 inhibits the expansion of neutrophil progenitors. B7-H4KO neutrophil progenitors transit through the cell cycle more rapidly than their wild-type counterparts within culture **(Fig. 6A)**. In addition, B7-H4Ig inhibited the differentiation of Lin- bone marrow cells to neutrophils in the presence of G-CSF *in vitro* **(Fig. 6B)**.

Therefore, B7-H4 appears to directly affect the generation of neutrophils from its progenitors by inhibiting their proliferation. This may contribute, at least partially, to increased resistance to bacterial infection in B7-H4KO mice. However, other factors such as those affecting the entry and exit of neutrophils in lymphoid and peripheral organs could not be excluded at the present time. Shortly after *Listeria* infection (1-2 days), T and B lymphocytes, as well as neutrophils, undergo rapid apoptosis (data not shown), which may be responsible for the decline of neutrophil numbers after a brief expansion **(Supplemental Table 1)**. In DKO mice, apoptosis appears to outweigh expansion. However, the percentage of neutrophils often increased (data not shown) contributing to the resistance observed against *Listeria*.

While our studies using a *Listeria* infection model emphasize the critical role of B7-H4 in the negative regulation of neutrophil-mediated innate immunity, the potential role of B7-H4 on T and B cell responses cannot be completely excluded at the present time. B7-H4KO mice in immunocompetent B6 background have more long-term survivors than DKO mice upon challenge by lethal dose Listeria **(Fig. 2A vs. Fig. 5D)**. The most likely explanation for this result is that both arms of the adaptive immune response, both T and B cells, play an important adjunctive role in thorough clearance of *Listeria*- most likely at a later stage when infection has established itself. An alternative consideration is that B6/B7-H4KO mice have enhanced adaptive immunity in addition to innate immunity due to B7-H4's negative influence on T cells. This observation of enhanced adaptive immunity in B7-H4KO mice could be the tangential consequence of enhanced innate immunity, which may strengthen the T cell response. This remains to be examined experimentally. It is also

evident that DKO mice have significantly more neutrophils than single KO mice in virtually all organs examined so far, even without infection. It is interesting that one of the mechanisms of B7-H4 function in previous experimental models as discussed above is inhibition of cell cycle progression in the adaptive immune system (Sica et al., 2003). It is therefore possible that the lack of B7-H4 on T, B, and/or NKT cells (absence in Rag-1-/- mice) affects normal turnover or homeostasis of neutrophils by a similar or analogous process.

Our results indicate that a mechanism behind resistance to *Listeria* infection in the absence of B7-H4 is due to increased neutrophils in peripheral organs. Although there are slight increases in neutrophil counts in uninfected B6/B7-H4KO mice (Fig. 4B), more significant increases of neutrophils occur upon *Listeria monocytogenes* infection **(Fig. 2C and supplemental Table 1)**. A similar increase was also observed in DKO mice **(Supplemental Table 1)**. In addition, depletion of neutrophils by anti-Gr-1 mAb efficiently eliminated this resistance **(Fig. 3A, 5E and 5F)**. In contrast to the increased number of neutrophils to infection, phagocytosis and respiratory burst, two major bactericidal functions of neutrophils, do not have obvious modifications in the absence of B7-H4 **(Fig. 3C)**. It is clear that while phagocytosis and respiratory burst contribute to neutrophil function, they are not the only functions of significance in neutrophilic bacterial eradication. It is quite possible that other effector systems may be influenced by B7-H4. Our assay systems may not detail those effects. Therefore, our data support a role for B7-H4 in modulating neutrophil levels as the its key mechanism for the resistance to *Listeria* infection.

Previous experiments have shown that B7-H4, upon binding to its putative receptor, inhibits cell cycle progression on T cells (Kryczek, Zou, et al., 2006; Sica et al., 2003). In our cell culture system, division of neutrophil progenitors in WT bone marrow is clearly slower than division from B7-H4KO mice **(Fig. 6A)**. In addition, recombinant B7-H4 inhibited proliferation **(Fig. 6B)** and division of Gr-1+CD11b+ cells **(Fig. 6C)**. These results indicate that B7-H4 also inhibits cell cycle progression of neutrophils. We did not observe significant changes in cell apoptosis in the culture of bone marrow from B7-H4KO mice up to 5 days (unpublished data), thereby excluding the role of cell death. Our data therefore support a direct role of B7-H4 in the inhibition of neutrophils. However, the mechanisms and biochemical details underlying this inhibition is yet to be clarified. It is tempting to speculate that neutrophils express the receptor for B7-H4, thus transmitting inhibitory signal directly to neutrophils. However, the receptor for B7-H4 has not yet been identified, therefore preventing further analysis. While we failed to detect binding of B7-H4Ig fusion protein to the surface of neutrophils (unpublished data), B7-H4Ig inhibits proliferation of Lin- neutrophil progenitors from bone marrow **(Fig. 6B)**. This could be interpreted by either low level expression of B7-H4 receptor or low affinity/avidity of B7-H4Ig fusion protein. Neutrophils do not express B7-H4 in our experiments by RT-PCR, flow cytometry or immunohistochemistry analyses (data not shown), indicating that B7-H4 is provided by other stromal cells in bone marrow.

In summary, neutrophils are the first line of host immune defense against infection. Our findings show an increased resistance to Listeria infection in B7-H4KO mice implicating a new approach to enhance innate immunity against infection by *Listeria* and possibly other

pathogens. Therefore, the method of selectively blocking B7-H4 expression by neutralizing mAb or appropriately engineered B7-H4 protein with antagonistic activity may represent a new approach to increase neutrophils and enhanced innate immunity against pathogen infection.

Areas for future investigation

How might the experiments we conducted inform and direct us toward the next stage of investigation? One intriguing possibility involves tumor cells and the tumor microenvironment. The tumor microenvironment is composed of numerous cells, both immune and non-immune, that shape and are shaped by their interactions with cancer cells. In addition to the role of lymphocytes, NK cells, fibroblasts, macrophages, endothelial cells, and pericytes in influencing the tumor microenvironment, neutrophils are becoming established as an influential member of this unique milieu.

Neutrophils and chronic inflammation are inextricably linked and the association between inflammation and cancer development is irrefutable (Coussens & Werb, 2002; Mantovani, Allavena, Sica, & Balkwill, 2008). Examples of inflammatory disease states associated with neoplasia include human papilloma virus (HPV) infection resulting in cervical cancer, *Helicobacter pylori* infection and the development of both gastric cancer and lymphoma, and the increased risk of colon cancer as a result of chronic inflammation by ulcerative colitis (Mantovani et al., 2008). Leukocytes, including neutrophils, can induce DNA damage in host tissue by the generation and release of reactive oxygen and nitrogen species that form peroxynitrite, a mutagenic agent (Maeda & Akaike, 1998). Furthermore, chronic inflammation and tissue damage lead to an increased rate cell turnover that results in stochastic mutagenic events culminating in malignant transformation aside from the mutagenic effects of the aforementioned reactive species.

Conversely, studies show that the use of non-steroidal anti-inflammatory medications is believed to reduce the risk of neoplastic development and tumor progression. In a

population-based cohort study of patients free of cancer and colorectal adenoma, the incidence rate of colorectal cancer was reduced in patients on nonaspirin NSAIDS with an adjusted relative risk of 0.5 (Garcia-Rodriguez & Huerta-Alvarez, 2001). Interestingly, the risk reduction disappeared one year after medication discontinuation suggesting once again that inflammation even at low levels is an ever present phenomenon at the epithelial layer (Garcia-Rodriguez & Huerta-Alvarez, 2001). Reduction in colorectal cancer risk was only seen in users of aspirin at doses of 300mg daily, a dose that is higher than that usually prescribed for prophylaxis against heart disease (Garcia-Rodriguez & Huerta-Alvarez, 2001). Maybe not surprisingly, the protective effect was shown for gastric and esophageal cancer as well (Baron & Sandler, 2000). The proposed mechanism by which this occurs is through the effect of NSAIDs on cyclo-oxygenase inhibition which decreases production of prostaglandin mediators which play a critical role in driving inflammation.

While it is possible that neutrophils contribute to combating and eradicating tumor cells in select circumstances, it is more likely that cancer cells harness neutrophils' pleiotropic functions to assist in modifying the tumor microenvironment, enhancing their growth, invasiveness, and ability to metastasize. This would seem to make intuitive sense as neutrophils play an essential, intrinsic role in wound healing by storing and selectively releasing large cargoes of enzymes for tissue breakdown while also possessing sophisticated systems to ingest and destroy foreign and native tissue- all functions that can be readily manipulated for use by tumor cells at their discretion.

Do experimental examples or clinical studies exist to inform us of the negative impact of neutrophils in cancer? It is irrefutable that neutrophils are present in within tumors. More

importantly, the presence of intratumoral neutrophils has been shown to be a negative prognostic factor in recurrence free, cancer-specific, and overall survival in patients with localized clear cell renal cancer (Jensen et al., 2009). Positive correlations have been indentified between the blood and recurrence-free survival (Perez et al., 2013). Patients with low neutrophil-to-lymphocyte ratio have improved 1- and 5-year recurrence-free survival compared to patients with high neutrophil-to-lymphocyte ratio. A multivariate analysis of patients with bronchioalveolar carcinoma revealed neutrophil percentage in the bronchioalveolar fluid as an independent predictor of clinical outcome (Bellocq et al., 1998). The use of an anti-granulocytic antibody to deplete neutrophils was shown to inhibit tumor growth as well as decreased the incidence of metastasis of a murine fibrosarcoma cell line (Pekarek, Starr, Toledano, & Schreiber, 1995; Tazawa et al., 2003).

The presence of tumor-associated neutrophils and worse prognosis could lead one to theorize that cancer cells actively pursue the assistance of neutrophils. Could tumor cells have developed mechanisms to recruit neutrophils to the tumor microenvironment through the release of cytokines and chemokines in much the same way as injured host tissue? Leukocyte chemoattractant molecules, such as IL-8, are expressed in gastric carcinoma and their level of expression was correlated with more aggressive gastric cancer subtypes and the presence of increased number of tumor vessels (Eck, Schmausser, Scheller, Brandlein, & Muller-Hermelink, 2003). IL-8 binds CXCR-2 receptors on endothelial cells and neutrophils, thereby promoting angiogenesis and neutrophil recruitment. In bronchioalveolar carcinoma, it has been shown that the cancer cells recruit neutrophils through the expression of IL-8 which results in neutrophils releasing biologically active hepatocyte growth factor

(HGF), a cytokine associated with poor prognosis in several cancers (Bellocq et al., 1998; Wislez et al., 2003).

This recruitment of tumor-associated neutrophils may be linked to mutational events in specific signaling pathways within tumor cells. Many tumors, including lung adenocarcinoma, ductal carcinoma of the pancreas, and colorectal cancer, express mutant K-ras which has been shown to induce IL-8 expression (Sparmann & Bar-Sagi, 2004). The supplementation of human neutrophils after melanoma cell injection into nude mice was associated with a 3-fold increase in tumor cell retention. The melanoma cells expressed high levels of IL-8 and increased beta-2 integrin expression which enhanced anchoring of melanoma cell to endothelium (Huh, Liang, Sharma, Dong, & Robertson, 2010).

Secreted enzymes from neutrophils may also contribute to tumor progression. Using tumor extracts from 463 breast tumors, high elastase levels were associated with a poor response rate to tamoxifen treatment compared with those with low levels. High neutrophil elastase levels were correlated with a poor progression-free survival and post-relapse survival (Foekens et al., 2003a). Levels of neutrophil elastase have been correlated with lengths of metastasis-free survival as well (Foekens et al., 2003b). Furthermore, in a mouse model of lung adenocarcinoma, it was shown that tumor burden was reduced in mice lacking neutrophil elastase and led to longer survival. It was shown that neutrophil elastase gained access to the tumor endosomal compartment and degraded insulin receptor substrate-1 allowing for increased interaction between phosphatidyl inositol 3-kinase and the platelet-derived growth factor receptor (Houghton et al., 2010). Matrix metalloproteinase-9 (MMP-9), expressed by neutrophils, macrophages, and mast cells, has been shown to contribute to

squamous carcinogenesis (Coussens et al., 2000). Transgenic mice lacking MMP-9 exhibited reduced keratinocyte proliferation in the human papilloma virus 16 epidermal tumor model.

It is clear from these studies that neutrophils are not only found at the site of tumor development and progression, but that they are active participants in tumor progression, recruited and manipulated into a polarized cell functioning hand in hand with cancer cells. The presence of activated neutrophils correlates with expedited tumor progression and worse outcomes. The studies on the role of neutrophil elastase in releasing and activating PI3K would suggest that tumor cells are driven toward increased rates of proliferation through their interaction with neutrophils and activation of selective neutrophil functions. Further connecting this intimate link between cancer cell growth and neutrophils are the findings of chemokine expression related to K-ras mutational status. No longer can it be assumed that neutrophils play an innocent bystander effect at the tumor microenvironment or that their main role is tumoricidal. While our studies indicate that B7-H4 modulates the quantity of neutrophils and not the quality, it is very well possible that the increased availability of neutrophils for tumor cells facilitates a pro-tumor environment.

It would appear then that therapeutic modulation of neutrophil numbers could have a bearing on tumor growth rate and virulence. Neutrophils could be therapeutically modulated at many different checkpoint stages. To some extent this is occurs routinely in the clinical setting in chemotherapy patients. Most chemotherapy backbones rely on drugs that affect bone marrow production. Clinically, patients develop neutropenia, and in many instances, profound neutropenia may develop resulting in severe microbial infections that require neutrophil replenishment through the use of recombinant G-CSF (Neupogen). Unfortunately,

because of the toxic and variable effect of chemotherapy, this form of treatment cannot be reliably used to suppress neutrophil production for an extended period. Antibody depletion of circulating neutrophils (Pekarek et al., 1995) is another form of therapy that has inherent limitations for some of the same reasons as chemotherapy. Instead, neutrophil progenitors in the bone marrow could be blocked using recombinant soluble B7-H4. While we are unsure of the side effects of this potential therapy, it would certainly mitigate or entirely eliminate the toxic side effects that plague the long-term viability of systemic chemotherapy. Our studies suggest a possible role for B7-H4 in altering tumor dynamics through its effect on neutrophil homeostasis.

B7-H4 has been identified in a variety of cancer types. Interestingly, B7-H4 expression coincides with the cancers described above that are influenced by neutrophils. B7-H4 expression was detected in the cytoplasm and on the membrane of primary serous, endometrioid, and clear cell carcinomas (I. Simon et al., 2007; Tringler et al., 2006). Hyperplastic and malignant endometrial epithelium showed overexpression of B7-H4 (Miyatake et al., 2007). B7-H4 was found in invasive ductal carcinomas and associated with decreased number of tumor infiltrating lymphocytes (Mugler et al., 2007). B7-H4 was detected in 92% of pancreatic ductal adenocarcinomas, a cancer well known to have K-ras mutations (Awadallah et al., 2008). B7-H4, along with B7-H3 were found to be highly expressed in human prostate cancer and associated with disease spread and poor outcome (Zang et al., 2007). 60% of renal cell carcinoma tumor specimens exhibited B7-H4 expression and the presence of this molecule correlated with adverse clinical and pathologic features. Patients with tumors expressing B7-H4 were three times more likely to die from

RCC compared to tumors lacking B7-H4 (Krambeck et al., 2006). Cultured tumor cells from human gliomas and medulloblastomas were found to express B7-H4 (Yao et al., 2008). B7-H4 expression was found in 45% of gastric cancer specimens and was positively correlated with cancer invasiveness and lymph node metastasis (Jiang et al., 2010). The median overall survival time of patients with lower B7-H4 expression was 13 months longer than that of patients with higher expression (Jiang et al., 2010). Despite this association between B7-H4 expression, malignant transformation, and adverse outcome, the effect of B7-H4 on cancer cells is unknown. It will be important experimentally determine whether the relationship seen between B7-H4 and oncologic outcome is related to neutrophil, and not T cell, biology.

Our results suggest a role for B7-H4 on neutrophil numbers that may bear an impact on malignant progression. It will be interesting to correlate the presence and levels of B7-H4 expression in these tumor types with the presence of neutrophil numbers and function. One possibility that would need to be determined is whether B7-H4 is getting cleaved from the cell surface and acting as a soluble blocking agent. Studies in our lab with rheumatoid arthritis provide hints that this could in fact be the case. Soluble B7-H4 was shown to be more commonly elevated in the sera of patients with rheumatoid arthritis and was associated with increased disease severity (Azuma et al., 2009). Therefore, it would be essential to collect serum samples and measure levels of soluble B7-H4 in patients with malignancy. It would also be interesting to find out if neutrophils that are recruited to Ras mutated tumor sites by the expression of the chemokine IL-8 by Ras mutant cancers results in the release of proteases that cleave active B7-H4 from the cell surface. Our immunohistochemical studies indicate that at least one place for B7-H4 expression is on the surface of various ductal

tissues. These ductal tissues are highly prone to neoplastic development. Quite possibly, cancer cells recruit and activate neutrophils leading to cleavage and soluble expression of B7-H4 resulting in a positive feedback loop that promotes further neutrophil progenitor proliferation for recruitment to tumor sites. Modulation of soluble B7-H4 through the use of blocking antibody or small molecule agents or through the blockade of its cognate receptor could result in a dialing down of the neutrophilic response and tumor progression. It would also be important to attempt to address this by transplanting tumor into single and double knockout mice through tumor transplantation experiments. Furthermore, it would be important to determine if released neutrophil products including neutrophil elastase and/or specific matrix metalloproteases assist directly in this process. There are numerous available metalloprotease inhibitors that may then be used to modulate soluble B7-H4 levels.

Materials and methods

Antibodies, recombinant proteins, and flow cytometry analysis:

Primary and secondary antibodies against murine Gr-1 and CD11b, which are directly conjugated with FITC, PE, or APC, were purchased from BD Pharmingen (San Diego, CA) or eBiosciences (San Diego, CA). Non-conjugated primary antibodies were purified from hybridoma culture supernatant. All cells were stained using standard protocols as previously described and were analyzed on a FACSCalibur flow cytometry (Sica et al., 2003). The data was analyzed with Software CellQuest (BD) or FlowJo (Tree Star, Inc., Ashland, OR). For *in vivo* studies, mAbs were prepared and purified as previously described (Sica et al., 2003). Anti-Gr-1 hybridoma (RB6-8C5) was a generous gift from Dr. Hans Schreiber at the University of Chicago. Depleting antibodies against NK cells (clone NK1.1) and plasmacytoid dendritic cells (clone PDCA1) were described previously (Blasius et al., 2006; Melero, Johnston, Shufford, Mittler, & Chen, 1998). Control mouse IgG, rat IgG, and hamster IgG were purchased from Sigma (St. Louis, MO) and further purified as previously described (Sica et al., 2003). Carrageenan was purchased from Sigma. All cell culture media and antibiotics were purchased from Cellgro (Herndon, VA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT).

Mice:

6~8-week-old B6 mice were obtained from the Jackson Laboratory. RAG-1 KO mice were purchased from Taconic Farms. Both female and male mice were used for the

experiments. All mice were housed under specific pathogen-free conditions in the Johns Hopkins Animal Facility with all protocols approved by the Institutional Animal Care and Use Committee.

The general strategy to generate gene KO mice by homologous recombination was previously described (F. Dong et al., 1995; Tamada et al., 2002). To generate B7-H4KO mice, a 5.09 kb DNA fragment upstream of the IgV domain (exon 3) of the murine B7-H4 genomic DNA was PCR amplified from a 129SvJ bacterial artificial chromosome (BAC) library (Invitrogen, Carlsbad, CA) and was cloned into the 5'-arm position of the pKO scrambler vector NTKV-1907 (Stratagene, La Jolla, CA). A 5.57 kb DNA fragment downstream of the IgC domain (exon 4) of B7-H4 genomic DNA was PCR amplified from the same library and was cloned into the 3'-arm position of the same vector to generate a targeting plasmid resulting in removing IgV and IgC domains from the B7-H4 gene (Fig. 1A). The targeting fragment containing the 5'- arm and the 3'- arm sequences of the B7-H4 gene, a positive selection marker NEO, and a negative selection marker TK was transfected into 129Sv/E embryonic stem (ES) cells. ES cell transfectants underwent neomycin drug selection. The targeted clones were identified by Southern blot analysis using a 3′ external probe (Fig. 1B). Chimeric mice were produced by injection of targeted ES cells into blastocysts of B6 hosts. Heterozygous B7-H4 (+/-) mice were obtained from breeding chimeric mice with B6 mice. PCR analysis was employed to distinguish the wild-type and deficient B7-H4 allele. The sequences of the three PCR primers are: (1) 5'-GTTAGATAGGGTCTCACTGGGTAGC, (2) 5'-CCTACAGCCTTCAGTATGCCAGAGA, (3) 5'-AGACTAGTGAGACGTGCTACTTCCA. Homozygous mice were produced by

back-crossing to B6 for more than ten generations before use for further analysis. B7-H4 KO/RAG-1 KO mice were obtained by backcrossing B7-H4 KO and RAG-1 KO mice.

Cell depletion in vivo:

Wild-type or B7-H4KO mice of 6-9 weeks old were used for all experiments. All depletion reagents and control reagents were administrated by intraperitoneal injection in 0.5 ml volume. Depletion of subset of cells was confirmed by flow cytometric analysis with subset-specific antibody. At day 0, mice were given $1x10^6$ CFU of Listeria by intraperitoneal injection. Two days after infection, mice were sacrificed and the liver Listeria load was evaluated by colony plating assay. For neutrophil depletion, 150 ug/mouse of anti-Gr-1 antibody (clone RB6-8C5) or Rat IgG was used at day -1 before Listeria infection (day 0). For macrophage depletion: Carrageenan at 2 mg/mouse or 0.5 ml PBS was applied on day -3 and day -1. For NK cell depletion, 500 ug/mouse of anti-NK1.1 antibody (clone PK136) or mouse IgG was administered on day -1. For plasmacytoid dendritic cell depletion, 250 ug/mouse of anti-pDC antibody (clone PDCA-1) or Rat IgG was injected on day -6, -3, and -1.

Listeria infection and colony counting:

Listeria monocytogenes strain DP-L4056 was kindly provided by Dr. Thomas W. Dubensky Jr. from Cerus Corp. To prepare *Listeria* stock, *Listeria* cells were grown in DIFCO *Listeria* Enrichment Broth (Becton Dickinson Co., Sparks, MD) to 0.8-1 at OD600 nm. Culture was harvested by centrifugation and was washed twice with PBS. Pellets were

then re-suspended in stock solution (PBS with 15-20% glycerol) and aliquoted to 200 ul per microtube for storage at -80˚C. The colony-forming units (CFU) of Listeria stock were determined by counting colonies of series dilutions of the aliquots growing on BBL CHROMagar *Listeria* plates (Becton Dickinson Co., Sparks, MD). Prior to infection, *Listeria* stock was thawed and diluted in PBS to appropriate concentration of CFU/ml and applied to mice or cells as indicated. 6-8 week old mice were infected by intraperitoneal or intravenous (for colony formation assay only) injection of indicated CFU of *Listeria*. At indicated time points post-infection, a piece of mouse liver or spleen was cut, weighed, and ground in PBS. The liver suspension was plated on BBL CHROMagar *Listeria* plates or on agar plates of *Listeria* Enrichment Broth. Colonies were counted 2 days post plating, and adjusted to CFU/g of liver or spleen.

Listeria infection of neutrophils in vitro:

Neutrophils were isolated similar to the methods previously described (L. Y. Chen et al., 2003). Briefly, mice were injected i.p. with 3% thioglycollate broth. Four to five hours post injection, peritoneal cavities of each mouse were washed with 5 ml PBS and cells were harvested by centrifugation. By this method, >90% harvested cells are Gr-1^{+}CD11b+ neutrophils. 1 x 10^{6} neutrophils were incubated with 1 x 10^{8} CFU of LM for 10 min at 37˚C. The cultures were terminated by adding Penicillin-Streptomycin (Cellgro). Subsequently, cells were harvested by centrifugation, plated in 96-well plates. The plates were incubated at 37˚C and harvested at indicated time points. Cells were lysed immediately by resuspending

in 1 ml of sterile water. Cell lysates or diluted cell lysates were plated on agar plates of Listeria Enrichment Broth for colony counting.

Respiratory burst and phagocytosis of neutrophils:

Neutrophil phagocytic activity and oxidative burst activity were measured as described (Radsak et al., 2003; Radsak, Salih, Rammensee, & Schild, 2004). Briefly, 1 x 10^6 neutrophils were incubated with 5 x 10^7 of red-fluorescent micro-beads (Polysciences, Inc. Warrington, PA) and 25 uM of H2DCFDA (2',7',-dihydrochlorofluoresein diacetate, Sigma-Aldrich) for 30-60 min at 37˚C. Cells were washed twice with FACS buffer (1% FBS in PBS) and fixed in 1% paraformaldehyde in PBS. Viable cells were first gated and the respiratory burst activity was quantified by green mean fluorescence intensity (MFI) and phagocytotic capacity was quantified by red MFI. The number in upper right gate represents % of cells with both phagocytic and oxidative capacity.

Assays for neutrophil growth inhibition:

BM cells were aspirated and prepared as previously described (Wilcox et al., 2004). Cells were then incubated in 2 uM of carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA) in PBS for 10 minutes. After extensive washing, cells were plated at 2.5 x 10^5/well. Cells were harvested at indicated time points and cell numbers were counted with Beckman Coulter Counter (Beckman, Fullerton, CA). The numbers of dividing neutrophils in each sample were decided by gating Gr-1+ mAb and CFSE dilution using flow cytometry analysis as shown previously (Sica et al., 2003). Lin- BM cells were prepared as

described previously (Mermel et al., 2006). Briefly, BM cells were collected from wild-type B6 mice (8-10 wks). Lineage+ cells were removed by PE-labeled anti-Gr-1, anti-CD11c, anti-Ter119, anti-CD11c, anti-CD3, anti-B220, and anti-CD19 (PharMingen) followed by MACS anti-PE microbeads and LD columns (Miltenyi Biotec). Lin^- BM cells were then labeled with CFSE and were plated in alpha-MEM supplemented with 20% FBS, 100 ng/ml SCF, 10 ng/ml G-CSF. Recombinant B7-H4 fusion proteins (B7-H4Ig) were added at the indicated concentrations. Cells were harvested daily for cell counting and flow cytometry analysis.

Statistical analysis:

Statistical analysis was performed by Student's T-test method for two parameters and Anova test for multiple parameters.

Figure Legends

Figure 1: Generation of B7-H4 KO mice

(A) Strategy for disruption of the B7-H4 gene. A 4.7 kb DNA fragment containing exons encoding the IgV and IgC domains of murine B7-H4 gene is substituted by a 1.7 kb fragment encoding the neomycin resistant (Neo) gene. Closed boxes represent B7-H4 coding exons. Lines between exons represent intron sequences. Open boxes represent untranslated exons. The Neo is represented by a shaded box.

(B) Screen of targeted ES cells by Southern blot analysis. Genomic DNA of ES cells were digested with SpeI and probed with a fragment (Probe) as indicated in (A).

(C) Lack of B7-H4 gene expression in B7-H4 KO mice. Liver RNAs were prepared from B7-H4 KO (-/-) and littermates (+/+). RT-PCR was performed with primers corresponding to the IgV domain of B7-H4 gene. RT-PCR of actin gene was used as positive control in the analysis.

Figure 2: B7-H4KO mice are resistant to Listeria infection with an accompanied increase in neutrophils

(A) B7-H4 KO mice are more resistant to Listeria infection than wild type mice. Fifteen WT or B7-H4 KO mice were i.p. injected with 2 x 10^6 of CFU of Listeria. Survival of mice was monitored daily. Data represents a pool of two independent experiments. *$P<0.05$.

(B) Decreased Listeria colony formation in spleens from B7-H4KO mice. WT or B7-H4KO mice (KO) were i.v. injected with 1 x 10^5 CFU of Listeria. Day 1 to 3 post infection, whole spleens were weighted, homogenized in 5 ml of PBS, and tissue lysates were plated on

agar plates of Listeria Enrichment Broth for colony counting. The results from individual mice are presented. Data represents at least 3 independently performed experiments. *P<0.05.

(C) Spleen neutrophils increase after Listeria infection in B7-H4 KO mice. Three B7-H4KO or littermate control mice were grouped and i.v. injected with 1 x 10^5 of CFU of Listeria. Splenocytes were isolated 1, 2 and 3 days post infection and were stained with Gr-1 and CD11b mAb. The same numbers of littermates were included as controls. Each point represents results from pool of three mice. Data represents at least 3 independent experiments. *P<0.05.

Figure 3: Increased neutrophils in B7-H4KO mice are required for resistance to infection but the bactericidal functions of neutrophils are normal

(A) Wild-type (Wt) B6 mice or B7-H4KO (KO) mice of 6-9 week-old at groups of 3 were used for all experiments. The mice were injected i.p. with anti-Gr-1, anti-NK1.1 and anti-pDC mAb to deplete neutrophils, NK cells and plasmacytoid cells, respectively. Injection of Carrageenan was used to deplete macrophages. Depletion of subset of cells was confirmed by flow cytometry analysis. After depletion, mice were challenged with 1x10^6 CFU of Listeria by i.p. injection. Two days after Listeria infection, mice were terminated and the liver Listeria load was evaluated by colony plating assay. Listeria colonies in each mouse were shown. The data is expressed as CFU/gram of liver. Data are representative of at least 3 independent experiments for each treatment. *P<0.05. Open symbols: control reagents, close symbols: treatment by antibody or Carrageenan.

(B) Neutrophils from B7-H4 KO mice have normal capacity to intake and digest Listeria. Three mice of B7-H4 KO or littermate control mice were i.p. injected with l ml of 3% thioglycollate. Mice were terminated 4-5 hours after injection and peritoneal cells were harvested and incubated with Listeria for 10 minutes. Listeria infection was stopped by washing cells with medium containing antibiotics in large volumes. Neutrophils were then cultured. At indicated time points, cultured cells were lysed and the cell lysates were plated for CFU counting as described in Materials and Methods.

(C) Normal respiratory burst and phagocytosis of neutrophils from B7-H4KO mice. Neutrophils were harvested as described in (B). 1 x 10^6 of neutrophils were incubated with 5 x 10^7 of red-fluorescent micro-beads and 25 uM of H2DCFDA for 30-60 minutes at 37˚C. Red and green fluorescence were analyzed by flow cytometry and the results were expressed as % of cells with oxidative and/or phagocytic capacity. The data is representative of 2 separate experiments. There is no statistically significant difference between neutrophils with both oxidative and phagocytic capacity from WT and B7-H4KO mice.

Figure 4: Phenotypes of B7-H4xRAG-1 double knockout mice (DKO)

(A and B) Enlarged and increased weight of spleens from the DKO mice. Spleens were obtained from 6-8 week old mice and the representative of their appearance (A) and weights of average of 4 spleens (B) are shown. DKO: B7-H4 x RAG-1 double KO; RKO: RAG-1 KO; B7-H4 KO: B7-H4 KO mice in B6 background; WT: normal B6. *P<0.05.

(C) Flow cytometry analysis of Gr-1+CD11b+ neutrophils in the absence of B7-H4. Cells were isolated from bone marrows, spleens, livers, and peripheral blood mononuclear

cells (PBMC) of indicated mice and stained with Gr-1 and CD11b mAb. Data was analyzed by flow cytometry.

Figure 5: Innate resistance against Listeria infection in B7-H4KO mice is independent of adaptive immunity

(A) Altered colony formation of Listeria in liver from B7-H4 x RAG-1 DKO mice. Mice were i.p. injected with 3 x 10^6 CFU of *Listeria*. Forty-eight hours post infection, liver tissues at 0.2 mg from each mouse were homogenized in 10 ml of PBS. 50 ul of tissues were plated on agar plates of *Listeria* Enrichment Broth for colony counting. Data represents at least 3 independently performed experiments. DKO, B7-H4 X RAG-1 double KO; RKO: RAG-1 KO littermate.

(B and C) Enumeration of *Listeria* colonies in livers (B) and spleens (C) on agar plates. Livers and spleens were prepared from RKO or DKO mice and cultivated as described in (A) to quantify *Listeria* colonies. Data represents 5 independently performed experiments. At day 3, differences of *Listeria* colonies in the organs from RKO and DKO are significant. *$P<0.05$.

(D) Resistance of DKO mice to *Listeria* infection. Five male RKO and eight male DKO were i.p. injected with 4 x 10^6 CFU of *Listeria*. Survival of mice was monitored daily for 15 days. Data represents 2 independently performed experiments. $P<0.05$

(**E and F**) Gr-1+ cell depletion eliminated resistance to *Listeria* infection equally in both RKO and DKO mice. Three mice of RKO or DKO were i.p. injected with 250 ug anti-Gr-1 mAb or isotype control Rat IgG 24 hours prior to *Listeria* infection. Mice were then i.v.

injected with 0.1 x 10^6 CFU of *Listeria*. Twenty-four hours post infection, mice were terminated and *Listeria* in liver was counted as described previously. The data is expressed as CFU/gram of liver (E) and spleen (F). Data represents 2 independent experiments. *Significantly different from the control (Cont. mAb), $P<0.05$). **Significantly different from other control Ab group. No significant differences were found in anti-Gr-1 mAb-treated groups between RKO vs. DKO, $P>0.05$.

Figure 6: B7-H4 inhibits growth of neutrophil progenitors from bone marrow

(**A**) B7-H4 deficient neutrophil progenitors have increased cell division. Two x 10^6 of BM cells from indicated mice were labeled with CFSE and cultured for 3 days. Cells were harvested and doubly stained with anti-Gr-1/CD11b mAb. The dilution of CFSE in gated Gr-1+CD11b+ neutrophils was analyzed by flow cytometry. Data represents at least 3 independently performed experiments.

(B) BM was harvested by flushing both femoral bones of wild-type B6 mice. Lineage+ cells were removed by PE-labeled anti-Gr-1, anti-CD11c, anti-Ter119, anti-CD11c, anti-CD3, anti-B220, and anti-CD19 (PharMingen) followed by MACS anti-PE microbeads and LD negative selection columns (Miltenyi Biotec). The c-Kit+ Lin- Sca+ cells in the BM were quantified and normally with 75-85% purity. Gr-1+/CD11b+ cells were completely depleted and were plated with 100 ng/ml SCF, 10 ng/ml G-CSF and 40 ug/ml B7-H4Ig. Cells were harvested on 1-3 days for cell counting. The results were presented as triplicates of mean numbers with standard deviation. The data is a representative of four independent experiments. *$P<0.05$.

(C) Lin- BM cells were prepared and in vitro differentiated as described in (B). Cells were harvested from day 1 to day 3 and analyzed by staining with anti-Gr-1 and anti-CD11b mAb. Upper panel: treated by control Ig; Middle panel: treated by B7-H4Ig. Cell division was also monitored by flow cytometry analysis of CFSE dilution (lower panel). Close symbol: control Ig; Open symbol: B7-H4Ig.

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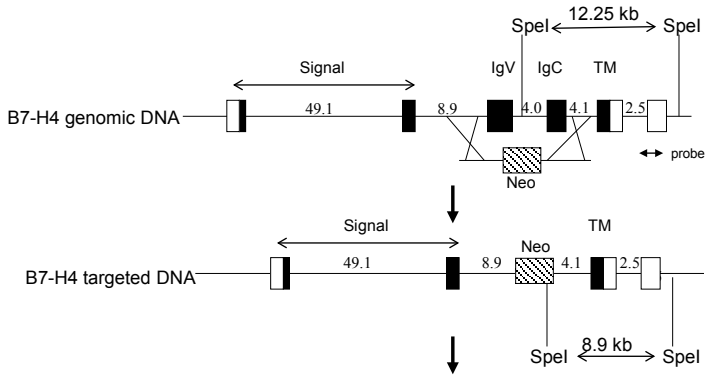
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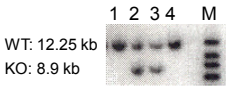
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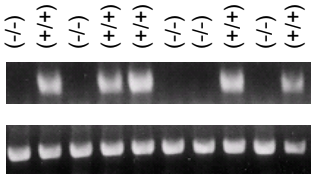

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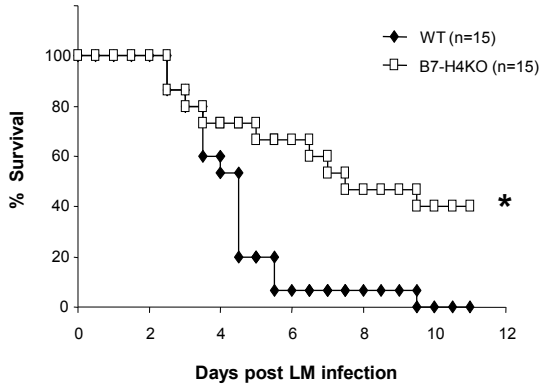
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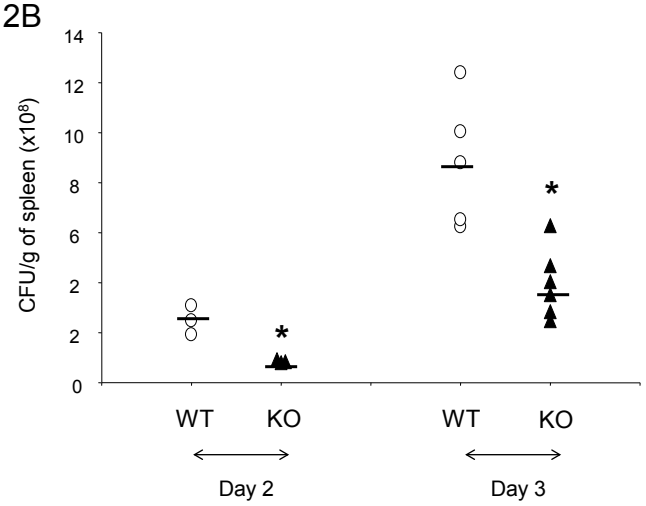

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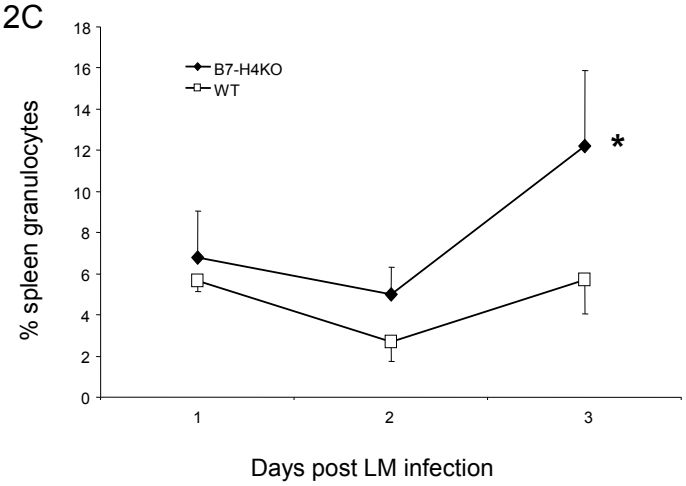
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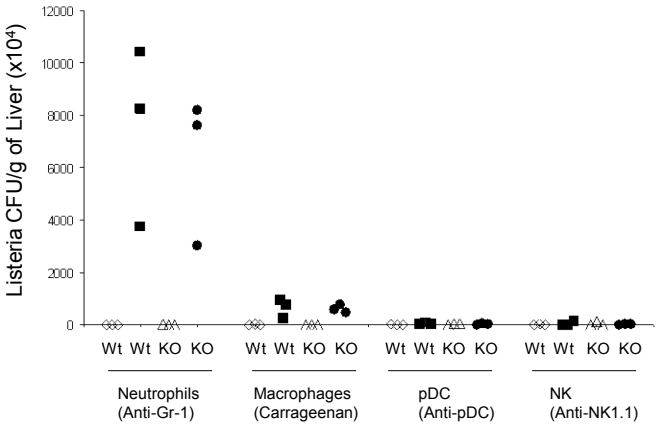
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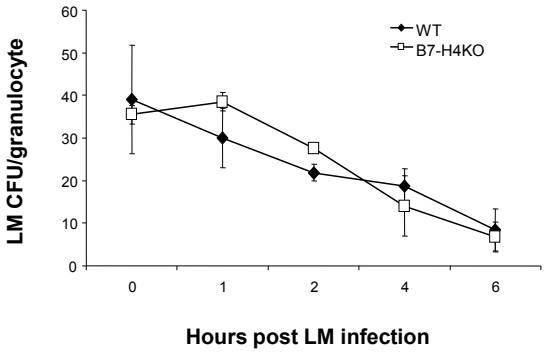
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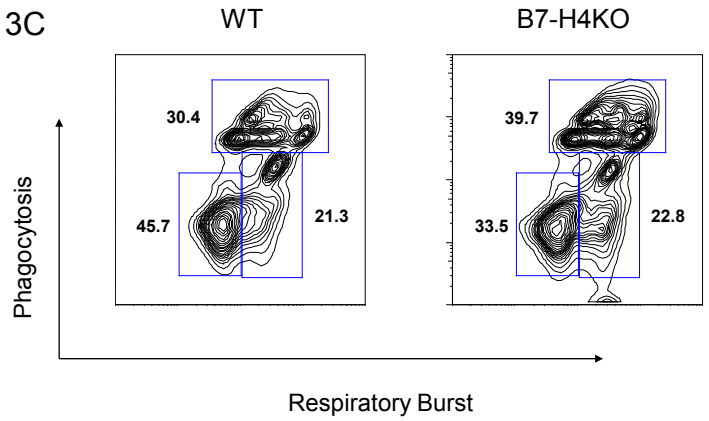
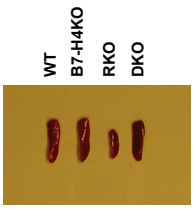
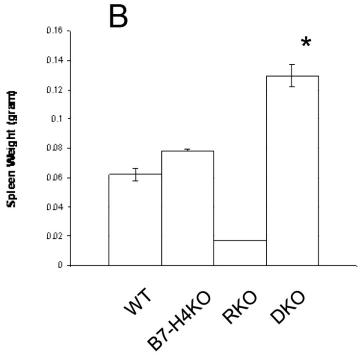
6
Hours post LM infection

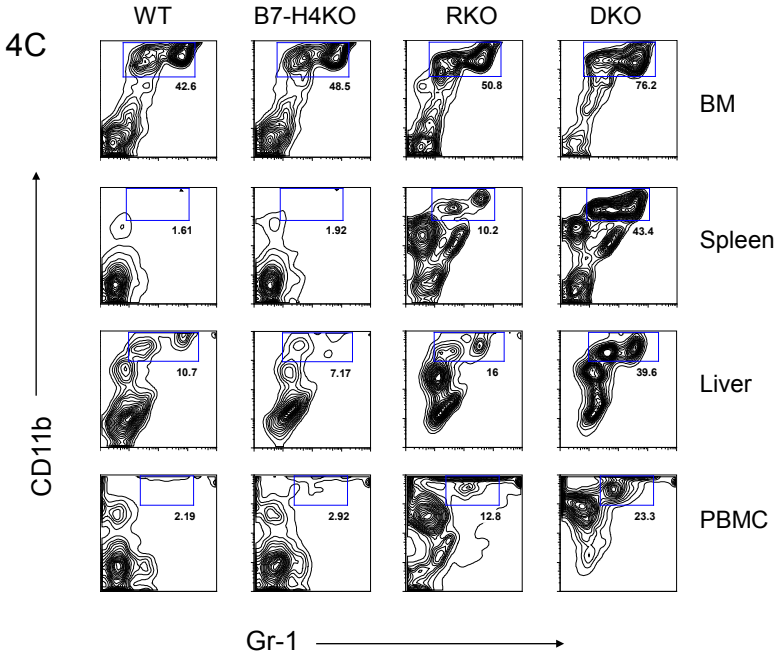

3C
WT
B7-H4KO
30.4
45.7
21.3
39.7
33.5
22.8
Phagocytosis
Respiratory Burst

4A

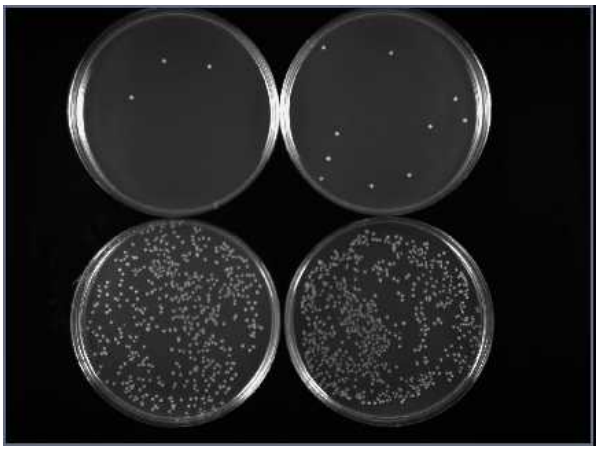

WT
B7-H4KO
RKO
DKO

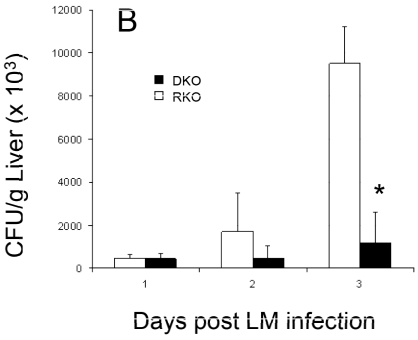
B

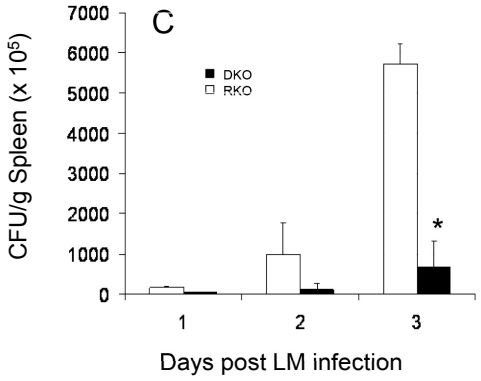

Spleen Weight (gram)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
WT
B7-H4KO
RKO
DKO

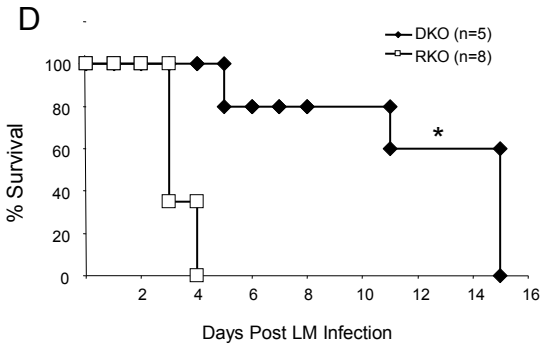

4C
WT
B7-H4KO
RKO
DKO
BM
Spleen
Liver
PBMC
42.6
48.5
50.8
76.2
1.61
1.92
10.2
43.4
10.7
7.17
16
39.6
2.19
2.92
12.8
23.3
CD11b
Gr-1

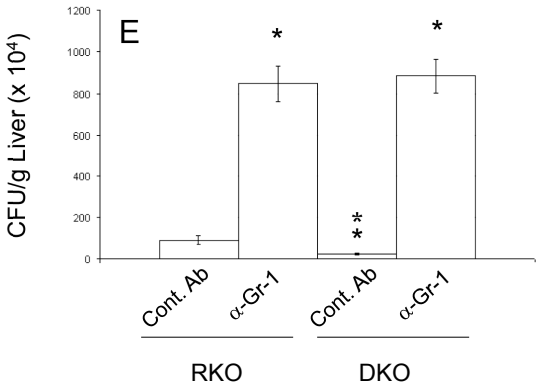
5A

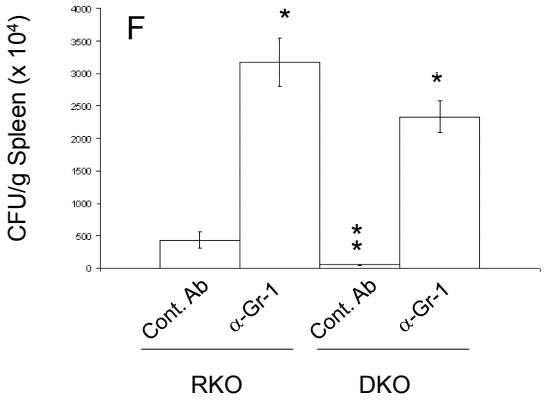



B
DKO
RKO
CFU/g Liver (x 10³)
12000
10000
8000
6000
4000
2000
0
1
2
3
*
Days post LM infection

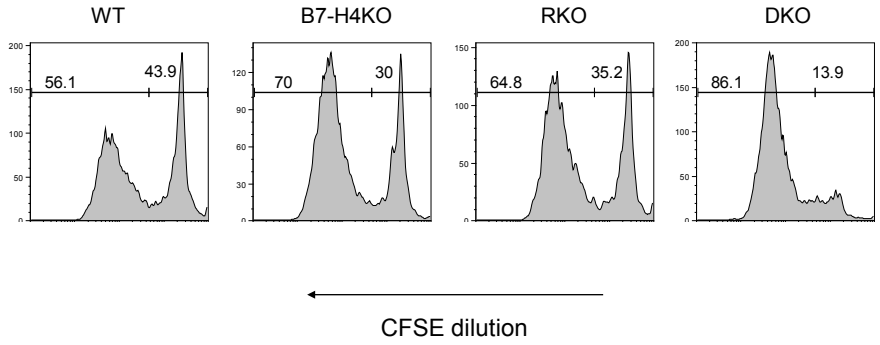

C
DKO
RKO
CFU/g Spleen (x 10^5)
7000
6000
5000
4000
3000
2000
1000
0
1
2
3
*
Days post LM infection


D
DKO (n=5)
RKO (n=8)
100
80
60
40
20
0
% Survival
*
2
4
6
8
10
12
14
16
Days Post LM Infection

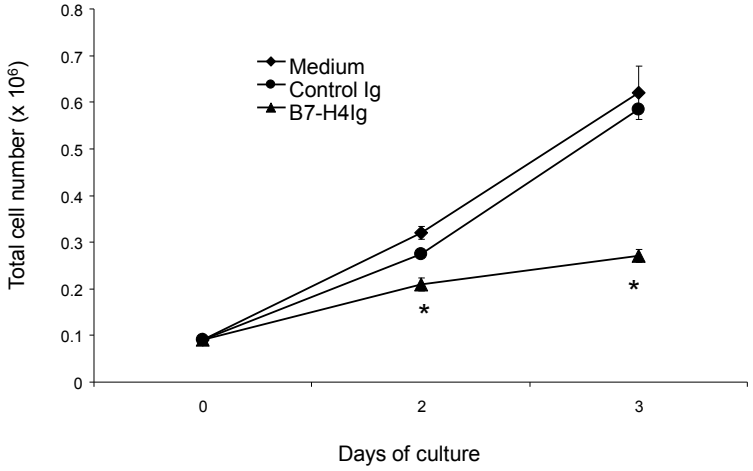

E
CFU/g Liver (x 10⁴)
1200
1000
800
600
400
200
0
*
*
**
Cont. Ab
α-Gr-1
Cont. Ab
α-Gr-1
RKO
DKO

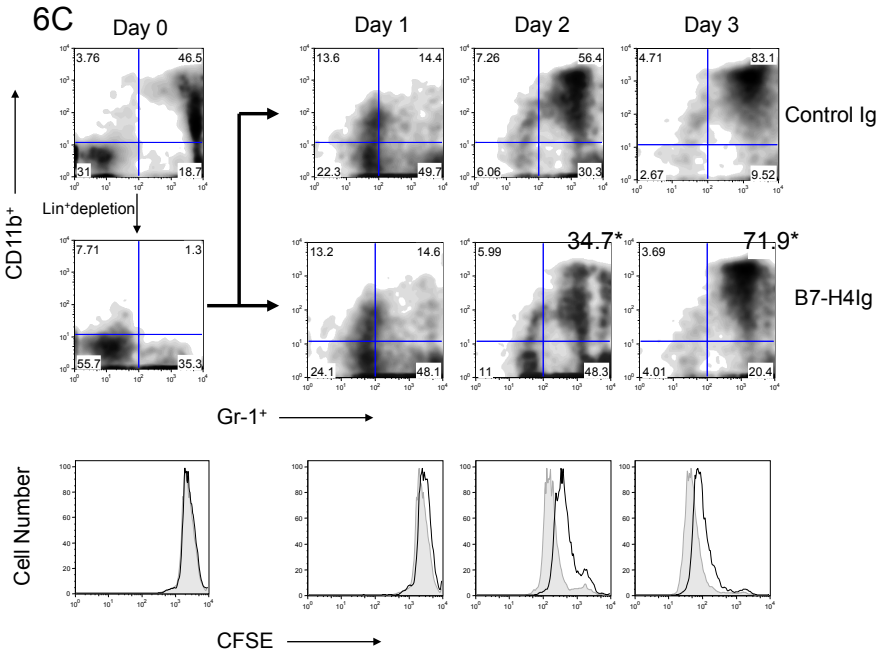

F
CFU/g Spleen (x 10⁴)
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
**
Cont. Ab
α-Gr-1
Cont. Ab
α-Gr-1
RKO
DKO

6A



6B


Total cell number (x 10^6)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Medium
Control Ig
B7-H4Ig
*
*
0
2
3
Days of culture


6C
Day 0
Day 1
Day 2
Day 3
3.76
46.5
31
18.7
13.6
14.4
22.3
49.7
7.26
56.4
6.06
30.3
4.71
83.1
2.67
9.52
Control Ig
CD11b+
Lin+depletion
7.71
1.3
55.7
35.3
13.2
14.6
24.1
48.1
5.99
34.7*
11
48.3
3.69
71.9*
4.01
20.4
B7-H4Ig
Gr-1+
Cell Number
CFSE

Supplemental Table 1

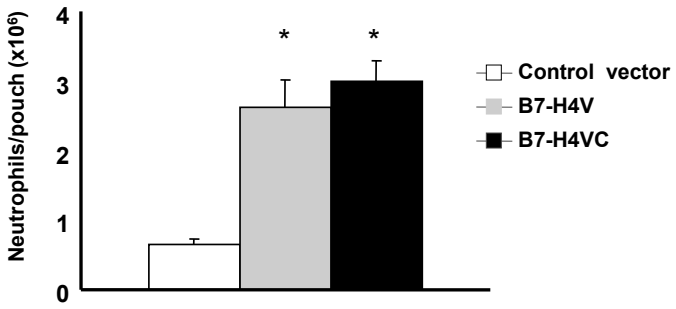
Table 1. Absolute number of neutrophils in spleens prior and post *Listeria Monocytogene* infection#

Day	WT^	B7-H4KO	RKO	DKO
0	1.74 ± 0.32	3.74 ± 0.82*	0.30 ± 0.02	36.5 ± 1.30*
1	12.52 ± 5.10	12.08 ± 0.93	2.56 ± 0.45	6.21 ± 2.55*
2	5.24 ± 1.73	7.40 ± 1.92*	1.57 ± 0.53	5.82 ± 2.67*
3	0.83 ± 0.03	1.34 ± 1.09	1.55 ± 0.27	6.08 ± 2.29*

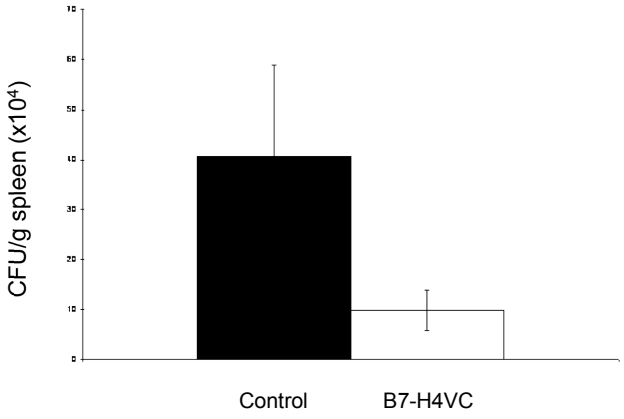
#Female mice of 6-10 weeks in groups of 3 were i.v. inoculated with 1 x 10^5 CFU of LM. Naive mice (day 0) and LM infected mice (days 1-3) from each group were terminated on day 1, day 2, and day 3 after the infection. Total splenocytes were numerated. Neutrophils were determined by staining of anti-Gr-1 and anti-Mac1 mAb followed by flow cytometry analysis. The absolute number of neutrophils were calculated by multiplying percentage of spleen Gr-1+CD11b+ cells with total spleen cell numbers. The values were averaged from 3 mice and the standard deviations were shown.

*Significantly different from corresponding control groups, P<0.05.

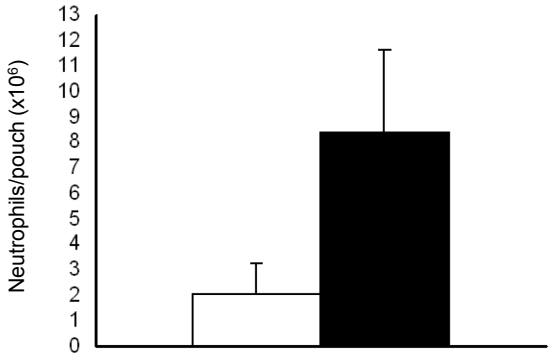
^WT: wild-type B6. B7-H4KO: B7-H4-/-. RKO: RAG-1KO. DKO: B7-H4KO x /RAG-1 double KO



Supplemental Fig. 1. Decoy B7-H4 enhances neutrophil response to LPS in B6 mice. To generate decoy B7-H4V and B7-H4VC plasmids, 2 primers flanking 5 and 3' of IgV fragment of murine B7-H4 were designed with XhoI and EcoRI restriction sites, respectively (5'primer; 5'-ccgctcgagccaccatggcttccttggggcag-3', 3'primer for B7-H4V; Similar primers for IgVC fragment of B7-H4 (5' primer, 5'-cggaattccgctaatttatctctggcatact-3' and 3'primer, 5'-cggaattccgctaagagttcagcaactgcag-3') were also obtained. Upon amplification, PCR product was digested with XhoI and EcroRI and ligated into pcDNA3.1 vectors (Invitrogen, Carlsbad, CA). Purified plasmids were injected i.v. by hydrodynamic method. Briefly, 20 μg of plasmid DNA in 3 ml PBS was injected into the tail vein of B6 mice within 10 seconds one day before air pouches were generated as described in Supplemental Fig. 1. Gr-1+ neutrophils were quantified by flow cytometry. Each bar represents the means ± s.d. of six to eight mice in each group. *Significantly different from control vector, $P<0.05$.



Supplemental Fig. 2. Decoy B7-H4 enhances neutrophil response to Listeria in B6 mice. Wild-type B6 mice of 6-8 week-old at groups of 5 were injected i.v. by hydrodynamic method as described in Fig. S1. with 30ug/2ml endotoxin-free plasmid DNA. One day later, mice were infected with $0.05x10^6$ CFU of Listeria by i.v. injection. Mice were terminated two days post Listeria infection. Spleen Listeria load was analyzed by colony plating. Each bar represents the means ± s.d. of five mice in each group. *Significantly different from control vector, $P<0.05$.



Supplemental Fig. 3. B7-H4KO mice have increased neutrophil response to LPS. The air pouch assay was performed as previously described (Edwards et al, J. Pathol. 134:147, 1981). Briefly, B7-H4KO or WT mice were anesthetized with 2, 2, 2-tribromoethanol (Sigma-Aldrich, St. Louis, Missouri) and subcutaneous dorsal pouches were created by injection of 5 ml of air. After 3 day, pouches were re-injected with 3 ml air. On day 6 after the first injection, 50 μg LPS in 1 ml PBS was injected into the pouches. Five hours later, Gr-1+ neutrophils were quantified by flow cytometry of cells rinsed from the pouch with sterile saline. Each bar represents the means ± s.d. of six to eight mice in each group. *Significantly different from WT, $P<0.05$.

Curriculum Vitae for PhD Candidates
The Johns Hopkins University School of Medicine
Mathew M. Augustine, MD
Date of Birth: February 29, 1976
Location of Birth: New York, NY

Educational History

PhD expected	2014	Program in Immunology	Johns Hopkins School of Medicine
MD	2003		Yale University School of Medicine
BA	1998	Biology Major/Psych Minor	Johns Hopkins University

Other Professional Experience

Surgical Oncology Fellow	2012-2014	Memorial Sloan Kettering Cancer Center
Chief Resident	2011-2012	The Johns Hopkins Hospital Dept. of Surgery
House Staff	2003-2011	The Johns Hopkins Hospital Dept. of Surgery
HHMI Research Fellow	2000-2001	Yale University School of Medicine
HHMI Research Fellow	1997	Johns Hopkins University

Scholarships, fellowships, or external funding

T32 NIH Surgical Oncology Research Training Grant. 2008-2009.
Fellowship for research conducted in the department of surgery in the immunology lab of Dr. Lieping Chen, MD/PhD.

Niarchos Surgical Research Fellowship. 2005-2008.
Fellowship for research conducted in the department of surgery in the immunology lab of Dr. Lieping Chen, MD/PhD.

Howard Hughes Medical Institute Medical Student Research Fellowship. 2000-2001.
Award given to 52 medical students from around the country to conduct basic science research. Research was performed in the immunobiology lab of Dr. Charles A. Janeway Jr., MD.

Howard Hughes Medical Institute Medical Student Continuing Fellowship. 2001-2003.
2-year scholarship provided to 10 HHMI Medical Student Research Fellows toward completion of medical school and research project.

Yale University School of Medicine Research Fellowship. 1999.

Fellowship award supports basic science research toward completion of thesis project in the immunobiology lab of Dr. Charles A. Janeway Jr., MD.

Howard Hughes Medical Institute Undergraduate Research Scholarship. 1997.
Award given to 10 Johns Hopkins undergraduates for year of research in biologic sciences. Research was performed in the neuroscience lab of Dr. Solomon H. Snyder, MD.

Johns Hopkins University Provost Undergraduate Research Scholarship. 1997-1998.
Award supports 15 Johns Hopkins undergraduates for year of research in biologic sciences, engineering, and humanities. Research was performed in the neuroscience lab of Dr. Solomon H. Snyder, MD.

Academic Honors

2003 Alpha Omega Alpha Yale University School of Medicine

2003 cum laude Yale University School of Medicine

1997 Phi Beta Kappa (junior year) The Johns Hopkins University

1997 Omicron Delta Kappa. The Johns Hopkins University

1997 Honor Society for Neuroscience The Johns Hopkins University

1997 Psi Chi Psychology Honor Society The Johns Hopkins University

1997 Golden Key National Honor Society The Johns Hopkins University

2012 Best American Board of Surgery Inservice Trainee Examination Score
The Johns Hopkins Hospital Department of Surgery

2010-2011 Anthony L. Imbembo Department of Surgery Resident Teaching Award
The Johns Hopkins Hospital Department of Surgery

2011-2012 Anthony L. Imbembo Department of Surgery Resident Teaching Award
The Johns Hopkins Hospital Department of Surgery

2009-2010 Johns Hopkins School of Medicine Medical Student Teaching Award – Junior Resident
The Johns Hopkins Hospital Department of Surgery

2011-2012 Johns Hopkins School of Medicine Medical Student Teaching Award –Chief Resident
The Johns Hopkins Hospital Department of Surgery

2010 Johns Hopkins Surgical Nursing Resident Award
The Johns Hopkins Hospital Department of Surgery

2003 American College of Surgeons Award
Connecticut Chapter of the American College of Surgery

2003 Yale University School of Medicine Department of Surgery Award
Yale University School of Medicine

2003 The Dr. Louis H. Nahum Prize Yale University School of Medicine

1996-1997 Johns Hopkins University Award for Outstanding Service to the Johns Hopkins and Baltimore Community Johns Hopkins University

1998 JHU Honor Society for Neuroscience Scholarship/Award Johns Hopkins University

1996-1998 Dean's List Johns Hopkins University

1996, 1997, 1998 JHU Office of Multicultural Student Affairs Certificate of Recognition Johns Hopkins University

2012 Best American Board of Surgery Inservice Trainee Examination Score Johns Hopkins Hospital

T32 NIH Surgical Oncology Research Training Grant. 2008-2009.
Fellowship for research conducted in the department of surgery in the immunology lab of Dr. Lieping Chen, MD/PhD.

Niarchos Surgical Research Fellowship. 2005-2008.
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Award supports 15 Johns Hopkins undergraduates for year of research in biologic sciences, engineering, and humanities. Research was performed in the neuroscience lab of Dr. Solomon H. Snyder, MD.

Research Experience

Johns Hopkins School of Medicine. 2005-2009. Graduate Program in Immunology. Graduate student in the lab of Dr. Lieping Chen, MD/PhD. Identification of the function and receptor underlying the co-stimulatory molecule B7-H4 in innate immunity.

Yale University School of Medicine. 2000-2003. Department of Immunobiology. Research student in the lab of Dr. Charles A. Janeway Jr., MD. Identification of the mechanism for the expression of the CD8 co-receptor molecule on CD4+ intestinal intraepithelial lymphocytes-Th2 dependent process and immunosuppressive function.

Yale University School of Medicine. 1999. Department of Immunobiology. Research student in the lab of Dr. Charles A. Janeway Jr. Identification of the T cell receptor CDR3 alpha chain region to establish the presence of positive selection of CD8 T cells involved in the pathogenesis of Type I diabetes in the beta chain transgenic NOD mouse.

The Johns Hopkins University. 1996-1998. Department of Neuroscience. Research student in Dr. Solomon H. Snyder's Lab. Development of a method for the identification of downstream targets of Akt, a serine-threonine protein kinase.

The Johns Hopkins University. 1995. Research student in the lab of Dr. A. Hari Reddi. Research lab focused on functional role of bone morphogenetic proteins in bone regeneration and cartilage development.

Publications, Peer Reviewed

Epidemiology of Liver and Biliary Tract Tumors.
Augustine MM, Fong, Y.
Surgical Clinics of North America. 2014 Apr:23(2): 171-188.

B7-H5 costimulates human T cells via CD28H
Zhu Y, Sheng Y, Iliopoulou BP, Han X, Augustine MM, Xu H, Phennicie RT, Flies SJ, Broadwater M, Ruff W, Taube JM, Zheng L, Luo L, Zhu G, Chen J, Chen L.
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Surgical treatment of primary hyperparathyroidism.
Augustine MM, Bravo PE, Zeiger MA.
Endocrine Practice. 2011 Mar-Apr;17 Suppl 1:75-82.

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Park JJ, Omiya R, Matsumura Y, Sakoda Y, Kuramasu A, **Augustine MM**, Yao S, Tsushima F, Narazaki H, Anand S, Liu Y, Strome SE, Chen L, Tamada K.
Blood. 2010 Aug 26;116(8):1291-8. Epub 2010 May 14.

Proximal cholangiocarcinoma: Tumor Depth Predicts Outcome.
De Jong MC, Hong SM, **Augustine MM**, Goggins M, Wolfgang CL, Hirose K, Schulick R, Choi M, Anders R, Pawlik TM.
Arch Surg. 2010 in press.

Palliation of advanced gastrointestinal malignancies using minimally invasive strategies.
Augustine MM, Pawlik, TM.
Progress in Palliative Care. 2009. *17*(5), 250-260.

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Zhu G, **Augustine MM**, Azuma T, Luo L, Yao S, Anand S, Rietz AC, Huang J, Xu H, Flies AS, Flies SJ, Tamada K, Colonna M, van Deursen JM, Chen L.
Blood. 2009 Feb 19;113(8):1759-67. Epub 2008 Dec 24.

An important regulatory role for CD4+CD8 alpha alpha T cells in the intestinal epithelial layer in the prevention of inflammatory bowel disease.
Augustine MM*, Das G*, Das J, Bottomly K, Ray P, Ray A.
Proc Natl Acad Sci U S A. 2003 Apr 29;100(9):5324-9. Epub 2003 Apr 14.

Qa-2-dependent selection of CD8alpha/alpha T cell receptor alpha/beta(+) cells in murine intestinal intraepithelial lymphocytes.
Das G, Gould DS, **Augustine MM**, Fragoso G, Sciutto E, Stroynowski I, Van Kaer L, Schust DJ, Ploegh H, Janeway CA Jr.
J Exp Med. 2000 Nov 20;192(10):1521-8. Erratum in: J Exp Med 2001 Feb 5;193(3):following 411.

Publications, Chapters
Augustine MM and Chen L. Cancer Immunotherapy: Untapping the potential of costimulatory molecules beyond CTLA-4. 243-268. Orentas RJ, Hodge JW, Johnson, BD, editors: *Cancer Vaccines and Tumor Immunotherapy,* Hoboken, 2008, John Wiley and Sons, Inc.
Augustine MM, Kamel IR, Pawlik TM. Morphometric Imaging Techniques and the Functional Liver Remnant. 1-11. Madoff DC, et al. editors: *Venous Embolization of the Liver*, London, 2011, Springer-Verlag London Ltd.
Augustine MM and Anders R. Technical Complications of Liver Transplantation. Liapas H and Wang HL editors: *Pathology of Solid Organ Transplantation*, Berlin Heidelberg, 2011, Springer-Verlag Berlin Heidelberg Ltd.

Service

1999 Co-Director Yale University School of Medicine Project Hope
1999 Co-Director Yale University School of Medicine Hunger and Homelessness Lecture Series
1999 Director Yale University School of Medicine Douglas House
1998-9 Volunteer Yale University School of Medicine Douglas House
1999 Teacher Career High School Summer Science Enrichment Program
1999 Volunteer Yale University School of Medicine Buddies for Kids Program
1997-8 Founder and Director Johns Hopkins University Safe and Smart Homework Tutorial Program
1996-7 Director Johns Hopkins University Job Skills Program

1995-6	Organizer	Johns Hopkins University Tutorial Project
1997	Head Delegate	Lieutenant Governor's Forum on Youth Serving Youth
1994-6	Tutor	JHU Tutorial Project
1995	Tutor	JHU Computer Job Skills Project
1997	Volunteer	Keswick Nursing Home
1996-8	Tutor	JHU Academic Advising- Biochemistry and Organic Chemistry