Role of Neurokinin 1 Receptor in Modulating the Immune-Stimulatory Function of Dendritic Cells

by

Brian Mark Janelsins

B.S. Biology, Mary Washington College, 2004

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This dissertation was presented

by

Brian Mark Janelsins

It was defended on

January 15, 2010

and approved by

Lawrence P. Kane, Ph.D.,
Associate Professor, Department of Immunology

Paul D. Robbins, Ph.D.,
Professor, Department of Biochemistry and Molecular Genetics

Russell D. Salter, Ph.D.,
Professor, Department of Immunology

Walter J. Storkus, Ph.D.,
Professor, Departments of Dermatology and Immunology

Dissertation Advisor: Adriana T. Larregina, M.D., Ph.D.,
Associate Professor, Departments of Dermatology and Immunology

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There is a growing body of evidence indicating that the nervous and immune systems cross-talk during inflammatory and immune responses. Secretion of pro-inflammatory neuropeptides from the tachykinin family, including substance P (SP) and hemokinin-1 (HK-1), favors cellular immunity by binding the neurokinin 1 receptor (NK1R) to promote immune cell survival and activation. Dendritic cells (DCs) are essential for the stimulation of cellular immunity; however, the ability of pro-inflammatory tachykinins to affect the immune-stimulatory function of DCs remains elusive. Since DCs home strategically to peripheral and lymphoid tissues where tachykinins are secreted, we hypothesized that signaling via the NK1R enhances DC longevity and their T cell-stimulatory function, including the induction of type-1 $CD4^+$ T cell helper (Th1) and $CD8^+$ cytotoxic T cell (CTL/Tc1) responses.

Using DCs generated from murine bone marrow precursors (BMDCs), I describe that BMDCs express functional NK1R, and agonistic signaling via the receptor rescues BMDCs from apoptosis. The immunological relevance of these findings were validated in vivo, as I demonstrate that adoptive transfer of NK1R-signaled BMDCs loaded with antigen (Ag) migrate efficiently to tissue-draining lymph nodes (DLNs) where they survive longer and induce superior type-1 DTH responses compared to adoptive transfer of control Ag-loaded BMDCs.

Secondly, I investigated the mechanisms by which NK1R-signaled BMDCs favor cellular immunity, including their ability to generate Th1 and CTL/Tc1 responses. I show that agonistic signaling via the NK1R promotes the maturation of BMDCs and inhibits their secretion of IL-10, and adoptive transfer of NK1R-signaled BMDCs elicits enhanced Ag-specific Th1 and CTL/Tc1 responses. The individual roles of adoptively transferred NK1R-signaled BMDCs and endogenous DCs were further addressed by comparing the development of type-1 immunity in wild-type, IL-12 knockout (IL-12p35$^{-/-}$) and Diphtheria Toxin Recetor (DTR) transgenic (inducible depletion of CD11c^{+} DCs) mice. With these models, I demonstrate that generation of robust Ag-specific Th1 and CTL/Tc1 responses requires secretion of IL-12p70 by endogenous DCs and inhibition of IL-10 production by transferred BMDCs. Collectively, our data strongly suggest that adoptive transfer of NK1R-signaled BMDCs promotes enhanced type-1 immunity by mechanisms involving both exogenous and endogenous DC populations.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

The personal and professional relationships I developed as a graduate student in the Immunology Graduate Program have reinforced my love of creative science; facilitated my ability to address scientific problems and instilled a sense of accomplishment and confidence. I am extremely grateful for you all who have contributed to my scientific upbringing.

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1.0 CHAPTER ONE: INTRODUCTION

1.1 DENDRITIC CELLS

Dendritic cells (DCs) are potent 'professional' antigen (Ag)-presenting cells (APCs) that serve as central conductors in bridging innate and adaptive immune responses. Strategically located in areas of pathogen exposure, DCs are specialized to recognize pathogens and internalize foreign antigenic material, ultimately leading to DC maturation and migration into secondary lymphoid tissues where they generate and polarize T cell immunity.[1,2,3,4] It is now appreciated that DCs migrating from peripheral tissues can transfer Ag to lymphoid-resident DCs, allowing for efficient induction of T cell responses.[5] Due to their unique ability to stimulate biased naïve and memory T cell responses, DCs are currently being investigated for their capability to serve as adjuvants in immunotherapy to promote robust cellular immunity against tumors and intracellular infections, including the polarization of type-1 T cell responses.[6]

1.1.1 Plasticity of DCs

DCs are defined by their phenotype, morphology and function. In mice, DCs are phenotypically identified by expression of the CD11c integrin molecule, while the phenotype of human DCs is not entirely defined. Despite ambiguity in phenotype, all DCs at some point in their life-span exhibit dendritic morphology and undergo functional conversion from an Ag-capturing mode to an immune-stimulatory Ag-presenting capability; a process termed 'maturation'.[7] According to their maturation state, DCs can be classified as immature or mature cells. In the steady-state, DCs reside in peripheral and secondary lymphoid tissues as immature DCs where they promote immunological tolerance. In the presence of a pro-inflammatory environment, immature DCs

undergo maturation to become mature DCs, which are fully activated and acquire potent T cell stimulatory function. Alternatively, DCs are capable of encountering activation signals that only induce partial stimulation, rendering them as semi-mature. Similarly to immature DCs, semi-mature DCs induce immunological tolerance[8] and are receptive to full activation by pro-inflammatory signaling to become potent stimulators of T cell function (Figure 1).[9,10]

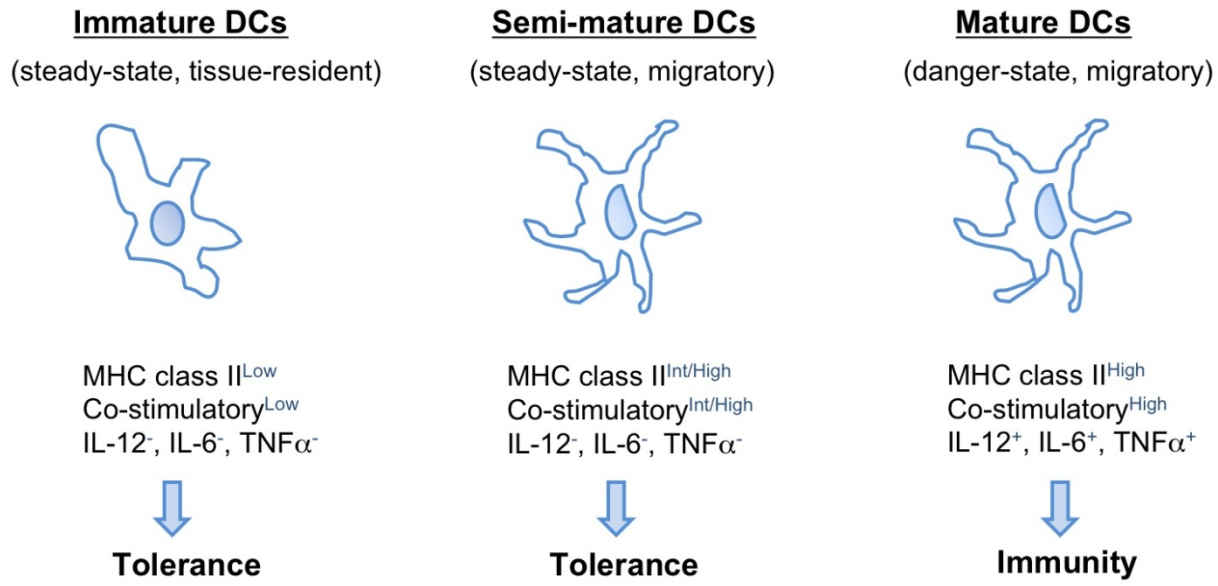

Figure 1: Immature, semi-mature and mature DCs differentially regulate immunity.

Three stages of DC activation during steady-state and danger-state. DCs exist as (i) immature (low levels of cell-surface MHC class II and co-stimulatory molecules), (ii) semi-mature (intermediate to high levels of cell-surface MHC class II and co-stimulatory molecules with low secretion of pro-inflammatory cytokines) or (iii) mature (high levels of MHC class II and co-stimulatory molecules and secretion of pro-inflammatory cytokines) cells.

1.1.1.1 DC maturation

Serving as sentinels for danger signals in peripheral and lymphoid tissues, immature DCs are quiescent and exhibit a high endocytic capacity to continuously sample foreign Ag via a variety of uptake mechanisms, including receptor-mediated endocytosis, phagocytosis and macropincytosis. Indeed, immature DCs express high levels of C-type lectins and other endocytic receptors specific for Fc, complement, scavenger and heat-shock proteins.[10,11] Due to the fact that immature DCs do not secrete pro-inflammatory cytokines and express low levels of co-stimulatory molecules and DLN-specific chemokine receptors on their cell-suface,[2] immature DCs are poor stimulators of T cell responses. Thus, it has been postulated that immature DCs derived from the periphery and resident in lymphoid tissues collectively induce and reinforce tolerance to T cells specific to harmless Ag (i.e., tissue-derived self Ag).

When peripheral tissues become inflamed or penetrated by microbes, haptens or allergens, DCs sense danger by encountering exogenous (pathogen-associated molecular patterns, PAMPs) and endogenous (e.g., alarmins) activation signals that promote their maturation from immature Ag-capturing DCs to mature immune-stimulatory DCs. PAMPs represent a diverse set of microbial molecules, which share a number of different recognizable biochemical features that inform DCs of invading pathogens. Such PAMPs are recognized by pattern recognition receptors (PRRs), including the well-described Toll-like receptor (TLR) family. TLRs have been demonstrated to recognize and respond to specific microbial products: lipoteichoic acid, peptidoglycan and zymosan are recognized by TLR-2; double-stranded RNA, viral RNA and polyinosinic:polytidylic acid (polyI:C) by TLR-3; lipopolysaccharide (LPS) by TLR-4; flagellin by TLR-5 and cytosine-phosphate-guanine (CpG) motifs of bacterial DNA by TLR-9.[12] Ligation of most TLR agonists induces DC maturation and secretion of pro-

inflammatory cytokines (i.e., IL-6, IL-1β and IL-12p70).[13,14] In addition to invading microbes, endogenous factors including neuropeptides,[15,16] anti-microbial peptides[17] and ATP[18] are often released during infection and damage to tissues, resulting in further activation of DCs.

DCs undergoing maturation down-regulate most of their endocytic receptors while up-regulating cell-surface MHC class I and II molecules, co-stimulatory molecules including members of the B7 (CD80, CD86 and ICOSL) and TNF (RANK, CD40L, 4-1BBL, OX40L and CD70) families, chemokine receptors (CCR5 and CCR7) and adhesion molecules (ICAM-1,-2 and LFA-1,-3), as well as enhancing the production of pro-inflammatory cytokines (IL-12p70, IL-6, IL-1β and TNF-α).[2] Upon the up-regulation of CCR7, maturing DCs respond to chemokines CCL19 and CCL21,[19] leading to lymph-trafficking into T cell-rich areas of lymphoid tissues. Mature DCs are then capable of presenting Ag in the context of MHC class I and II molecules for the activation of Ag-specific naïve T cells into effector and memory $CD8^+$ and $CD4^+$ T cells, respectively (Section 1.1.1.2).

Interestingly, some DCs resembling the mature phenotype have been described to promote T cell tolerance.[20-22] These DCs were classically distinguished as mature DCs due to intermediate to high levels of CCR7, MHC class II and co-stimulatory molecules on their cell surface. However, these DCs do not produce pro-inflammatory cytokines (IL-6, IL-1β and IL-12p70) but enhance the secretion of anti-inflammatory cytokine IL-10, ultimately leading to the induction of Ag-specific T regulatory cells (T regs). Thus, these partially activated DCs are called semi-mature DCs, and recent studies suggest that interaction with gut flora, apoptotic cells and TNF-α induces partial activation of DCs.[9,10] Interestingly, semi-mature DCs become fully immunogenic when challenged with typical pro-inflammatory stimuli, including LPS and agonistic CD40 ligation. Thus, it is likely that semi-mature DCs represent an intermediate bridge

to migrate into secondary lymphoid organs for the purpose of tolerizing self during the steady-state, while becoming fully mature to promote immunity against invading pathogens during infection.[9]

1.1.1.2 T cell activation

Mature DCs egress via the lymph into T cell-rich areas of DLNs, then induce $CD4^+$ and $CD8^+$ T cell activation by communicating a series of signals through the 'immunological synapse'; a network of collaborative membrane-bound and secreted signaling events between the Ag-presenting DC and the engaging T cell.[23,24] In the center of the synapse, DCs present Ag in the context of MHC class I and II molecules, which is recognized by an Ag-specific TCR (signal 1) expressed on naïve $CD8^+$ and $CD4^+$ T cells, respectively. In addition to adhesion interactions mediated by ICAM-1, ligation of co-stimulatory molecules CD86 and CD80 with the molecule CD28, expressed on the cell-surface of T cells, allows for efficient activation of $CD4^+$ and $CD8^+$ T cells to prevent tolerance against the presented Ag. Additionally, engagement of co-stimulatory molecule CD40 with CD40L expressed on $CD4^+$ T cells further activates DCs to induce potent $CD8^+$ T cell responses. Excessive T cell stimulation is avoided by co-stimulatory (CD80 and CD86) and inhibitory (PDL-1/-2) molecule interaction with CTLA-4 and PD-1/-2, respectively, on activated $CD4^+$ and $CD8^+$ T cells. As shown in Figure 2, DCs emit a 3^{rd} signal to T cells that serves to polarize the type of Ag-specific T cell response generated, via secretion of cytokines or ligation of membrane-bound molecules (IL-12p70, Th1; IL-4 and OX40L, Th2; IL-6 and TGF-β1, Th17; and TGF-β1 and IL-10, T regs). Recently, Kalinski and colleagues proposed that a 4^{th} signal transmitted through the immunological synapse determines the

efficiency of activated $CD4^+$ and $CD8^+$ T cells to migrate into peripheral tissues and elicit Ag-specific effector functions.[24] In contrast to naive T cells, effector and memory T cells require signal 1 with only little co-stimulation for re-activation.

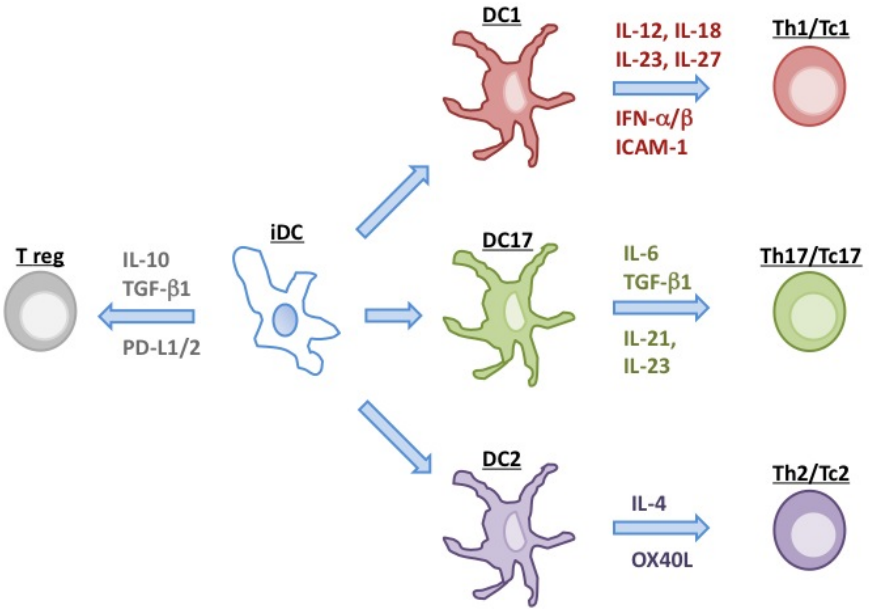

Figure 2: DC polarization of Ag-specific T cell responses.

Intracellular pathogens signal DCs to exhibit a type-1 biasing phenotype (DC1), which polarizes Th1/Tc1 responses via IL-12p70 production. Alternatively, DCs employ a DC2 phenotype when signaled with extracellular pathogens (e.g., helminths), ultimately leading to the generation of Th2/Tc2 responses associated with robust signaling via OX40/L and IL-4 with minimal production of IL-12p70. ATP and certain neuropeptides have been proposed to play roles in inducing DCs to bias Th17/Tc17 responses (DC17) by favoring IL-6 and TGF-β1 secretion. In contrast, DCs interact with factors that prevent their full maturation, ultimately leading to secretion of anti-inflammatory cytokines IL-10 and TGF-β1 and interaction with programmed death ligands (PDLs). Such immature DCs inhibit Ag-specific effector populations (anergy and apoptosis) and promote Ag-specific T reg populations.

1.1.2 Induction of type-1 T cell responses

DCs have been shown to elicit robust $CD4^+$ Th1 and $CD8^+$ CTL/Tc1 responses, which are necessary for the generation of potent cellular immunity targeting intracellular infections and tumors.[25-27] Current research is focused on stimulating DCs with adjuvants that polarize DC1s to preferentially elicit Th1/Tc1 responses. In addition to DC1 bias, the longevity of DCs serves as another crucial parameter for the ability of DCs to induce long-lasting robust Th1/Tc1 responses in vivo.

1.1.2.1 Bias of type-1 DCs

The molecules produced by DC1s that drive Th1/Tc1 polarization, and the factors that induce their long-lasting expression, are the center of investigation for studies utilizing DCs for generation of cellular immunity. Twenty years after its discovery, IL-12p70 is now appreciated as the major cytokine produced by DCs that polarizes Th1 responses in $CD4^+$ T cells and contributes to the generation of potent $CD8^+$ CTL/Tc1 responses.[28-31] Additional studies indicate that DCs employ other mechanisms to promote type-1 T cell responses, including the secretion of IL-23,[32] IL-27,[33] IL-18[34] and IFN-α/β[35] and membrane-bound ligation of CD70,[36] ICAM-1[37] and notch ligand, delta-like 4.[38] Most reports suggest that these factors act in concert with IL-12p70 to enhance Th1/Tc1 induction, but further investigation is warranted to determine if they promote Th1/Tc1 responses via IL-12p70-independent mechanisms.

TLRs are the most well studied PRRs that instruct DCs to secrete high levels of IL-12p70 for the generation of Th1/Tc1 responses. Indeed, agonistic signaling via TLR-3, -4 and -9 promotes IL-12p70 production in most human and mouse DC subtypes.[39-43] However, DCs

become exhausted in their ability to produce high levels of IL-12p70 unless maintained by other factors, such as those provided in the DC-$CD4^+$ T cell synapse.[44] Likewise, as TLR-signaled DCs mature and travel to DLNs, DCs interact with $CD4^+$ T cells via CD40 ligation to optimize IL-12p70 production for extended periods of time.[43] Enhanced secretion of IL-12p70 in the DC-$CD4^+$ T cell synapse potently stimulates IFN-γ production via induction of the transcription factor T-bet in naïve $CD4^+$ and $CD8^+$ T cells, leading to generation of potent Th1 and CTL/Tc1 responses. Furthermore, DC populations in lymphoid tissues have been observed to be critical for cross-priming and inducing high levels of IL-12p70 after systemic delivery of Ag.[45] These results suggest that in additional to instructional factors in peripheral tissues, conditions in secondary lymphoid tissues also drive and optimize IL-12p70 signaling to favor Th1/Tc1 immunity.

Interestingly, most TLR agonists such as LPS and CpG not only generate potent levels of IL-12p70 in DCs, but also promote abundant secretion of the anti-inflammatory cytokine IL-10.[46-48] IL-10 signaling serves to abrogate Th1/Tc1 responses by inhibiting the immune-stimulatory function of DCs, including their maturation and secretion of IL-12p70, while also inhibiting T cell responsiveness of IL-12p70 and secretion of IFN-γ and IL-2.[49,50] Thus, investigators are searching for other DC adjuvants that promote secretion of IL-12p70 without favoring the production of anti-inflammatory cytokines that would circumvent the optimal induction of Th1/Tc1 responses.

Emerging data from the last decade demonstrate that inflammatory mediators IL-1β, IFN-γ and polyI:C, in conjunction with interactions from immune cell populations, including memory $CD8^+$ T cells and properly activated NK cells, promote DCs to secrete higher levels of IL-12p70 in comparison to signaling DCs with TLR agonists alone, ultimately leading to robust Th1/Tc1

immune responses.[25,51,52] It is unknown whether these DC1s lead to better Th1/Tc1 responses by producing higher levels of IL-12p70 coupled with reduced secretion of IL-10 or via other mechanisms, such as enhanced chemotaxis and longevity.

1.1.2.2 Survival of DCs

The longevity of DCs in vivo is another critical parameter in generating robust Th1/Tc1 immune responses. DCs in peripheral and lymphoid tissues encounter a variety of pro-apoptotic signals, including tissue-derived factors and immune cell populations.[53] Indeed, upon adoptive transfer into mice, activated DCs loaded with Ag are short-lived in tissue-DLNs, and phagocytosis of dying DCs leads to tolerance towards the immunizing Ag.[54,55]

Due to these observations, investigators have aimed to identify ligands and their intracellular signaling pathways that promote enhanced DC survival, while still preferentially inducing Th1/Tc1 responses. It is well-established that granulocyte-macrophage colony stimulating factor (GM-CSF) and prostaglandin E2 (PG-E2) serve as growth factors that favor the survival of DCs. However, GM-CSF and PG-E2 also promote high levels of IL-10, which inhibits Th1/Tc1 responses.[56-59] In contrast, stimulation of DCs via membrane-bound molecules (CD40, TRANCE and CCR7) or secreted peptides (leptin) enhances DC survival and immune-stimulatory function to favor Th1/Tc1 responses.[60-62]

Pharmacological studies utilizing such pro-survival factors have implicated certain intracellular signaling pathways that preferentially regulate DC longevity. Utilizing murine D1 cells (splenic DC cell-line), it was initially reported that LPS activates nuclear factor- kappa B (NF-κB) to promote DC maturation, while signaling via extracellular signal-regulated kinase (Erk), from the mitogen-activated protein kinase (MAPK) family, to favor cell survival.[63]

Studies utilizing more physiologically relevant DCs suggest versatility of intracellular pathways involved in favoring DC longevity. Ardeshna et al. demonstrated that human monocyte-derived DCs cultured with LPS utilize PI3K (phosphoinositide-3-kinase)-Akt (protein kinase B; referred as Akt) pathways to promote survival.[64] Other studies have observed that TNF family ligands CD40 and TRANCE activate NF-κB to sustain cell longevity in bone marrow-derived DCs (BMDCs) and splenic DCs.[65] It is becoming increasingly evident that PI3K-Akt and NF-κB intracellular signaling pathways are related; activation of PI3K-Akt signaling enhances the degradation of IκB, allowing for full activation of NF-κB to synergistically inhibit pro-apoptotic molecules (BAD, BID and caspases). Indeed, activated DCs derived from human peripheral blood mononuclear cells (PBMCs) utilize both PI3K/Akt and NF-κB intracellular signaling pathways to up-regulate anti-apoptotic machinery and promote DC survival.[66-68] Similar to NF-κB, PI3K-Akt signaling has recently been shown to activate pathways that are responsible for DC activation [i.e., c-Jun-N-terminal kinase (MAPK/Jnk)], leading to elevated production of IL-12p70.[69] Further studies illustrate that the balance of pro-survival and pro-activation pathways ultimately dictate the fate of DC longevity. These studies demonstrate that pro-activation signaling pathways (MAPK/Jnk) leads to apoptosis of DCs when pro-survival pathways are compromised.[70]

Given the importance of PI3K-Akt activation in enhancing DC survival and immune-stimulatory function, Spencer and colleagues have demonstrated that sustained activation of Akt enhances the immune-stimulatory function and longevity of DCs in DLNs after adoptive transfer, augmenting their ability to promote robust CTL responses against tumor-associated

Ag.[54] Thus, signaling DCs with agonists that mediate PI3K-Akt activation might enhance the longevity and bias of adoptively transferred DCs for the purpose of generating robust Th1/Tc1 responses in vivo.

1.1.3 Adoptively transferred DCs and induction of Th1/Tc1 responses

As stated above, immune-stimulatory function and longevity are important parameters of adoptively transferred DCs to elicit Th1/Tc1 responses in vivo;[52,71-73] however, the mechanisms by which adoptively transferred DCs induce such responses in vivo are not entirely known. Emerging data support crucial roles for both adoptively transferred DCs and endogenous DC populations to promote robust Th1/Tc1 immunity. Indeed, it has recently been shown that endogenous DCs are required to induce Ag-specific T cell responses mediated by subcutaneous delivery of adoptively transferred DCs,[74] while respiratory mucosal delivery elicits Ag-specific T cell responses independent of endogenous DCs.[75] Generation of acquired Th1/Tc1 responses in vivo could be due to the enhanced longevity and immune-stimulatory function of adoptively transferred DCs to directly activate and polarize Ag-specific Th1/Tc1 cells,[76] or indirectly, by spreading Ag in an immune-stimulatory manner to activate endogenous DC populations in peripheral and/or lymphoid tissues.[77] While there are few reports investigating how adoptively transferred DCs utilize endogenous DCs to promote Th1/Tc1 responses, various endogenous DC populations, including subtypes located in peripheral and lymphoid tissues, have been described to play essential roles in promoting Th1/Tc1 responses when Ag is administered via intravenous (i.v.) or subcutaneous (s.c.) injection.

1.1.3.1 Possible role of peripheral tissue DCs in generating Th1/Tc1 responses

Peripheral tissue DCs are found in areas of pathogen exposure, such as skin, gut and lungs. The skin is one of the well-studied peripheral organs and harbors two main DC populations: Langerhans cells (LCs) in the epidermis and dermal DCs (dDCs) in the dermis. While LCs are generally identified by expression of CD11c, Langerin (CD207), $EpCAM^{Hi}$ (CD326), CD1a and birbeck granules; dDCs are characterized by expression of CD11c and other C-type lectins such as mannose receptor (CD206), and several subtypes are capable of expressing low levels of CD1a and Langerin.[78-81] Despite differences in phenotype, both LCs and dDCs are fully capable of capturing Ag in the skin, migrating to DLNs and generating T cell immunity.[82-85] Indeed, blockade of epidermal and dermal DC migration after subcutaneous delivery of Ag in the skin prevents efficient activation of $CD4^+$ T cell immunity.[86] Further studies illustrate important roles for both LCs and dDCs to transfer Ag to lymphoid-resident DCs, and alternatively, directly stimulate $CD4^+$ T cells in DLNs.[86-89] In contrast, migratory DCs from the skin have thought to serve strictly as sources of Ag for $CD8^+$ T cell immunity, enabling lymphoid-resident DCs to capture Ag and directly prime $CD8^+$ T cells[87]. Recent reports challenge this view, demonstrating that novel populations of migratory DCs ($CD103^+$ and $Langerin^+CD8\alpha^+$ subtypes) are capable of migrating to DLNs and directly cross-presenting Ag for the induction of $CD8^+$ T cell immunity.[45,90]

In other peripheral tissues, migratory DCs have also been demonstrated to be crucial in regulating T cell tolerance and immunity.[91] Although their potential to serve as sources of Ag to lymphoid-resident DCs or directly prime $CD4^+$ and $CD8^+$ T cell responses warrants further investigation.

1.1.3.2 Possible role of lymphoid-resident DCs in generating Th1/Tc1 responses

In contrast to migratory DCs from peripheral tissues, lymphoid-resident DCs in secondary lymphoid organs are believed to be the main inducers of $CD8^+$ T cell immune responses. Lymphoid-resident DCs are identified in mice by differential expression of CD8α and/or CD4 molecules. The spleen possesses 3 major DC populations of $CD8\alpha^+CD4^-CD11b^-$, $CD8\alpha^-CD4^+CD11b^+$ and $CD8\alpha^-CD4^-CD11b^+$ phenotypes; while skin-DLNs contain an additional DC population identified by $CD8\alpha^{lo}CD4^-CD11b^+$ expression, which is thought to represent migratory DC populations from the skin.[92] It should be noted that human DCs found in lymphoid tissues lack CD8α, but do display differential regulation of CD4 and CD11b.

$CD8\alpha^+$ DCs in secondary lymphoid organs have been implicated in the induction of type-1 T cell responses. Initial studies demonstrated that $CD8\alpha^+$ DCs are capable of internalizing dying cells in vitro and in vivo, leading to Ag processing and presentation into MHC class I and II molecules.[93] Indeed, $CD8\alpha^+$ DCs lie in T cell-rich areas of lymphoid tissues and serve as potent producers of IL-12p70,[94,95] ultimately leading to the generation of robust Th1/CTL responses.[96] Carbone and colleagues proposed that lymphoid-resident $CD8\alpha^+$ DCs internalize Ag from migrating DC populations, and subsequently, prime naïve $CD8^+$ T cells.[97] $CD8\alpha^+$ DCs were also shown to be equally important in directly priming $CD4^+$ T cells.[98] The role of lymphoid-resident $CD8\alpha^-$ DCs, excluding DCs derived from peripheral tissues, is less understood but these DCs are believed to participate in eliciting $CD4^+$ Th2 responses.[96,99]

Studies dissecting the immune-stimulatory role of DCs derived from peripheral and lymphoid tissues based on CD8α expression are complicated by the recent observation that different DC populations can up-regulate the CD8α molecule after homing into DLNs.[100]

Indeed, it has recently been demonstrated that migrating Langerin$^+$ DCs enhance CD8α expression and are vital to cross-present Ag and produce IL-12p70 in secondary lymphoid tissues,[45] suggesting the possibility that CD8α$^+$ DCs used in the experiments mentioned above may represent such migratory DC populations. Furthermore, Morelli et al. have demonstrated that CD8α$^-$ DCs in the spleen are capable of up-regulating the CD8α molecule then migrating into the follicular T cell-rich area.[101]

Adoptively transferred DCs are exposed to pro-apoptotic signaling in peripheral injection sites, and subsequently, the remaining viable DCs (<1%) then migrate to DLNs where they are found to accumulate near lymphoid-tissue DCs. Thus, it is likely that short-lived adoptive transferred DCs interact with endogenous DC populations from peripheral and lymphoid tissues to mediate their Th1/Tc1 responses.[74] Studies described above, utilizing regional or systemic delivery of Ag, demonstrate that subtypes of peripheral and lymphoid tissue DCs serve as a collaborative network to elicit CD4$^+$ and CD8$^+$ T cell immunity. Whereas, the survival and immune-stimulatory function of adoptively transferred DCs are appreciated, it will be interesting to determine the role of endogenous DC populations in mediating the effects of adoptively transferred DCs.

1.2 MAMMALIAN TACHYKININS AND IMMUNITY

Mammalian tachykinins comprise of a group of bioactive neuropeptides that are secreted in peripheral and lymphoid tissues, regulating a wide variety of biological functions, ranging from smooth muscle contraction, pain transmission, hematopoiesis, vasodilation, neurogenic

inflammation and immunity.[102] Whereas most neuropeptides, including somatostatin and products of proopiomelanocortin (POMC) cleavage, inhibit developing immune responses;[103-105] tachykinins that bind the neurokinin 1 receptor (NK1R), such as substance P (SP) and hemokinin-1 (HK-1), represent some of the few neuropeptides that are immune-stimulatory.[106-109] Despite these observations, it is still unknown whether immune-stimulatory tachykinins modulate the ability of DCs to promote cellular immunity.

1.2.1 Mammalian tachykinins

Mammalian tachykinins are short hydrophobic peptides defined by expression of the C-terminal motif: Phe-X-Gly-Leu-Met-NH_2 [X represents an aromatic (Phe or Tyr) or hydrophobic (Val or Ile) residue] (Table 1). The C-terminal sequence of tachykinins is highly conserved and essential in mediating their biological functions.[110] Historically, the tachykinin family has included SP, neurokinin A (NKA), neurokinin B and two elongated forms of NKA; neuropeptide K (NPK) and neuropeptide-γ (NP-γ). The recent identification of a novel gene precursor, and its splice variants, has led to the discovery of new tachykinins, including HK-1 and elongated forms of HK-1 termed endokinins (EKs). Tachykinins are derived from 3 genes named preprotacykinins (referred to as TAC1, TAC3 and TAC4; HUGO Gene Nomenclature Committee). TAC1 encodes SP, and through alternative splicing, gives rise to transcripts also encoding NKA, NPK and NP-γ. TAC3 generates NKB transcripts, while the newly identified TAC4 encodes HK-1, and in humans, undergoes alternative splicing to give rise to transcripts encoding tachykinins EKA and EKB and tachykinin-like peptides EKC and EKD.[111]

Table 1: The Mammalian Tachykinins.

Tachykinin	Amino Acid Sequence	Ref.
Substance P	R P K P Q Q **F** F **G** **L** **M** -NH_2	112
Neurokinin A	H K T D S **F** V **G** **L** **M** -NH_2	113,114
Neurokinin B	D M H D F **F** V **G** **L** **M** -NH_2	115
Neurokinin-γ*	H K T D S **F** V **G** **L** **M** -NH_2	116
Neuropeptide K*	H K T D S **F** V **G** **L** **M** -NH_2	117
Hemokinin-1	R S R T R Q **F** Y **G** **L** **M** -NH_2	118-120
Hemokinin-1 (h)	T G K A S Q **F** F **G** **L** **M** -NH_2	119
Endokinin A* (h)	G K A S Q **F** F **G** **L** **M** -NH_2	118
Endokinin B* (h)	G K A S Q **F** F **G** **L** **M** -NH_2	118

* Abbreviated sequence of the last 10 amino acids. Sequences of human tachykinins are distinguished from mice by indication of (h).

1.2.1.1 Distribution of tachykinins in tissues

Historically, tachykinins were thought to be strictly of neuronal origin. Initial studies detected tachykinins in neurons within the central nervous system (CNS) and primary afferent neurons innervating peripheral and lymphoid tissues.[108,121-125] The dogma that tachykinins are restricted to nerve-fiber terminals has been challenged repeatedly in the past 20 years, and it is now well-understood that tachykinins are expressed throughout the mammalian body in both neuronal and non-neuronal sources. Indeed, immunoassays utilizing antibodies specific to the C-terminal motif of tachykinins have implicated peptide expression in tissues devoid of innervation, including the placenta.[118,126] Granulomas are also absent of innervation and express tachykinins. For example, SP mRNA and protein have been found in eosinophils and neutrophils from

granulomas in murine schistosomiasis mansoni[127] and periadicular granulomas after LPS treatment,[128] respectively. Mononuclear immune cells, including macrophages, DCs and T cells produce SP and express transcripts also encoding NKA, NPK and NP-γ.[129-133] TAC4-derived HK-1[134-137] and human EKs[118,138] are also expressed in a variety of immune cells present in peripheral and lymphoid tissues. In contrast, TAC3-derived NKB is widely expressed in the CNS,[139] although recent reports have detected expression in several immune cells.[135]

1.2.1.2 Metabolism of tachykinins

The biological functions of tachykinins are tightly regulated by the activity of a variety of tissue-specific membrane-bound and soluble peptidases. Inhibition of such peptidases prevents proper control of tachykinin function, leading to exacerbation of various inflammatory diseases (e.g., psoriasis,[140] IBD,[141] asthma[142] and rhinosinusitis[143]). Whereas the metabolism of most tachykinins is not entirely known, degradation of SP and NKA by neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) are well-studied. Both kinase type-II enzymes target and cleave the C-terminal region of SP, yielding the generation of small peptide fragments that are biologically inactive in peripheral tissues.[144] Dipeptidyl aminopeptidase IV (DAP IV) is a homodimeric type 11 transmembranic glycoprotein that also targets SP, leading to truncations in the N-terminal region.[145] Since the C-terminal region is still intact, these peptide fragments are still biologically active but are highly susceptible to further degradation by aminopeptidase M (AmM).[146] In contrast, NKA has been demonstrated to be generally resistant to AEC and DAP IV cleavage, but still susceptible to NEP degradation.[146] The unique ability of NKA to be resistant to most SP-degrading enzymes might allow NKA signaling to predominate, while SP

would be cleared faster to avoid excessive stimulation. Thus, understanding the expression and regulation of tachykinins and their degrading enzymes during the course of infection and inflammation might be relevant in the control of immunity.

1.2.2 Neurokinin receptors

Tachykinins mediate their function through three mammalian tachykinin receptors (known as neurokinin receptors), belonging to the family of rhodopsin-like G protein-coupled receptors (GPCRs). Currently, three distinct neurokinin receptors (NK1R, NK2R and NK3R) have been cloned and are expressed in tissues of different species including human, mouse and rat. Although all tachykinins act as full agonists through each neurokinin receptor, deviations in the N-terminus allow tachykinins to preferentially bind certain neurokinin receptors with greater affinity than others. For example, NK1R exhibits high affinity for SP, while NK2R preferably binds NKA, NPK and NP, and NK3R favorably ligates NKB.[147] Recent studies demonstrate the capability of HK-1 and human EKs to behave as SP-like peptides and exhibit high selectively towards NK1R in comparison to NK2R and NK3R.[111] However, it has been observed that HK-1 stimulates the proliferation of IL-7-expanded B cell precursors and promotes the survival of early B cells, whereas SP has minimal effect.[134] Future studies are needed to determine whether there exist novel HK-1- and EK-specific neurokinin receptors, or whether these SP-like tachykinins differentially bind the NK1R.

Neurokinin receptors are usually co-expressed with their ligands. Likewise, NK1R and NK2R are widely expressed throughout the nervous system and periphery (peripheral and lymphoid tissues), whereas NK3R is largely confined to the CNS, with exceptions including the placenta and uterus.[147]

1.2.2.1 Signal transduction and cellular responses following agonistic binding of NK receptors

Agonistic signaling via neurokinin receptors, as with all GPCRs, leads to the recruitment and activation of G proteins that potentiate cellular responses (Figure 3). Depending on the type of agonist and conformation state of the receptor, all tachykinin receptors have been described to temporally activate $G\alpha_{q/11}$ and $G\alpha_{s}$ proteins to some extent.[148] NK2R and NK3R typically couple to $G\alpha_{s}$ proteins that activate adenylyl cyclase (AC) to elicit cyclic adenosine monophosphate (cAMP) production. In contrast, NK1R signaling predominately couples to $G\alpha_{q/11}$ proteins that stimulate phospholipase C-γ (PLC-γ) activity, leading to the generation of second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).[149,150] IP3 releases calcium (Ca^{2+}) from intracellular stores via binding specific receptors on the endoplasmic-reticulum (ER). DAG activates conventional protein kinase C (PKC) isoforms to potentiate Ca^{2+} release from intracellular stores and induce a variety of signaling networks, including MAPK/Erk stimulation. Likewise, induction of PKC-Ca^{2+}-MAPK/Erk pathways has been demonstrated to prevent cell death in neurons when signaled with NK1R agonists.[151]

Recent reports have indicated that agonistic signaling via the NK1R participates in additional intracellular signaling pathways. Studies utilizing human colonocytes demonstrate that NK1R signaling activates PI3K and Akt to promote cell longevity.[152] It is currently

unknown whether NK1R stimulation utilizes PI3K-Akt activation downstream or independently of G protein signaling. In addition to intracellular signaling via PI3K/Akt, Bhatia and colleagues have described that NK1R signaling induces the activation of PKC (atypical and novel isoforms) and MAPK (Erk and p38) pathways, which converge to elicit NF-κB activity for enhanced chemokine production in mouse macrophages.[153] Furthermore, β-arrestins have been documented to interact with agonistic-bound NK1R to serve as scaffolds for MAPK/Erk activation, ultimately leading to cell survival.[154] Thus, it is likely that agonistic signaling via the NK1R utilizes multiple pathways that cross-talk to modulate cellular responses.

Importantly, it should be noted that a sub-population of NK1R has been described to exist in peripheral tissues with a truncation in the C-terminal region, preventing sufficient coupling to G proteins. Indeed, truncated-NK1R does not activate conventional G protein-dependent signaling pathways, nor stably interacts with β-arrestins, to promote cellular responses. [155,156] However, Douglas and colleagues have proposed that the truncated-NK1R exhibits functionality, as observed by enhancement of monocyte chemotaxis after SP treatment.[157] Further information is required to elucidate the disparity between NK1R variants in modulating cellular responses mediated by NK1R agonists.

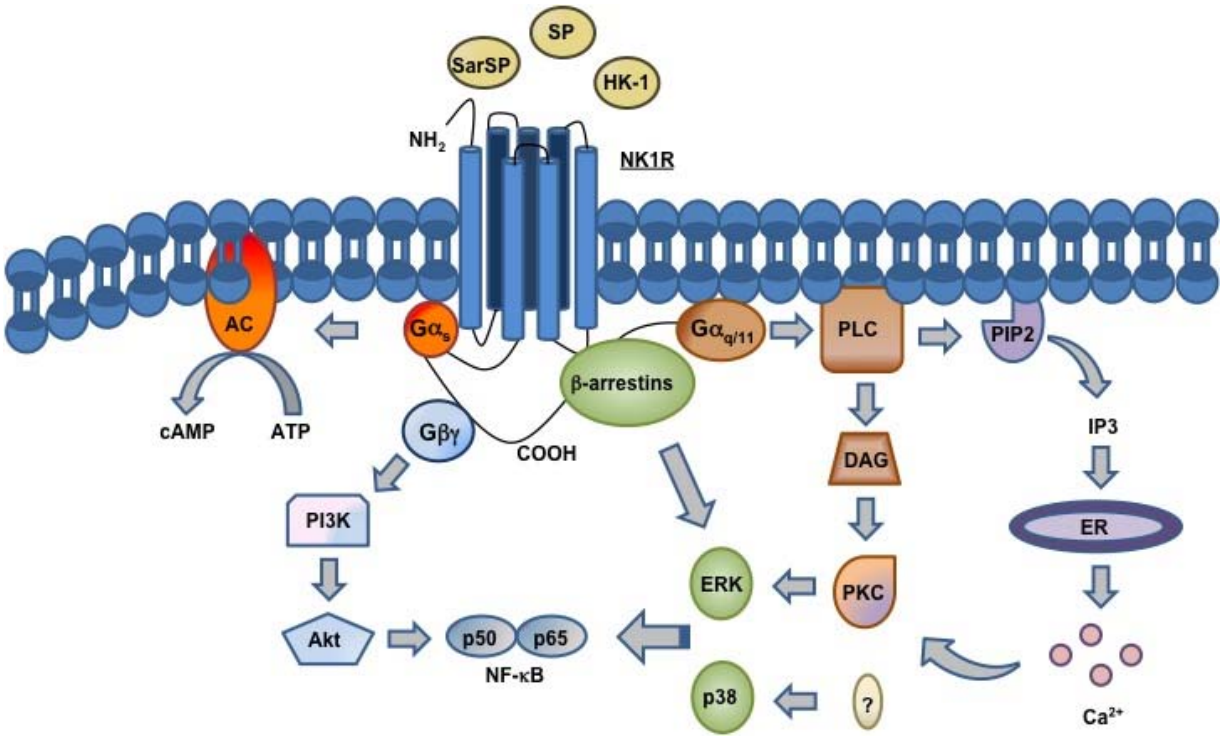

Figure 3: Classical and novel signaling pathways via the neurokinin 1 receptor.

Agonistic signaling via the NK1R leads to the activation of a variety of intracellular signaling pathways. Classically, agonistic signaling via the NK1R enables receptor coupling to $G\alpha_{q/11}$ and $G\alpha_s$ proteins, leading to elicitation of PLC-γ and AC activity, respectively. Activation of PLC results in a transient increase in intracellular IP3, DAG, Ca^{2+} and PKC, whereas AC induces cAMP production. Alternatively, agonistic signaling via the NK1R has been shown to activate PI3K/Akt and β-arrestin pathways, resulting in NF-κB and MAPK (Erk and p38) activation, respectively.

1.2.3 NK1R signaling and immune regulation

Tachykinins are becoming recognized as crucial regulators of immune responses. Lymphoid organs and peripheral tissues are highly innervated with tachykinin-expressing nerve-fiber endings that lie in close proximity to various immune cell populations expressing neurokinin receptors.[123,158] In contrast to other neurokinin receptors, there is increasing evidence that

signaling via the NK1R plays essential roles in promoting development and survival of immune cells, inflammatory responses and cellular immunity.[159-162] Thus, the rest of this chapter will be focused specifically on the role of agonistic signaling via the NK1R in modulating immunity.

1.2.3.1 Role of NK1R in hematopoiesis and cell survival

Within the past two decades, endogenous (SP and HK-1) and synthetic agonists for the NK1R have been demonstrated to participate in an integrated network of factors that promote the hematopoiesis and survival of lymphoid and myeloid cells. Preliminary reports have described that mice treated with capsaicin, a neurotoxin that depletes SP-containing sensory neurons in peripheral tissues, or synthetic NK1R antagonists, exhibit defects in thymic weight and elimination of $CD4^+CD8^+$ double positive (DP) cells in the thymus, suggesting impairment of T cell development.[163,164] Further reports have demonstrated that application of exogenous HK-1 to fetal thymic organ cultures increases the number of $CD4^+CD8^+$ DP cells during T cell development, which was prevented when cultures were supplemented with NK1R antagonists.[137,165] Similar to T cell development, administration of NK1R antagonists in vivo results in a reduction of early B cell populations in mice, suggesting NK1R relevance in the hematopoiesis of B cells.[134] Consistent with these results, addition of HK-1 to a pre-B cell line (70Z/3) or primary bone marrow B lineage cells enhances survival and proliferation in the absence of IL-7, which is a crucial growth factor for B cell development. As expected, NK1R antagonists further enhances apoptosis induced by IL-7 withdrawal.[134,165] Collectively, these results demonstrate the involvement of NK1R agonistic signaling in promoting the development and survival of developing lymphocytes.

Additional investigations have suggested a role of NK1R agonistic signaling in the development and survival of myeloid cells. Likewise, physiological concentrations of SP (10^{-8} to 10^{-11}M) have been reported to replace or act in concert with suboptimal concentrations of various hematopoietic growth factors (e.g., GM-CSF) in stimulating the colony formation of myeloid lineages from bone marrow cultures.[159,166] Detailed experiments have elucidated the ability of SP to ligate the NK1R on stromal cells to induce secretion of a variety of hematopoietic growth factors (IL-3, IL-6, GM-CSF and M-CSF)[167] and directly signal hematopoietic stems in the absence of stromal cells to generate myeloid lineages.[168] In addition to differentiation of myeloid cells, agonistic signaling via the NK1R has been demonstrated to promote the survival of neutrophils[169] and macrophages.[170,171] The significance of NK1R-mediated survival of granulocytes for the outcome of immunity is currently being investigated, but most likely contributes to sustaining inflammatory responses.

1.2.3.2 Role of NK1R in inflammation

It is established that agonistic signaling via the NK1R promotes neurogenic inflammation in the skin, gut and lungs.[106,108,110] During inflammatory responses, chemotaxis is a crucial process to ensure that phagocytes respond quickly to internalize foreign Ag and relay antigenic information for the generation of T cell immunity in DLNs. Likewise, SP has been shown to participate in the chemotaxis of granulocytes[162,172] and mononuclear cells.[161] In vivo administration of SP leads to the adherence of granulocytes to vascular endothelial and bronchial epithelial cells, allowing for extravasation into peripheral sites of inflammation.[162,173] Extravasation of granulocytes involves the up-regulation of selectin molecules (membrane glycoproteins) on the endothelium and integrins (carbohydrates) on migrating granulocytes, ultimately leading to cell

adhesion and migration. SP enhances the expression of β2-integrin ligands ICAM-1 and VCAM-1 on vascular endothelial cells.[174] There is evidence that SP may increase the expression of integrins on migrating granulocytes.[175] In addition to up-regulating adhesion molecules, SP may also enhance the expression of chemokine receptors and promote the induction of chemotactic cytokines, including IL-8 and MIP-2, which lead to the accumulation of neutrophils in inflamed tissues.[176] As granulocytes migrate into inflamed tissues, SP is well known to induce degranulation of mast cells[177] and respiratory bursts in neutrophils,[178] ultimately perpetuating inflammatory responses.

The chemotaxtic effect of SP is also observed for mononuclear cells, including DCs (Section 1.2.4), macrophages and T cells. Indeed, several studies describe that splenic T cell populations migrate towards SP release via LFA-1 and ICAM-1 interactions.[179] In addition, monocyte and macrophage stimulation with SP enhances the induction of chemokines (MIP-2 and MCP-1)[180] and pro-inflammatory cytokines (IL-12p70, IL-1, IL-6 and TNF-α),[181] while diminishing production of the anti-inflammatory cytokine TGF-β1.[182] It is likely that SP release, via nerve-fiber terminals or immune cells, favors chemotaxis of granulocytes, APCs and effector T cells to sites of inflammation.

1.2.3.3 Role of NK1R in the generation of type-1 T cell responses

Due to the observation that SP secretion and NK1R expression are enhanced in inflammatory and autoimmune disorders characterized by a type-1 T cell bias,[183-186] there has been a recent focus on the ability of NK1R signaling to promote the development of $CD4^+$ Th1 and $CD8^+$ CTL/Tc1 T cell responses. As observed in vitro and in vivo, SP stimulation of murine and human $CD4^+$ T cells leads to the polarization of Th1 cells secreting IFN-γ.[187] Interestingly, SP

stimulation has been reported to drive established Th2 clones to a Th1 profile,[188] and suggested to switch Th2-driven atopic dermatitis to a chronic Th1-mediated disease.[189] The capability of NK1R signaling to promote type-1 T cell immunity has been suggested to occur for $CD8^+$ T cell responses. A recent study demonstrates that administration of SP in vivo enhances $CD8^+$ CTL secretion of IFN-γ.[190] Furthermore, mice that are defective in NK1R signaling mount weak CTL responses and are highly susceptible to intracellular pathogens, including gamma herpes virus 68[191] and Salmonella.[192] We have reported that application of the selective NK1R agonist [$Sar^9Met(O_2)^{11}$]-SP (SarSP, 10^{-9} M) in vivo leads to the development of enhanced Th1 and Tc1 responses.[84] Cytokines that are associated with Th1/Tc1 immunity, including IL-12p70, IL-18 and IFN-γ, have been demonstrated to enhance SP and NK1R expression in T cells and macrophages.[130,193] In contrast, the anti-inflammatory cytokines IL-10 and TGF-β1 diminish SP and NK1R expression in T cells and macrophages.[185] Collectively, these results demonstrate an association of NK1R agonistic signaling and the induction of type-1 T cell responses. However, it is not completely understood if NK1R signaling promotes type-1 T cell responses via stimulation of DCs.

1.2.4 NK1R signaling and DC function

Increasing evidence suggests that SP may directly regulate the immune-stimulatory function of DCs. Previous studies have determined that DCs lie in close proximity to SP-expressing nerve-fiber terminals innervating lymphoid[194] and peripheral tissues,[122,195] and express full-length

NK1R.[196] Additionally, it has been reported that DCs express TAC1 mRNA and SP protein,[197] although secretion of SP has yet to be published. Therefore, DCs might serve dual roles in inducing and reinforcing NK1R-mediated immunity.

Not surprisingly, initial studies investigating the role of NK1R signaling in modulating DC function have implicated NK1R agonists as chemoattractants for DCs migrating into inflamed sites in peripheral tissues. Kraid et al. have demonstrated that physiological concentrations of SP (10^{-10} M) enhance the migration of rat pulmonary DCs to sites of inflammation during responses to inhaled Ag.[198] Likewise, Kanieder et al. have reported that murine DCs signaled via the NK1R augment prion-protein (PrP)-induced chemotaxis of DCs.[199] In contrast, one study describes that only high concentrations of SP (10^{-4} M) induce modest chemotaxis of DCs in vitro, which may indicate a non-physiological finding or an emergency case scenario where SP is secreted from nerve-fibers following pro-inflammatory stimuli.[200]

During infection, DCs in peripheral tissues take up Ag, mature and migrate to DLNs as they process and present Ag in the context of MHC class molecules for the presentation to naïve T cells. Our laboratory has observed that administration of SarSP (10^{-9} M) in vivo enhances the ability of LCs loaded with Ag to migrate from the periphery into skin-DLNs.[84] Furthermore, we report that SarSP induces the generation of lymphatic vessels expressing the DC chemoattractant CCL21 in skin-DLNs, which correlated with a quicker disappearance of DCs in the epidermis and their redistribution in skin-DLNs. It can be speculated that SP signaling via the NK1R attracts and arrests immature DCs to inflamed sites as they recognize danger signals and undergo maturation. Acquiring the capacity to migrate to DLNs, SP-mediated chemotaxis might serve to guide DCs into DLNs, allowing them to relay antigenic information to T cells for the elicitation of adaptive immunity.

Due to the association of NK1R signaling and their ability to generate Th1/Tc1 responses, our lab has postulated that NK1R agonists signal DCs to elicit type-1 T cell responses. As discussed above, application of SarSP (10^{-9} M) in vivo promotes LC activation in the epidermis and their subsequent migration to the DLNs, where they polarize robust Ag-specific Th1 and Tc1 immune responses. Further studies are needed to determine whether SP directly activates LCs, or if neighboring cells, such as keratinocytes, bind SP and secrete IL-6 and IL-12p70 to induce the ability of LCs to promote Ag-specific type-1 T cell responses.

There are relatively few studies investigating the direct effects of signaling DCs with NK1R agonists in modulating T cell stimulatory function (Figure 4). Pharmacological studies conducted by Marriott and colleagues have demonstrated that treatment of DCs with SP promotes Ca^{2+} influx and NF-κB nuclear translocation.[201] NF-κB is a transcription factor that is responsible for up-regulating MHC class II molecules, co-stimulatory molecules and the Th1-biasing cytokine IL-12p70. Interestingly, it has yet to be reported whether NK1R agonists can induce DC maturation and IL-12p70 secretion. Lambrecht et al. have demonstrated that addition of a selective NK1R antagonist (SR140333), but not NK2R and NK3R antagonists, abrogates T cell proliferation in allogeneic and Ag-specific DC-$CD4^+$ T cell co-cultures, confirming the importance of endogenous NK1R signaling in promoting T cell responses.[197] However, it is still unclear whether NK1R agonists signal DCs to elicit type-1 cell responses, or if they signal T cells directly to enhance IFN-γ secretion.[187]

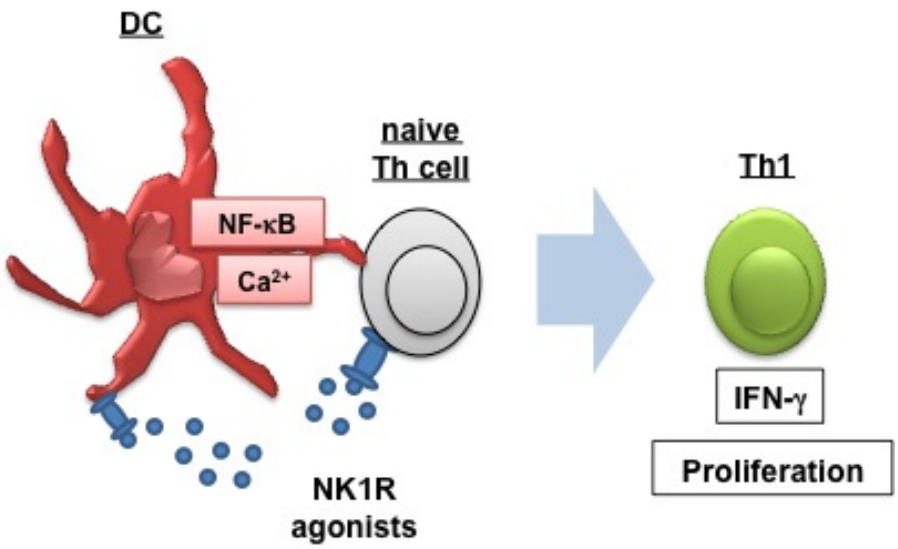

Figure 4: Current model representing NK1R signaling of DCs and CD4$^+$ T cells.

NK1R agonists elicit DCs to increase cytosolic Ca^{2+} concentration and nuclear translocation of NF-κB. In addition, CD4$^+$ T cells are capable of responding to SP, leading to greater T cell proliferation, and in some context, elevated secretion of IFN-γ. It is unknown whether SP signals DCs to induce NK1R-mediated induction of type-1 biased T cell responses.

1.3 CELLULAR IMMUNITY

Immune responses generated from immunization and infections have historically been divided into two arms of defenses, including humoral-mediated or cell-mediated immunity. While, humoral immunity provides protection found in the serum via secretion of antibodies that target extracellular pathogens; cellular immunity relies on the activation of cells to provide protection against intracellular infection and cancer. Successful cellular immunity is mediated by the induction of Ag-specific type-1 CD8$^+$ CTL/Tc1 and CD4$^+$ Th1 cell responses, along with phagocytosis and secretion of pro-inflammatory cytokines from macrophages and NK cells. In

most models, $CD8^+$ CTLs play crucial roles in eradicating tumor and intracellular infections.[202,203] Further studies have elucidated the importance of $CD4^+$ Th1 cells in providing aid to CTL responses, in addition to their unique roles in immunity.[204]

1.3.1 $CD8^+$ Tc1 responses

$CD8^+$ CTLs recognize infected or abnormal cells via Ag-specific interactions communicated through TCR and MHC class I/peptide complex signaling. As infected and tumor cells are poorly immune-stimulatory, mature Ag-loaded DCs migrating to DLNs serve as the main cell type to stimulate naive $CD8^+$ T cells. Activated CTLs then migrate to inflamed peripheral tissues where they eliminate intracellular bacteria, viruses and tumors by efficiently recognizing foreign Ag-encoded peptides presented in the context of MHC class I molecules on the cell surface of infected cells, leading to apoptosis via two possible distinct mechanisms. The first scenario includes the secretion of perforin and cytotoxic granules. The cytotoxic granule granzyme B (GrB) has been demonstrated to mediate CTL killing, and abrogation of either GrB[205] or perforin[206] leads to impaired CTL responses in several models of infections. Both GrB and perforin are secreted simultaneously in the area of the target cell and coordinate together to elicit apoptosis. GrB binds to the mannose-6-phosphate receptor and enters the target cell via the endocytic pathway. Perforin then disrupts the plasma membrane of the endosomes, allowing GrB to escape and initiate apoptosis by cleaving several substrates, including caspases. Alternatively, CTLs can also induce apoptosis via cross-linking death receptors on the surface of

target cells. Most notably from the TNF family, Fas-FasL signaling has been described to recruit pro-apoptotic adaptor proteins and activate caspase cascades to elicit apoptosis.[207] It is likely these pathways act together to elicit CTL function to clear pathogens and tumors.[208]

As described in section 1.1.1.2, type-1 polarized $CD8^+$ CTL/Tc1s preferentially secrete IFN-γ, TNF-α and IL-2 with little production of type-2 (IL-4, IL-5 and IL-13) and type-17 (IL-17 and IL-22) cytokines. Some studies report that $CD8^+$ Tc1 cells are superior in killing their targets, due to enhanced cytotoxicity coupled with prolonged survival and IFN-γ secretion.[209] Data from HIV models of infection suggest that $CD8^+$ T cells become less efficient in killing targets by developing into Tc2 cells, due to a deficiency in IL-12p70 signaling or conditions that favor excess IL-4 production.[210] In addition, Tc17 cells do not express GrB and other CTL effector molecules, and are subsequently less efficient at mediating cell lysis of targets in vitro. However, recent reports demonstrate that adoptive transfer of Tc17 cells can protect against influenza lethal challenge by accumulating more neutrophils and converting to a Tc1-like subset that secretes high levels of IFN-γ.[211]

In addition to short-lived effector populations, Tc1 central memory cells are able to persist in DLNs for long periods of time and become quickly re-activated to suppress re-challenge of tumor and infectious Ag. Memory Tc1 cells also promote cellular immunity by enhancing DC function. For example, memory Tc1 cells secrete IFN-γ to elevate the production of IL-12p70 in DCs[25,212,213] and TNF-α to protect DCs from CTL killing by enhancing expression of GrB inhibitor 9.[214] Thus, the ability of memory Tc1 cells to prevent DCs from CTL killing could allow for better generation of Ag-specific $CD8^+$ CTL/Tc1 responses.

1.3.2 $CD4^+$ Th1 responses

Similar to Tc1 cells, $CD4^+$ Th1 cells secrete significant levels of IFN-γ, TNF-α and IL-2, and additionally, play significant roles in aiding primary, secondary and memory CTL responses (Figure 5).[204] Upon Ag recognition and co-stimulation provided by mature DCs in DLNs, $CD4^+$ Th1 cells up-regulate cell-surface expression of CD40L and produce high amounts of IFN-γ and IL-2. CD40 ligation during the DC-$CD4^+$ T cell synapse fully activates DCs, enabling them to effectively provide the 'license to kill' to stimulate $CD8^+$ T cells specific for the presenting Ag.[215] CD40 signaling has been observed to enhance IL-12p70 and IL-15 secretion and co-stimulatory molecule (B7, CD70 and 4-1BBL) expression in DCs.[216,217] IL-2 and IL-15 have been demonstrated to provide maintenance for Ag-specific $CD8^+$ CTL effector and memory responses, respectively.[218,219]

Matzinger and colleagues have demonstrated that $CD4^+$ Th1 cells are more effective than $CD8^+$ T cells in eradicating tumors without the aid of exogenous cytokines or treatment.[220] In addition to helping $CD8^+$ CTL responses, it has been reported that $CD4^+$ Th1 cells promote anti-tumor responses via cytokine signaling. For example, adoptive transfer of $CD4^+$ Th1 cells can mediate transplanted, endogenous or β-cell tumor rejection by inhibiting tumor angiogenesis and proliferation while promoting apoptosis via secretion of IFN-γ and TNF-α.[221] Indeed, Th1 cells only induce tumor regression in these studies when IFN-γ and TNF-α signaling are intact, while impairment of these cytokines resulted in tumor growth. Other studies suggest that Th1 cells directly induce apoptosis of tumors cells via ligation of FasL and TRAIL or secretion of perforin and GrB.[222] In contrast, $CD4^+$ Th2 cells do not elicit tumor rejection due to their poor secretion of IFN-γ. Similar to their roles in favoring anti-tumor responses, Th1 cells are highly efficient in

eradicating intracellular infections. Mechanistic analysis demonstrates that IFN-γ secretion from Th1 cells, along with Tc1 cells, enhances the immune-stimulatory activity of phagocytes harboring intracellular pathogens by promoting phagosomal fusion, nitric oxide production and expression of molecules involved in the MHC class I and II Ag processing and presentation pathways.[223,224]

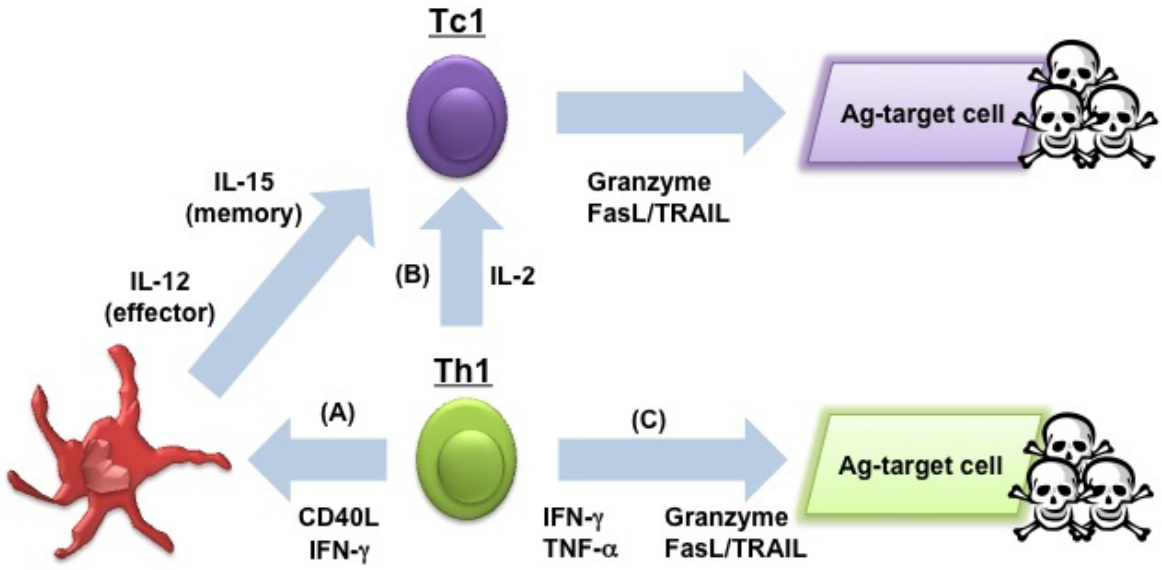

Figure 5: Role of CD4$^+$ Th1 cells in promoting cellular immunity.

Activated CD4$^+$ Th1 cells eradicate tumors and infected cells by (A) licensing DCs via CD40 ligation to favor CD8$^+$ CTL effector and memory responses, (B) secreting IL-2 to directly maintain developing CD8$^+$ T cell responses and (C) inducing apoptosis of target cells via secretion of IFN-γ, TNF-α and granzymes or ligation of FasL/TRAIL molecules.

1.4 SPECIFIC AIMS

The activation and survival of DCs are critical for generating robust cellular immunity, and for that purpose, these features are currently being exploited in DC-based immunotherapy protocols designed to elicit successful cellular-based vaccinations. It is becoming increasingly evident that the nervous system, via secretion of pro-inflammatory tachykinins that bind with high affinity to the NK1R, promotes immune cell survival and type-1 T cell responses. However, the ability of NK1R agonists to modulate the immune-stimulatory function of DCs remains unknown. In these studies, we formulate and investigate two specific aims addressing the immunological role of stimulating murine BMDCs via the NK1R to enhance DC longevity (Aim 1) and induce potent type-1 T cell responses (Aim 2) in vivo.

1.4.1 Specific Aim 1 (Chapter 2): To analyze the capacity of agonistic signaling via the NK1R to modulate the viability of DCs

Given the importance of DC longevity in mediating enhanced Th1 and Tc1 responses, we investigated the role NK1R agonists serve in modulating the viability of DCs when exposed to apoptotic signaling in vitro and in vivo. In this chapter, we describe that BMDCs express the full-length functional NK1R, and agonistic signaling via the NK1R activates a pro-survival PI3K-Akt intracellular pathway to rescue DCs from apoptosis induced by GM-CSF and IL-4 withdrawal. After adoptive transfer, we discovered that NK1R-signaled BMDCs loaded with or without Ag persist longer in DLNs, which was mediated by a combination of NK1R signaling in vitro and CD40 signaling in vivo, and promote robust cellular immunity as assessed in DTH

assays. These findings demonstrate for the first time that neuropeptides binding the NK1R are capable of modulating DC function, including their survival and ability to elicit cellular immunity.

1.4.2 Specific Aim 2 (Chapter 3): To assess the ability of NK1R-signaled DCs to promote Ag-specific $CD4^+$ Th1 and $CD8^+$ CTL/Tc1 responses

NK1R signaling favors the generation of Th1 and Tc1 responses.[187] Thus, we addressed the ability of NK1R agonists to modulate the immune-stimulatory function of DCs, and subsequently, their ability to tailor Ag-specific Th1/Tc1 responses. Initial results demonstrate that signaling DCs with NK1R agonists in vitro enhances their maturation, while significantly diminishing the production of anti-inflammatory cytokines IL-10 and TGF-β1. Furthermore, adoptive transfer of NK1R-signaled DCs promotes robust OVA-specific Th1/Tc1 responses in vitro and in vivo.

Mechanistic analysis demonstrates the necessity of endogenous IL-12p70 secretion and survival of endogenous DCs to amplify robust Ag-specific Th1/Tc1 responses in vivo mediated by adoptive transfer of DCs. These results suggest that endogenous DC populations play crucial roles in interacting with adoptively transferred DCs to generate Th1/Tc1 responses. As we illustrate and discuss, the unique ability of NK1R-signaled DCs to down-regulate the secretion of IL-10 allows for greater induction of Th1/Tc1 responses, which may be contributed to interactions with endogenous DC populations in peripheral or lymphoid tissues, and

alternatively, greater direct priming of $CD4^+$ and $CD8^+$ T cells. Collectively, these novel findings demonstrate the unique ability of tachykinins, acting via the NK1R, to promote immune-stimulatory DCs that initiate robust Th1/Tc1 responses in vivo.

2.0 CHAPTER TWO: PRO-INFLAMMATORY TACHYKININS THAT SIGNAL DENDRITIC CELLS VIA THE NEUROKININ 1 RECEPTOR PROMOTE CELL SURVIVAL AND INDUCTION OF CELLULAR IMMUNITY

Brian M. Janelsins, Alicia R. Mathers, Olga A. Tkacheva, Geza Erdos, William J. Shufesky, Adrian E. Morelli, and Adriana T. Larregina.

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 I collaborated with experimental design, performed most of the experimental work and wrote the manuscript. Alicia Mathers contributed with qRT-PCR design and assistance, while Olga Tkacheva performed BMDC cultures and other experimental techniques. William Shufesky performed immunofluorescence staining and analysis of tissues and Adrian Morelli contributed with experimental design and flow cytometric analysis. Lastly, Adriana Larregina coordinated the experimental approach and helped with data analysis and interpretation, as well as with manuscript preparation.

2.1 ABSTRACT

DCs are the preferred targets for immune-therapy protocols focused on stimulation of cellular immune responses. However, despite initial promising results, ex vivo-generated DCs do not always promote immune-stimulatory responses. The outcome of DC-dependent immunity is regulated by pro-inflammatory cytokines and neuropeptides. Pro-inflammatory neuropeptides of the tachykinin family, including SP and HK-1, bind the NK1R and promote stimulatory immune responses. Nevertheless, the ability of pro-inflammatory tachykinins to affect the immune functions of DCs remains elusive. In the present work, we demonstrate that murine BMDCs generated in the presence of GM-CSF and IL-4 express functional NK1R. Signaling via NK1R with SP, HK-1, or the synthetic agonist SarSP rescues DCs from apoptosis induced by deprivation of GM-CSF and IL-4. Mechanistic analysis demonstrates that NK1R agonistic binding promotes DC survival via PI3K-Akt signaling cascade. In adoptive transfer experiments, NK1R-signaled BMDCs loaded with Ag exhibit increased longevity in draining lymph nodes, resulting in enhanced and prolonged effector cellular immunity. Our results contribute to the understanding of the interactions between the immune and nervous systems that control DC function, and present a novel approach for ex vivo-generation of potent immune-stimulatory DCs.

2.2 INTRODUCTION

Due to their crucial role in initiation and control of innate and adaptive immunity, myeloid DCs are the preferred target Ag-presenting cells for positive cellular vaccination protocols.[225] During the past decade, ex vivo-generated DCs have been used in immunization approaches for prevention and treatment of cancer and infectious diseases.[226] Current DC-based vaccines focus on the adjuvant effect of pro-inflammatory mediators conferring DCs the capability to initiate and bias T cell immune responses.[27,43,227] However, in spite of initial promising results, DC-based vaccines do not always elicit potent T cell immunity.[73]

Generation of efficient T cell immunity using ex vivo-generated DCs requires a critical number of adoptively transferred DCs capable of surviving apoptosis.[54] Indeed, DCs used as cellular vaccines are exposed to pro-apoptotic stimuli at the injection sites and in DLNs.[54,228] Accordingly, exposure of DCs to pro-apoptotic stimuli triggered by lytic infections, UVB-irradiation, tumor mediators, and cytotoxic cells results in immune-suppression.[53] Additionally, interaction of DCs with cells in early apoptosis down-regulates the T cell-stimulatory ability of DCs and induces immunological tolerance.[101,229-231] Conversely, pro-inflammatory mediators and growth factors promoting DC survival, including GM-CSF,[57] PG-E_2,[56] LPS,[63] and CCR7[67] and CD40[232] ligands, correlate with enhanced T cell immunity.

The intracellular signaling involved in DC survival is currently being elucidated. Activation through CD40 promotes DC survival by favoring a positive balance of NF-κB vs. Jnk-AP-1 pathway,[70] whereas GM-CSF, LPS, and PG-E_2 prevent DC apoptosis by signaling via PI3K and Akt.[56,57,64] In contrast, the immune-suppressive drug Rapamycin induces DC death by antagonizing GM-CSF signaling via inhibition of the PI3K-Akt signaling cascade.[57,233]

Recently, it has become evident that the outcome of the immune response is highly regulated by neuropeptides. The balance between anti-inflammatory and pro-inflammatory neuropeptides is crucial to maintain the immune privilege of the CNS and the steady-state condition in peripheral tissues, and altering this delicate balance plays a role in the pathogenesis of chronic inflammatory and autoimmune diseases.[106,107,234-236] Pro-inflammatory neuropeptides like SP favor $CD4^+$ Th1 bias and cellular immunity, while vasoactive intestinal peptide (VIP) promotes $CD4^+$ Th2 bias.[84,110,160,187,237-240] Conversely, calcitonin gene-related peptide (CGRP) and the anti-inflammatory products of POMC cleavage, α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), are potent suppressors of cellular immunity.[235]

The pro-inflammatory tachykinins SP and HK-1 exert their immune-stimulatory functions by binding with high affinity to the NK1R, a seven transmembrane domain GPCR.[241] SP and HK-1 induce IFN-γ secretion and proliferation of T cells.[106,107,134,137,197] Our laboratory has recently reported that skin DCs express functional NK1R and elicit in vivo $CD4^+$ Th1 and $CD8^+$ CTL immunity in response to NK1R agonists.[84] Nevertheless, because the NK1R is expressed by several types of skin cells, the potential of NK1R agonists to modulate directly the immune-stimulatory function of DCs remains unknown.

In the present study, we examined the expression of functional NK1R by BMDCs and the ability of NK1R agonists SP, HK-1, and SarSP to rescue BMDCs from apoptosis induced by deprivation of GM-CSF and IL-4. We also investigated in vivo the immunological relevance of signaling BMDCs with these pro-inflammatory neuropeptides.

2.3 MATERIALS AND METHODS

2.3.1 Mice

Eight- to twelve-week old wild-type C57BL/6 mice (B6) (The Jackson Laboratory, Bar Harbor, ME) and B6 $NK1R^{-/-KO}$ mice (kindly provided by Dr. Christopher Paige, University of Toronto, Canada) were housed in the pathogen-free animal facility of the University of Pittsburgh and used according to institutional guidelines with approval of the University of Pittsburgh IACUC.

2.3.2 Generation of BMDCs

BMDCs were generated by culturing mouse BM-precursors for 6d in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA) (complete medium) and GM-CSF and IL-4 cytokines (both at 1,000 U/ml) (R&D, Minneapolis, MN), as previously described.[242,243] Total $CD11c^+$ BMDCs were purified by histodenz gradient (16% wt/vol, purity of $CD11c^+$ cells ≥85% as determined by FACS-analysis). The DC morphology was confirmed in cytospins of purified $CD11c^+$ cells obtained, as previously described,[244] and stained with Hema-3 (Fisher, Pittsburgh, PA).

2.3.3 Quantitative RT-PCR

The expression of NK1R mRNA was detected by SYBR green-based quantitative RT-PCR (qRT-PCR) using reverse transcribed cDNA isolated from $CD11c^+$ BMDCs cultured for 24h in

complete medium with or without LPS (500 ng/ml, Sigma). Total RNA was isolated using the RNeasy isolation kit (Qiagen, Valencia, CA) and was reverse transcribed using the QuantiTect Reverse Transcription Kit (Invitrogen, Carlsbad, CA), according to manufactures' instructions. cDNA template amplification was performed by using the Quantitect SYBR Green PCR kit with specifically designed NK1R and phosphoglycerate kinase 1 (PGK1) primers (Qiagen). qRT-PCR was conducted using the ABI Prism 7900HT system (Genomics and Proteomics Core Laboratories, Univ. of Pittsburgh, Pittsburgh, PA) with cycling conditions consisting of 15 min Taq activation at 95^{o}C followed by denature, anneal, and extension phases for 15 sec at 94^{o}C, 30 sec at 54^{o}C, and 30 sec at 72^{o}C, respectively, for 40 cycles. Analysis was performed using Sequence Detection System software 2.2.2 (Applied Biosystems, Foster City, CA), and the quantification of PGK1-normalized NK1R was expressed as mRNA fold increase.

2.3.4 Immunofluorescence microscopy and FACS-analysis

The expression of NK1R protein on the surface of BMDCs was analyzed in cytospins. BMDC suspensions were FcR-blocked with anti-CD16/32 mAb, fixed in 2% paraformaldehyde (PFA), and stained with a purified goat anti-mouse IgG specific for the N-terminus domain of the NK1R (Santa Cruz Biotechnology, Santa Cruz, CA), followed by donkey anti-goat FITC-IgG F(ab')$_2$ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After labeling, BMDCs were cytospun as described[242] and analyzed using an AxioStar Plus microscope equipped with epi-fluorescence, an AxioCamera, and the image analyzing software AxioVision (Carl Zeiss Vision Imaging Systems, Thornwood, NY).

Quantification of NK1R in BMDCs was performed by FACS-analysis (FACSCalibur™, Becton Dickinson, Palo Alto, CA). BMDCs were: i) stained with anti-mouse APC-CD11c and PE-CD86 or PE-CD83 mAbs (BD-PharMingen, San Diego, CA); ii) fixed in 2% PFA; iii) permeabilized in 0.1% saponin (Sigma); and iv) incubated with goat anti-NK1R IgG (recognizing the N-terminus domain) or rabbit anti-NK1R IgG (specific for the C-terminus domain) (Advanced Targeting Systems, San Diego, CA), followed by anti-goat or anti-rabbit FITC-IgG $F(ab')_2$ (Jackson). Negative controls included species-matched isotype IgG and secondary Abs.

Quantification of anti- and pro-apoptotic proteins in BMDCs was assessed by intracellular staining and FACS-analysis. BMDCs were cultured in complete medium or supplemented with SarSP (10^{-9} M) or GM-CSF and IL-4 (both at 1,000 U/ml). After 24h, cells were: i) labeled with FITC-CD11c mAb (BD-PharMingen); ii) fixed and permeabilized as described above; and iii) incubated with one of the following anti-mouse mAbs, Alexa Fluor 488-pAktSer473 (Abcam, Cambridge, MA), PE-active caspase 3 (BD-PharMingen) or PE-Bcl-2 (BD-PharMingen). For detection of pBad, BMDCs were incubated with goat anti-mouse pBadSer136 mAb (Santa Cruz Biotechnology) followed by anti-goat FITC-IgG $F(ab')_2$.

2.3.5 Induction and quantification of apoptosis

BMDCs were cultured for 24-72h in complete medium with or without the NK1R antagonist WIN-51708 (10^{-8} M) (Sigma) and/or one of the following NK1R agonists: i) SarSP (Sigma); ii) SP (Bachem, King of Prussia, PA); or iii) HK-1 (Bachem) (all at 10^{-9} or 10^{-5} M). Positive controls included BMDC cultures supplemented with GM-CSF and IL-4 or agonistic CD40 mAb

(10 μg/ml, BD-PharMingen).[244] At the indicated time points, BMDCs were harvested and cell viability was assessed by trypan blue exclusion. The morphology of BMDCs undergoing apoptosis was examined in cytospins stained with HEMA-3 (Fisher) or DAPI (Invitrogen). DNA fragmentation was further assessed by FACS-analysis in BMDCs immunostained using a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche, Indianapolis, IN), according to manufactures' protocol. For quantification of apoptosis, FACS-analysis was performed on BMDCs that were immunostained with APC-CD11c, FITC-Annexin V (BD-PharMingen), and propidium iodide (PI, Sigma). Percentage of apoptotic cell rescue was calculated by the formula: [1-(% of apoptotic NK1R-signaled BMDCs) / (% of apoptotic non-treated BMDCs)] x 100.

Experiments addressing specificity of signaling via NK1R utilized BMDCs generated from B6 $NK1R^{-/-KO}$ and B6 $NK1R^{+/+}$ mice, the later treated for 30 min with WIN-51708 (10^{-8} M or 10^{-5}M) prior to stimulation with SarSP (10^{-9} M).

2.3.6 Blockade of intracellular signaling

BMDCs were cultured in complete medium with one of the following cell-permeable inhibitors: i) LY294002 (PI3K inhibitor, 5 μM, Biomol, Plymouth Meeting, PA); ii) Akt inhibitor X (5 μM, Calbiochem, San Diego, CA); iii) PD98059 (ERK inhibitor, 20 μM, Biomol); iv) SN-50 (NFκB inhibitor, 18 μM, Calbiochem); or v) Jnk inhibitor II (10 μM, Calbiochem). Following 30 min blockade, BMDCs cultures were treated or not with SarSP (10^{-9} M) for 24h. Quantification of apoptotic BMDCs was analyzed by FACS as described above.

2.3.7 Adoptive transfer of BMDCs

BMDCs were cultured in complete medium with or without SarSP (10^{-9} M). After 24h, BMDCs were left untreated (DCs, control) or haptenized with trinitrobenzene sulfonic acid (TNBS, 1 mM, 25 min at 37°C) (Sigma) (TN-DCs). DCs and TN-DCs were then labeled with carboxyfluorescein succinimidyl ester (CFSE, 1 μM, 15 min at 37°C) (Invitrogen). After thorough rinsing, CFSE-labeled DCs and TN-DCs were adoptively transferred ($2x10^6$ cells / 50μl PBS, footpad, s.c.) into B6 mice (n=3 animals per group).

For in vivo studies analyzing the survival of DCs in the presence or absence of CD40 ligation, mice were treated (or not) with two doses of anti-CD154 blocking MR1 mAb (Bio Express, West Lebanon, NH) injected simultaneously and 2d after BMDC transfer (250 μg per dose, flank, i.p.). At indicated time points, mice were euthanized and local (popliteal) and distant (cervical and axillary) DLNs were dissected. The number of viable CFSE-labeled $CD11c^+$ BMDCs in DLNs was analyzed by FACS, as previously described.[84] An equal number of $CD11c^+$ DCs (10^6) per experimental situation was acquired by FACS and the percentage of CFSE-labeled $CD11c^+$ BMDCs was calculated with the following formula: [(number of $CFSE^+CD11c^+$ BMDCs) / (total number of $CD11c^+$ DCs) x100].

2.3.8 Delayed-type hypersensitivity (DTH) assays

B6 mice (n=6 animals per group) were sensitized with one dose of BMDCs ($2x10^6$ cells / 50μl PBS, footpad, s.c.) pre-treated as follows: i) TNBS haptenized (TN-DCs); ii) TN-DCs cultured with SarSP (10^{-9} M) (SarSP-TN-DCs); iii) TN-DCs cultured with CD40 mAb (CD40-TN-DCs);

iv) non-haptenized DCs (DCs); or v) non-haptenized DCs cultured with SarSP (SarSP-DCs). Elicitation of DTH was performed 6d after sensitization by applying trinitrochlorobenzene (TNCB, 1% in acetone:olive oil, 4:1) (Sigma) on the dorsal surface of the ears. The severity of the DTH responses was assessed by comparing the thickness of ears measured prior and after elicitation for up to 6d, using an electronic caliper (Mitutoyo, Aurora, IL). The DTH responses were expressed as the percent in ear thickness increase using the formula: [(thickness of challenged ear – thickness of control ear) / (thickness of control ear) x 100]. For histological analysis, mice were euthanized 6d after elicitation and samples from ear skin were fixed in 4% formaldehyde, embedded in paraffin, stained with H&E, and analyzed by microscopy or embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN) and snap-frozen in methyl-butane (Sigma). The composition of the cellular infiltrate 6d after elicitation was assessed by immunofluorescence microscopy of cryostat sections of the ears, as previously described.[84] Cryostat sections (8 μm) were mounted on slides pre-treated with Vectabond (Vector Laboratories, Burlingame, CA), fixed in cold 96% ethanol, and incubated with Alexa Fluor 488-CD4 mAb (Invitrogen) and biotin-CD8 (eBioscience, San Diego, CA) or biotin-F4/80 mAbs (Invitrogen), followed by Cy3-streptavidin (Jackson). Cell nuclei were stained with DAPI.

2.3.9 Statistical analysis

Statistical analysis was performed using the GraphPad Prism® 4 software (GraphPad software, San Diego, CA). Differences between more than two means ± 1SD were analyzed by a one-way ANOVA analysis followed by a Student Newman Keuls test. Comparisons between two different means ± 1SD were performed by a Student's "t" test. A "*p*" value <0.05 was considered significant.

2.4 RESULTS

2.4.1 Expression of NK1R in BMDCs

The population of d6 BMDCs was composed of immature and mature $CD11c^+$ DCs. Similar percentages of $CD11c^+$ DCs exhibited short dendrites and a round nucleus, features of immature DCs, or well-developed dendrites and a bean-shaped nucleus, indicative of mature DCs (Figure 6A). Likewise, FACS-analysis demonstrated that BMDC cultures contained $CD11c^+CD86^{-/low}CD83^-$ immature DCs and $CD11c^+CD86^{high}CD83^+$ mature DCs (Figure 6A).

To determine whether BMDCs might be targets of pro-inflammatory neuropeptides that signal via NK1R, we investigated NK1R protein and mRNA transcript expression in BMDCs. Presence of NK1R was analyzed by fluorescence microscopy (Figure 6B) and FACS (Figure 6C) using an Ab recognizing the N-terminus extracellular domain of the receptor. Both immature and mature BMDCs expressed surface NK1R (Figure 6B and C, upper panels).

Since NK1R exists as a full-length (functional) or a C-terminus truncated variant (deficient in signaling),[245] we investigated whether BMDCs express functional NK1R, utilizing an Ab recognizing the C-terminus motif of the receptor. As determined by FACS-analysis, immature and mature BMDCs expressed the functional variant of NK1R (Figure 6C, lower panels).

Next, we analyzed whether BMDCs up-regulate NK1R mRNA following exposure to the pro-inflammatory stimulus LPS by quantifying NK1R transcripts via qRT-PCR. The levels of NK1R mRNA increased 3-fold in BMDCs cultured with LPS ($p<0.05$) (Figure 6D). These results demonstrate that mature and immature BMDCs express surface functional NK1R, and transcription of NK1R mRNA was up-regulated by the DC-activating mediator LPS.

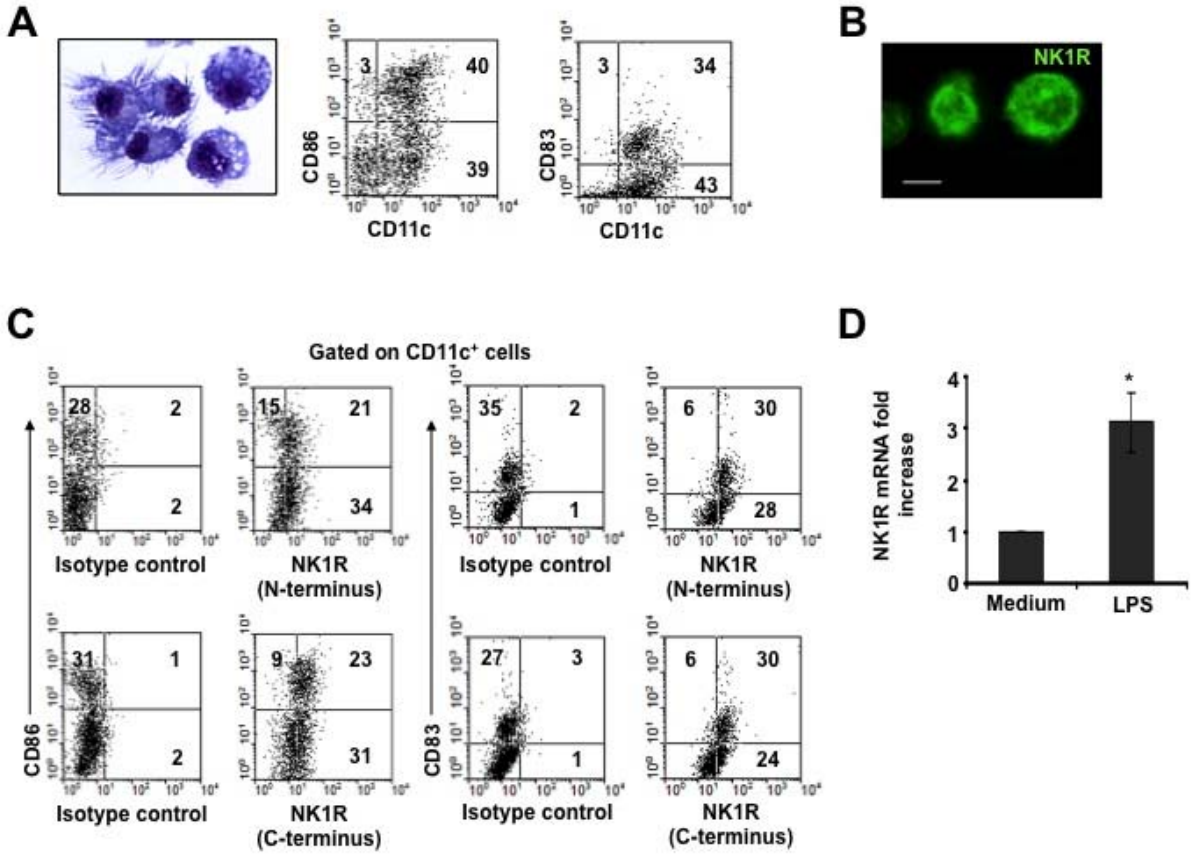


Figure 6: Mature and immature BMDCs express the full-length NK1R.

(A) Microscopic and phenotypic characteristics of d6 BMDCs. Cytospin showing immature DCs (arrows) identified by round nuclei and short membrane prolongations and mature DCs (asterisks) with long membrane processes and bean-shaped nuclei. Hema-3 stain, 500x; scale bar = 5μm. Dot plots of BMDCs illustrating the percentages (numbers in dot plots) of mature ($CD11c^{+}CD86^{high}$ or $CD11c^{+}CD83^{+}$) and immature ($CD11c^{+}CD86^{-/low}$ or $CD11c^{+}CD83^{-}$) DCs. (B and C) Expression of NK1R by BMDCs. (B) Cytospin of purified BMDCs showing the expression of NK1R on their cell surface. Fluorescence microscopy, 500x; scale bar = 5 μm. (C) NK1R expression by $CD11c^{+}CD86^{high}CD83^{+}$ mature and $CD11c^{+}CD86^{-/low}CD83^{-}$ immature DCs analyzed by FACS. Mature and immature BMDCs express the N-terminal (upper panels) and the C-terminal (lower panels) motifs of the NK1R, indicating expression of the full-length functional NK1R. (A-C) Data are representative of 5 independent experiments. (D) qRT-PCR analysis of NK1R mRNA transcripts in BMDCs cultured with or without LPS. *$P < 0.05$. Means (+ SD) of 3 independent experiments are shown.

2.4.2 Agonistic signaling via the NK1R rescues BMDCs from apoptosis

Withdrawal of GM-CSF and IL-4 from BMDC cultures triggers DC death.[57,246,247] Therefore, we investigated the effects of signaling through NK1R on the viability of BMDCs following cytokine withdrawal. BMDCs were cultured for 24h in complete medium with or without GM-CSF and IL-4, the synthetic NK1R agonist SarSP (10^{-9} M),[248] or agonistic CD40 mAb (DC survival signal, positive control). Withdrawal of GM-CSF and IL-4 significantly decreased the number of viable BMDCs ($p<0.001$), whereas addition of SarSP, CD40 mAb, or GM-CSF and IL-4 yielded similar high numbers of viable BMDCs (Figure 7A). GM-CSF and IL-4 withdrawal induced apoptosis of BMDCs characterized by plasma membrane blebs, nuclear condensation, and DNA fragmentation (Figure 7B and C). Conversely, DCs cultured in medium with SarSP or agonistic CD40 mAb exhibited the morphology of viable cells (Figure 7B). Accordingly, SarSP reduced DNA fragmentation in BMDC cultures deprived of GM-CSF and IL-4, as determined by fluorescent microscopy and FACS-analysis of nuclear staining with DAPI and TUNEL, respectively (Figure 7C).

The ability of SarSP to rescue BMDCs from apoptosis was quantified by FACS in BMDCs stained with Annexin V and PI. Withdrawal of GM-CSF and IL-4 increased significantly the percentage of apoptotic BMDCs, whereas addition of SarSP, at physiological concentration, reduced the level of apoptosis by 47% ($p<0.001$) (Figure 7D). The anti-apoptotic effect of SarSP was sustained up to 72h, and addition of higher concentrations of SarSP (from 10^{-7} to 10^{-5}M) did not result in further apoptotic cell rescue (not shown). Cultures of BMDCs supplemented with agonistic CD40 mAb showed a slightly less anti-apoptotic effect than

BMDCs treated with SarSP or GM-CSF and IL-4, a result that could be explained by the low percentage of immature BMDCs that do not express CD40.[242] These results indicate that NK1R agonistic signaling rescues BMDCs from apoptosis induced by GM-CSF and IL-4 withdrawal.

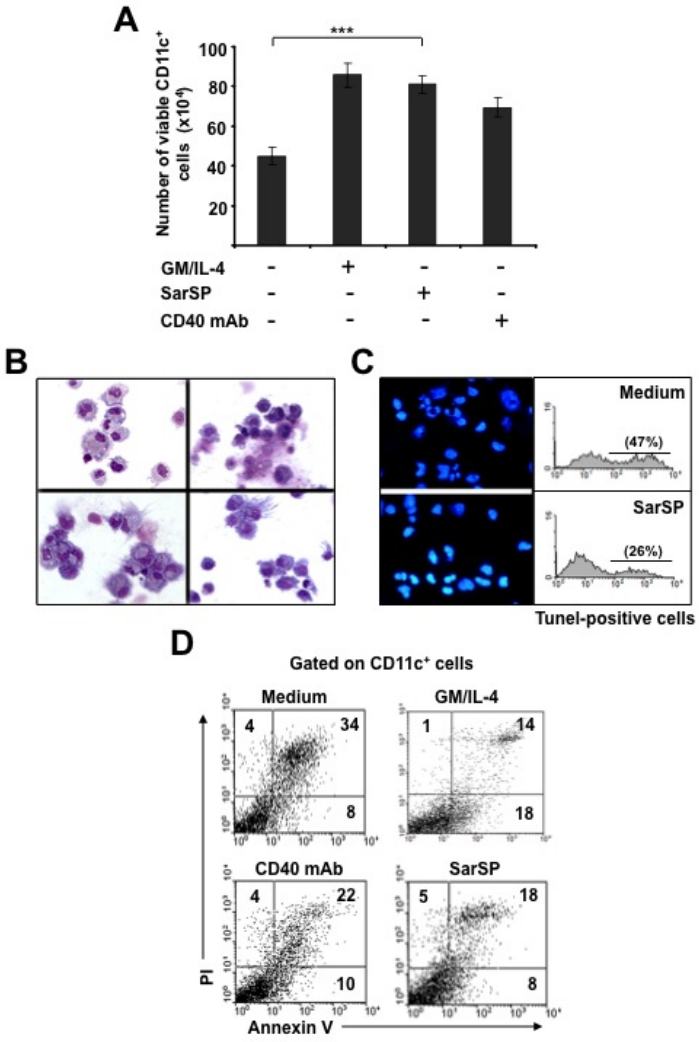


Figure 7: Agonistic signaling through the NK1R in BMDCs prevents apoptosis.

(A) Number of viable $CD11c^+$ BMDCs recovered 24 hours after culture in different conditions. ***P less than .001. Means (± SD) of 6 independent experiments are shown. (B and C) Morphological and nuclear characteristics of apoptosis by BMDCs harvested 24 hours after culture in different conditions. (B) Morphological analysis of apoptosis as evaluated by cytospins of $CD11c^+$ BMDCs stained with Hema-3. (C) Nuclear analysis of apoptosis as determined by cytospins of $CD11c^+$ BMDCs stained with the nuclear dye DAPI (left panels) or suspensions of $CD11c^+$ BMDCs stained with TUNEL (right panels). Withdrawal of GM-CSF (GM) and IL-4 (Medium) induces apoptosis of BMDCs characterized by surface membrane blebs (B, upper right panel, asterisk), nuclear breakage (B upper panel, arrows), chromatin condensation, and DNA fragmentation (C, upper panels, arrows), 500x; scale bar = 20 μm. (D) Quantification of apoptosis in $CD11c^+$ BMDCs by FACS analysis of Annexin V and PI labeling. Numbers in dot-plots represent percentage of cells. Signaling via NK1R with SarSP (10^{-9} M) prevents apoptosis of BMDCs to a similar extent as signaling via CD40 or addition of GM and IL-4 (positive controls). Data are representative of 10 independent experiments.

2.4.3 Pro-inflammatory tachykinins prevent apoptosis of BMDCs via signaling of the NK1R

The naturally-occurring NK1R agonists SP and HK-1 also ligate NK2R and NK3R,[249] which decreases their pro-inflammatory function. In addition, at very high concentrations, SP and HK-1 bind other neuropeptide receptors[250] or exert effects independently from receptor ligation[251]. Therefore, we investigated if similar to SarSP, SP and HK-1 induce anti-apoptotic effects on BMDCs and whether the apoptotic cell rescue induced by SarSP is mediated through NK1R signaling.

SP, HK-1, and SarSP exerted similar anti-apoptotic effects on BMDCs at their physiological concentration (10^{-9} M) ($p<0.001$), and a higher concentration (10^{-5} M) did not enhance this effect ($p>0.05$) (Figure 8A). The anti-apoptotic effect of SarSP was mediated by NK1R binding, as demonstrated by the lack of apoptotic rescue by SarSP, in NK1R blockade assays of BMDCs pre-treated with the selective NK1R antagonist WIN-51708 ($p<0.001$) or in BMDCs generated from B6 $NK1R^{-/-KO}$ mice ($p<0.001$) (Figure 8B and C, respectively). Blockade of NK1R in BMDC cultures, with WIN-51708 by itself, did not significantly increase the level of apoptosis induced by GM-CSF and IL-4 withdrawal (Figure 8D). Together, these results demonstrate that the synthetic NK1R agonist SarSP, SP, and HK-1 exert similar anti-apoptotic effects on BMDCs, which requires signaling via NK1R.

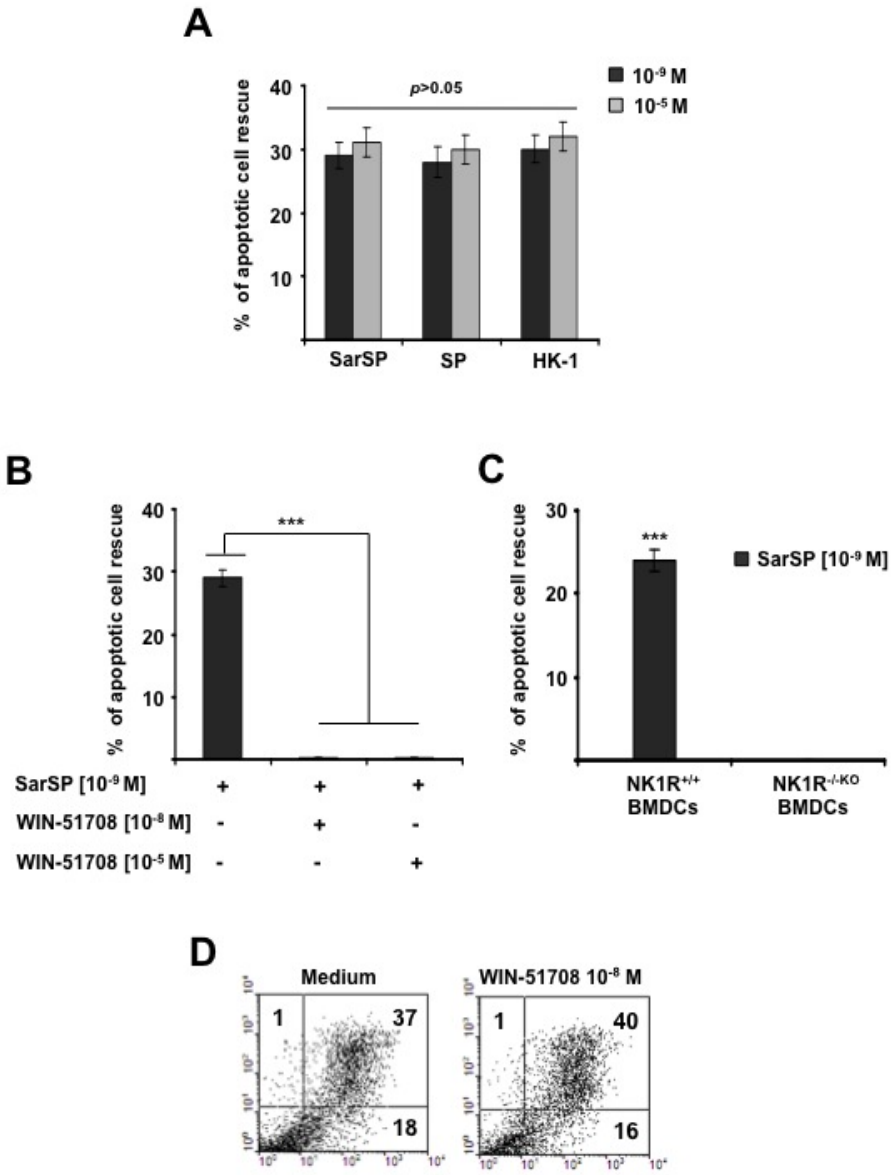


Figure 8: Pro-inflammatory tachykinins specifically prevent apoptosis of BMDCs by NK1R signaling.

Pro-inflammatory tachykinins prevent apoptosis of BMDCs by NK1R signaling. (A) Comparison of the anti-apoptotic effects of SarSP, SP, and HK-1 at physiological (10^{-9} M) and nonphysiological (10^{-5} M) concentrations on $CD11c^{+}$ BMDCs as determined by FACS analysis of Annexin V and PI labeling. (B and C) Bar-diagrams representing the anti-apoptotic effect of SarSP on $CD11c^{+}$ BMDCs is abrogated by (B) preincubation with the highly selective NK1R antagonist WIN-51708 or (C) in $NK1R^{-/-KO}$ BMDCs. ***P less than .001. (A-C) Means ($1 \pm$ SD) of 3 independent experiments are illustrated. (D) Dot-plots illustrating the percentage of apoptosis (numbers in dot-plots) of $CD11c^{+}$ BMDCs signaled with NK1R antagonist WIN-51708 alone. Data are representative of 4 independent experiments.

2.4.4 Agonistic binding of the NK1R activates the intracellular PI3K-Akt pathway to prevent apoptosis of BMDCs

The mechanisms by which NK1R agonists exert their anti-apoptotic effect on BMDCs were analyzed by investigating the role of intracellular pathways involved in DC survival in blockade assays using cell-permeable inhibitors specific for PI3K (LY294002),[54,64,252] NF-κB (SN-50),[65] Erk (PD98059),[63] and Jnk-AP-1 (JNK inhibitor II).[70] Blockade of PI3K decreased significantly the ability of SarSP to rescue BMDCs from apoptosis ($p<0.001$), whereas inhibition of Erk or NF-κB exerted lower but still significant decreases of BMDC apoptosis (Figure 9A). Conversely, inhibition of Jnk-AP-1 did not interfere with the anti-apoptotic effect of SarSP ($p>0.05$) (Figure 9A).

Since the previous experiment demonstrated that DC survival promoted by the NK1R agonist was abrogated by PI3K blockade, we further investigated the role of effector molecules downstream PI3K that could be involved in the anti-apoptotic effect of SarSP on BMDCs.[253] We analyzed whether signaling via NK1R in BMDCs activates Akt by intracellular labeling with an Ab recognizing phosphorylated-Akt at the Ser473 residue (pAKTSer473), an event that occurs downstream of PI3K and is necessary for full-activation of Akt. Signaling BMDCs with SarSP increased the percentage of BMDCs expressing pAktSer473 from 31% to 72%, as determined by FACS-analysis (Figure 9B). Moreover, blockade of pAktSer473 with the Akt Inhibitor X decreased the anti-apoptotic effect of SarSP ($p<0.001$) to a similar extent as observed with PI3K blockade (Figure 9C).

Next, we compared the abilities of SarSP and GM-CSF and IL-4 to: i) induce phosphorylation of Bad, reducing its pro-apoptotic activity; ii) increase anti-apoptotic Bcl-2 expression; and iii) decrease pro-apoptotic caspase 3 activity, which are the final effectors of the PI3K-Akt cascade.[254,255] Signaling BMDCs with SarSP or GM-CSF and IL-4 induced similar significant increases of intracellular levels of the Akt-dependent phosphorylated-Bad at the Ser136 residue (pBadSer136) and Bcl-2 (Figure 9D). Importantly, we observed a significant decrease of active caspase 3 levels, a protease that executes apoptosis by activation of nuclear-DNAses.[256,257] Collectively, these results demonstrate that SarSP promotes survival of BMDCs through activation of the PI3K-Akt pathway.

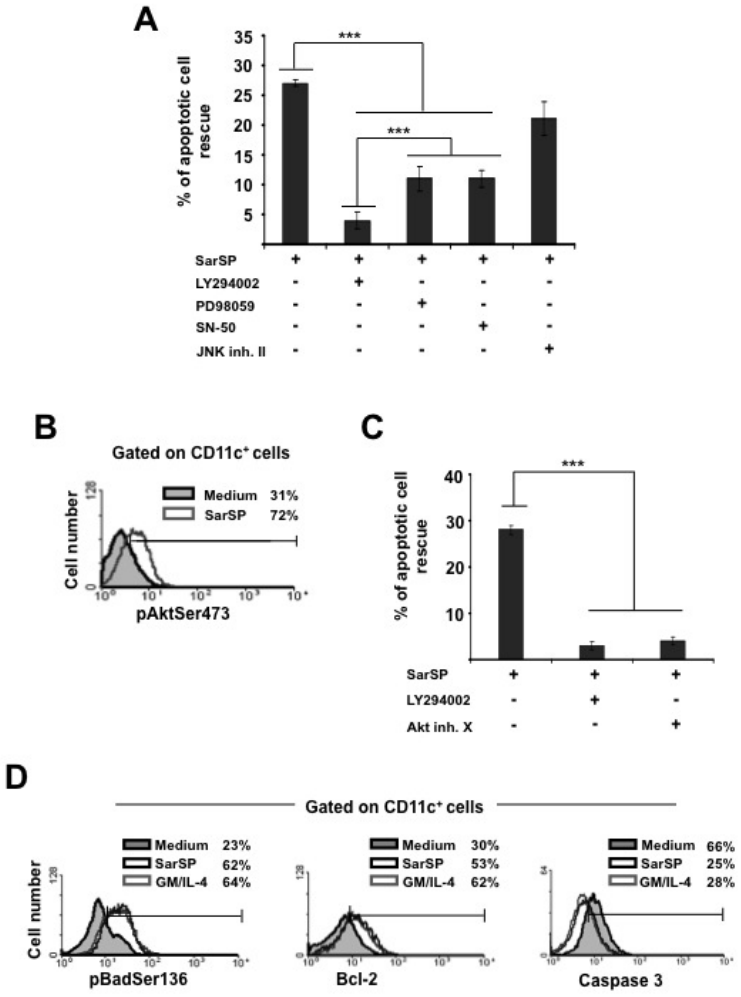


Figure 9: Signaling BMDCs via the NK1R prevents apoptosis through activation of the PI3K-Akt cascade.

(A) Histogram representing the effect of intracellular pathway blockade on the anti-apoptotic effect exerted by SarSP on $CD11c^+$ BMDCs. Before culture with or without SarSP, BMDCs were left untreated (control) or incubated with the cell-permeable inhibitors LY294002 (PI3K), PD98059 (Erk), SN-50 (NF-κB), or Jnk II (Jnk-AP1). ****P* less than .001. (B) FACS analysis showing the level of pAktSer473 expression by $CD11c^+$ BMDCs cultured in the presence of SarSP (gray empty histogram) or medium alone (gray filled histogram). (C) Comparative analysis of the effects of LY294002 and Akt inhibitor X (pAktSer473 inhibitor) on the anti-apoptotic effect of SarSP on BMDCs. ****P* less than .001. (D) FACS analysis illustrating the level of pBadSer136, Bcl-2, and active caspase 3 expression by $CD11c^+$ BMDCs cultured in the presence of SarSP (black empty histogram), GM, and IL-4 (gray empty histogram), or medium alone (gray filled histogram). (A and C) Means (± SD) of four independent experiments are shown. (B and D) Data are representative of 4 independent experiments. Numbers in histograms indicate percentage of $CD11c^+$ BMDCs.

2.4.5 NK1R-signaled BMDCs show enhanced survival in vivo

The relevance of our previous results was analyzed in vivo by studying the effect of signaling via NK1R on the survival of adoptively transferred BMDCs. CFSE-labeled BMDCs, pre-incubated or not with SarSP (SarSP-DCs and DCs, respectively), were injected (footpad, s.c.) into B6 mice, and the number of viable CFSE-labeled $CD11c^+$ BMDCs was quantified in local DLNs for a period of 7d by FACS. Two days after adoptive transfer, similar numbers of SarSP-DCs and control DCs were detected in DLNs, indicating that both DC subsets displayed equivalent DLN-homing capacities (Figure 10A). Five days after injection, 95% of SarSP-DCs remained in DLNs compared to 63% of control DCs and by d7 42% of SarSP-DCs were present vs. 24% of control DCs (Figure 10A). No CFSE dilution was detected in $CD11c^+$ BMDCs homed to local DLNs, indicating that the higher percentage of SarSP-DCs observed in the LNs was due to prolonged DC survival and not DC proliferation (Figure 10A).[258] Conversely, $CFSE^+$ cells were not detected in non-draining homolateral or collateral LNs (not shown).

These findings confirm that signaling via NK1R enhances survival of BMDCs in vivo. Next, we addressed whether SarSP prolongs survival of Ag-loaded BMDCs in DLNs. CFSE-labeled SarSP-DCs or control DCs were left untreated or haptenized with TNBS (SarSP-TN-DCs and TN-DCs, respectively), injected (footpad, s.c.) into B6 mice, and quantified by FACS-analysis of DLNs at d2, 5 and 7 following transfer. Regardless of the treatment, BMDCs showed similar DLN-homing capacities (not shown). However, the percentage of TN-DCs detected in DLNs 5 and 7d after DC administration was significantly lower than the number of non-

haptenized DCs ($p<0.001$) (Figure 10B). Importantly, the percentage of SarSP-TN-DCs homed in DLNs was significantly higher than that of TN-DCs, indicating that signaling via NK1R prolongs the survival of Ag-loaded BMDCs in DLNs.

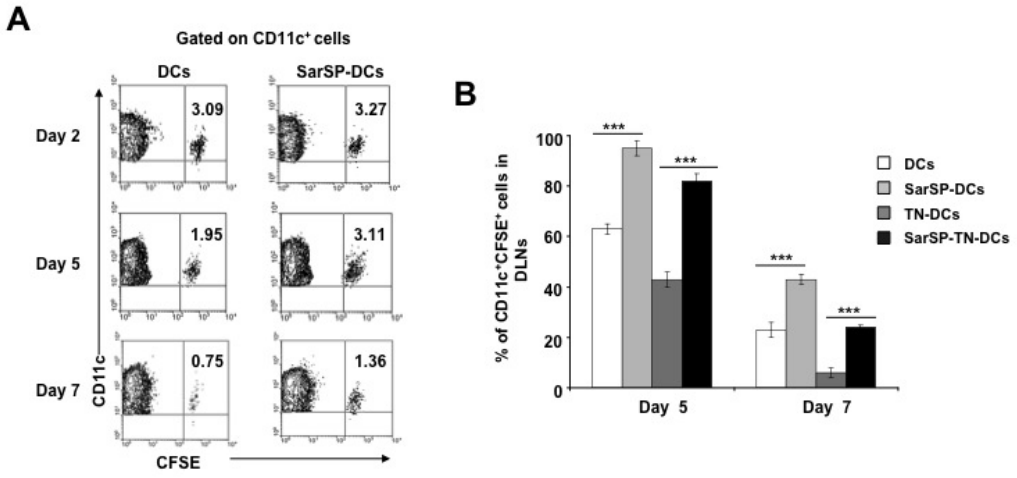


Figure 10: Agonistic signaling via the NK1R prolongs BMDC survival in vivo.

(A) Contour-plots illustrating the percentage of SarSP-DCs or control DCs ($CD11c^+CFSE^+$) in DLNs after their adoptive transference into B6 mice and quantification by FACS analysis at different time points. Data are representative of 3 independent experiments. (B) Comparative analysis of the percentages of $CD11c^+CFSE^+$ BMDCs in DLNs after adoptive transference of TN-DCs, SarSP-TN-DCs, SarSP-DCs, or untreated DCs as quantified by FACS analysis at different time points. ***$P < 0.001$. Means ($\pm$ SD) of three independent experiments are displayed.

2.4.6 NK1R and CD40 signaling act synergistically to prolong BMDC survival after adoptive transfer

The finding that SarSP-TN-DCs exhibit prolonged survival once they home in DLNs could be ascribed to sustained expression of anti-apoptotic molecules induced by SarSP or a combined

effect of NK1R and CD40 signaling, the later provided by DC-activated $CD4^+$ T cells in DLNs.[215,228,259-262] Thus, we analyzed the individual and combined contribution of NK1R and CD40 signaling in the expression of Bcl-2 and on the viability of BMDCs in vitro and in vivo, respectively.

For in vitro experiments, BMDCs were pre-treated (or not) with SarSP during the first 24h of culture, and signaled with agonistic CD40 mAb added at 24h of culture. Expression of Bcl-2 by $CD11c^+$ BMDCs was quantified up to 96h by FACS. As expected, NK1R signaling significantly increased the percentage of BMDCs expressing Bcl-2 after 24h of culture ($p<0.001$), and signaling via CD40 further enhanced Bcl-2 expression in both SarSP-DCs and control DCs 48h after culture ($p<0.001$) (Figure 11A). By 96h, the percentage of BMDCs expressing Bcl-2 diminished, regardless of the treatment employed; however, CD40 ligation of SarSP-DCs significantly enhanced the number of cells expressing Bcl-2 vs. DCs ($p<0.001$), SarSP-DCs ($p<0.01$) or DCs signaled only via CD40 ($p<0.05$) (Figure 11A).

Next, we compared in vivo the survival of adoptively transferred BMDCs homed in DLNs in the presence or absence of CD40-CD40L (CD154) interaction blockade. CFSE-labeled TN-DCs or SarSP-TN-DCs were injected (footpad, s.c.) into B6 mice treated or not with CD154 (CD40L) blocking MR1 mAb (250 μg, i.p.) simultaneously and 2d after DC administration. In vivo blockade of the CD40-CD154 interaction decreased the percentage of transferred TN-DCs and SarSP-TN-DCs in DLNs 5d after injection. Nevertheless, mice that were non-treated with CD154 blocking mAb had a significantly higher number of SarSP-TN-DCs remaining in DLNs compared to TN-DCs (Figure 11B). Together, these results suggest that the prolonged viability of BMDCs homed in DLNs was caused by a synergistic effect of NK1R and CD40 signaling.

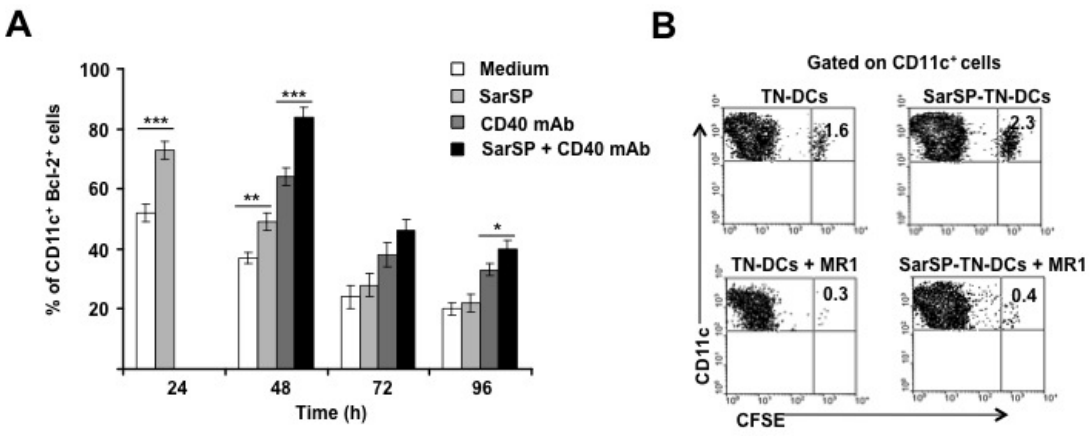


Figure 11: NK1R and CD40 signaling act synergistically to promote BMDC survival.

(A) Bar-diagram representing the percentage of $CD11c^+$ BMDCs expressing Bcl-2 after culture in medium alone or with SarSP for different time points. Agonistic CD40 mAb was added at 24 hours to cultures, and the percentage of $CD11c^+$ BMDCs expressing Bcl-2 was analyzed at each time point. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Means (± SD) of 3 independent experiments are shown. (B) Contour-plots illustrating the presence of SarSP-TN-DCs or TN-DCs ($CD11c^+CFSE^+$) in DLNs 5d after adoptive transference of DCs in B6 mice treated with or without the CD154 (CD40L) neutralizing mAb. Data are representative of 3 independent experiments.

2.4.7 BMDCs signaled via the NK1R promote enhanced cellular immunity

The previous results indicate that combination of NK1R and CD40 signaling enhances the interaction of BMDCs with T cells in DLNs, which might result in robust immune responses. To evaluate the immunological relevance of our findings, we compared the capability of TN-DCs and SarSP-TN-DCs to induce DTH responses. B6 mice were sensitized by administration of TN-DCs or SarSP-TN-DCs (footpad, s.c.) and DTH was elicited 5d after by topical TNCB application to the dorsal skin of the ears. Mice were injected with haptenized CD40-TN-DCs or non-haptenized (untreated or SarSP-signaled) DCs to be included as positive and negative controls, respectively. The effector cellular immune response was analyzed by measuring the

increase of ear thickness up to 6d post-elicitation. As expected, mice sensitized with TN-DCs, SarSP-TN-DCs, and CD40-TN-DCs exhibited a significant ear thickness increase vs. control mice 1d post-elicitation ($p<0.001$) (Figure 12A). In mice sensitized with TN-DCs, the ear thickness diminished significantly 2d after elicitation, and showed minimal differences vs. control mice 3d post-elicitation (Figure 12A). Conversely, mice sensitized with SarSP-TN-DCs and CD40-TN-DCs exhibited a further increase 2d after elicitation, and showed a significant prolonged increase in ear thickness in comparison to TN-DCs and control mice for up to 6d. We also characterized the cellular infiltrate in the ears of mice obtained 6d after elicitation by microscopic analysis. Mice sensitized with TN-DCs presented scarce cellular infiltrate, whereas mice sensitized with SarSP-TN-DCs exhibited severe inflammatory infiltrate composed of mononuclear cells (Figure 12B and C). Immunofluorescence-microscopy analysis revealed that the enhanced cellular inflammatory infiltrate consisted of epidermal $CD8^+$ T cells and dermal $CD4^+$ T cells and $F4/80^+$ macrophages (Figure 12C), demonstrating that the immune response generated was a type-1 DTH response.

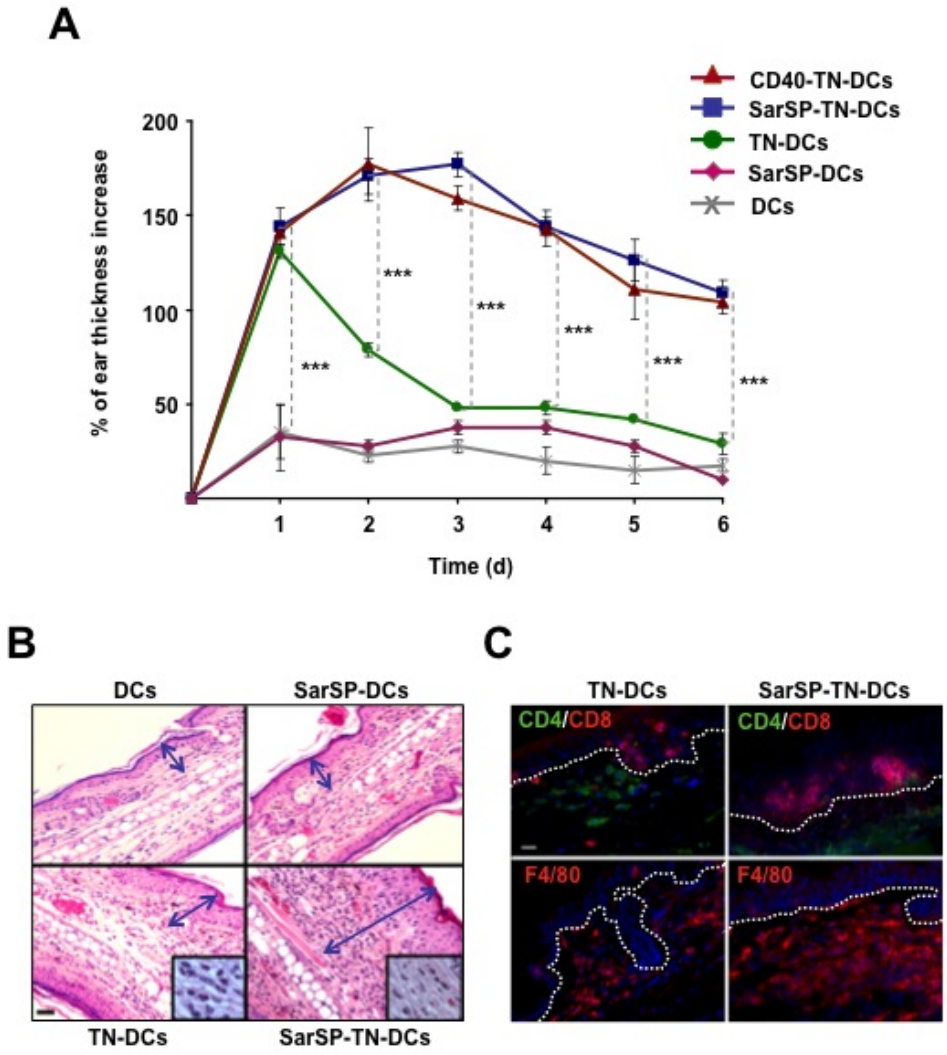


Figure 12: Signaling BMDCs via the NK1R prolongs effector cellular immune responses.

(A) Ear thickness increase of mice sensitized with adoptively transferred TN-DCs, SarSP-TNDCs, CD40-TN-DCs, or control (nonhaptenized) SarSP-DCs or untreated DCs, as analyzed at different time points after DTH elicitation. Means (± SD) of 6 mice per experimental group are illustrated. ***$P < 0.001$. (B) Histological analysis of ear skin sections dissected 6 days after DTH elicitation shows severe mononuclear cell infiltrate in samples obtained from mice sensitized with SarSP-TN-DCs compared with mice sensitized with TN-DCs or control SarSP-DCs or untreated DCs. The mononuclear cell infiltrate is shown at higher magnification in the insets. Arrows indicate the thickness of the skin (excluding the hypodermis). H&E, 200x (insets, 1000x); scale bar = 20 μm. (C) Characterization of the cellular infiltrate in ear skin of mice sensitized with TN-DCs or SarSP-TN-DCs. Doted-lines indicate the epidermal-dermal junction. Immunofluorescence, 500x; scale bar = 20 μm. (B and C) One representative skin section of 5 analyzed per experimental group is shown.

2.5 DISCUSSION

The survival of DCs transporting Ag from peripheral tissues to DLNs is crucial for initiation of cellular immunity, and administration of DCs unable to survive in the periphery or DLNs represents a major drawback for current DC-based immune-stimulatory therapies. Despite the relevance of DC survival for the purpose of positive vaccination, the mechanisms that control viability of adoptively transferred DCs have not been fully elucidated.

Recently, it has been demonstrated that the nervous and immune systems interact to modulate the immune response via secretion of neuropeptides. The balance of pro-inflammatory and anti-inflammatory neuropeptides in the CNS and peripheral tissues is crucial in maintaining self-tolerance under the steady-state. Indeed, a predominant secretion of the pro-inflammatory SP vs. anti-inflammatory neuropeptides favors chronic inflammatory and autoimmune disorders in the CNS and peripheral tissues.

We hypothesized that agonistic binding of NK1R promotes DC viability during their migration from peripheral tissues and in DLNs, which will result in a more efficient DC-T cell interaction. Likewise, it has been demonstrated that pro-inflammatory tachykinins that signal via NK1R promote survival of neutrophils, macrophages, and B and T cell precursors. Recent publications have demonstrated that SP recruits immature DCs to inflammation sites, and that signaling skin DCs via NK1R participates in Th1 polarization and induction of CTL/Tc1 bias. However, the mechanisms involved in the relevant DC-stimulatory activities of pro-inflammatory tachykinins remain largely unknown. Moreover, the ability of pro-inflammatory tachykinins to promote the survival of ex vivo-generated DCs relevant for DC-based immune-therapies has not been investigated.

In the present work, using a model of ex vivo-generated BMDCs, we demonstrated that regardless of their maturation stage, BMDCs expressed constitutively the functional variant of NK1R. Importantly, BMDCs increased the levels of NK1R mRNA following LPS stimulation, indicating that DCs are capable of up-regulating NK1R and serving as direct targets of the increased levels of pro-inflammatory neuropeptides that are secreted during inflammation.

Current approaches for adoptive transference of mouse or human DCs utilize BM precursor- or monocyte-derived DCs generated ex vivo in the presence of GM-CSF and IL-4, and deprivation of these cytokines results in DC apoptosis. Here, we confirmed that deprivation of GM-CSF and IL-4 results in significant apoptosis of BMDCs, and this effect is prevented by signaling BMDCs via NK1R with the natural agonists SP or HK-1 or the synthetic NK1R ligand SarSP.

At high concentrations, SP and SP analogs bind other neuropeptide receptors, including bombesin and serpin-enzyme complex receptors, or alternatively, exert their functions independently from receptor-ligation as described for mast cells. Under our conditions, the pro-inflammatory tachykinins induced their anti-apoptotic effects on BMDCs at physiological concentrations and this function was abrogated completely by the presence of NK1R antagonists or in BMDCs generated from $NK1R^{-/-KO}$ mice, demonstrating that the effect of the tachykinins on DC survival was mediated by NK1R binding. Moreover, our data suggest that antagonistic blockade of the NK1R does not exert apoptosis per se, but prevents NK1R agonistic apoptotic rescue by competing for NK1R binding. Additionally, BMDCs might not secrete significant levels of endogenous NK1R agonists to account for apoptotic rescue via an autocrine feedback loop.

Our results showed that signaling BMDCs through NK1R with pro-inflammatory neuropeptides activates the PI3K-Akt cascade to prolong DC survival. In addition, we observed that NF-κB or Erk signaling was less significant in the anti-apoptotic effect mediated by NK1R agonists. These findings agree with recent studies, which emphasized the relevance of the PI3K-Akt cascade in promoting DC survival, with less prominent roles of MAPK and NF-κB pathways. Under our conditions the relative lower contribution of Erk and NF-κB activation could be attributed to their interaction with the PI3K-Akt cascade since cross-activation of these pathways has been described.

Downstream effectors of the PI3K-Akt cascade include the anti-apoptotic proteins from the Bcl-2 family. The Bcl-2 family includes anti- and pro-apoptotic proteins with characteristic homology sequences (BH domains) that are crucial for regulating mitochondrial outer membrane permeability, leading to inhibition or activation, respectively, of terminal effector caspases. Accordingly, Bad, a pro-apoptotic Bcl-2 family protein, exerts its effects by sequestering the anti-apoptotic Bcl-2 through dimerization. Phosphorylation of Bad at the Ser136 residue by Akt disrupts its affinity for Bcl-2, allowing the free Bcl-2 to block the release of cytochrome C from the mitochondria and prevent the activation of caspases. Our results demonstrate that NK1R signaling of BMDCs promotes Akt-dependent pBadSer136 and enhances Bcl-2 expression, with a consequent decrease of caspase 3 activity.

DCs homing in secondary lymphoid organs become targets of $CD8^+$ CTLs and NK cells that eliminate DCs in Ag-dependent or -independent fashions, respectively. Conversely, cognate activation via CD40 provided by $CD4^+$ Th cells presents DCs with anti-apoptotic signals necessary to survive CTL killing. Our data demonstrate that signaling via NK1R prolongs survival of BMDCs adoptively transferred into mice and their subsequent homing into DLNs, in

both Ag-independent and Ag-dependent manners. Mechanistic analysis of the latter phenomenon revealed that signaling BMDCs via NK1R followed by CD40 stimulation exerted a synergistic effect to sustain in vitro Bcl-2 expression and prevent in vivo, the clearance of BMDCs homed in DLNs. The synergistic effect of signaling BMDCs via NK1R and CD40 was further confirmed in vivo by those results showing a significant increase of viable SarSP-TN DCs in the absence of the CD40L inhibition, compared to the number of TN-DCs that received CD40 signaling in the absence of exogenous NK1R agonist. The fact that inhibition of CD40-CD40L interaction diminished greatly the anti-apoptotic effects exerted by both DC survival signals demonstrates the relevance of DC-$CD4^+$ Th cell interaction in DLNs for prolonging survival of NK1R-signaled BMDCs.

Importantly, enhanced longevity of Ag-loaded BMDCs signaled via NK1R and CD40 resulted in elicitation of a potent and sustained cellular immunity mediated mainly by $CD8^+$ T cells and macrophages. Moreover, the observation that elicitation of DTH responses promoted by BMDCs signaled via NK1R persisted up to one week implies that the effector cells overcame the mechanisms of resolution of inflammation and remained active for the period that the Ag was present in the skin.

In conclusion, we have demonstrated that signaling BMDCs via NK1R with pro-inflammatory tachykinins enhances DC survival in vitro and in vivo, resulting in induction of potent immune-stimulatory DCs that promote robust cellular immunity. Our data provide insight into the mechanisms by which the nervous system regulates the function of DCs and the subsequent cellular immune responses, and helps to understand the requirements for the development of more effective immune-stimulatory DCs for cell-based vaccination.

3.0 CHAPTER THREE: PRO-INFLAMMATORY TACHYKININS SIGNAL DENDRITIC CELLS VIA THE NEUROKININ 1 RECEPTOR TO ELICIT ROBUST TYPE-1 $CD4^+$ AND $CD8^+$ T CELL RESPONSES

3.1 ABSTRACT

DCs generated ex vivo constitute as a desired target for cellular-based vaccine protocols due to their efficient ability to bias robust Ag-specific Th1 and Tc1 responses against cancer and intracellular pathogens. However, DC-based vaccines do not always promote immune-stimulatory responses, and thus, further understanding of the immune mechanisms that govern the ability of DCs to elicit Th1/Tc1 immunity is required. Previously, we demonstrated that signaling murine BMDCs with pro-inflammatory neuropeptides from the tachykinin family, including SP, HK-1 and SarSP, bind the NK1R to enhance DC longevity and their ability to induce cellular immunity. In the present work, we extend our findings to demonstrate that signaling BMDCs with NK1R agonists promotes DC maturation and low production of IL-12p70, while inhibiting the secretion of anti-inflammatory cytokines IL-10 and TGF-β1. Subsequently, adoptive transfer of NK1R-signaled BMDCs enhanced Ag-specific Th1/Tc1 responses in vivo by down-regulating the secretion of IL-10. The ability of NK1R-signaled BMDCs to generate robust Ag-specific Th1/Tc1 responses in vivo was dependent on the survival of endogenous DCs and the production of endogenous IL-12p70, suggesting the possible activation and transfer of Ag to endogenous DC populations. Collectively, our data demonstrate that pro-inflammatory tachykinins, signaling via the NK1R, enhance the immune-stimulatory function of BMDCs that allows for induction of potent type-1 T cell immunity in vivo, which include immune-stimulatory contributions from both exogenous and endogenous DC populations.

3.2 INTRODUCTION

Due to their crucial role in biasing robust Th1/Tc1 immunity, DCs are the preferred target APCs for positive cellular vaccination protocols for the prevention and therapy of cancer and infectious diseases.[76,225,226] However, in spite of initial promising results, current DC-based vaccines do not always elicit potent Th1/Tc1 immunity,[73] and further understanding of immune mechanisms is warranted to determine ideal adjuvants for DCs.

Generation of potent Th1/Tc1 responses in vivo requires signaling DCs with pro-inflammatory mediators that (i) favor cell longevity after adoptive transfer[54,71,72,228] and (ii) induce immune-stimulatory DCs,[263] including their ability to enhance the Th1-driving cytokine IL-12p70, while inhibiting anti-inflammatory cytokines IL-10 and TGF-β1. Adoptive transfer of DCs expressing Ag are short-lived and exposed to pro-apoptotic stimuli at the injection site and in DLNs,[53,54] allowing DCs to serve as sources of Ag to endogenous DC populations in peripheral and lymphoid tissues, respectively. Indeed, DCs are capable of transferring Ag to other DC populations in vivo.[264-266] Thus, it is becoming evident that endogenous DCs may play important roles in mediating the effects of DC-based vaccines; however, the means involved remain unclear.

Along with well-known pro-inflammatory mediators, various neuropeptides have recently been implicated in modulating T cell immunity.[15,108,239,267] Most neuropeptides released in peripheral and lymphoid tissues are immune-inhibitory; however, mammalian tachykinins SP and HK-1 promote robust immune-stimulatory Th1/Tc1 responses by binding the NK1R.[84,106,191,241]

Our laboratory has demonstrated that administration of a highly selective NK1R agonist (SarSP) in vivo leads to DC activation and migration into DLNs where they generate robust Ag-specific Th1/Tc1 responses.[84] However, it is unknown whether NK1R agonists directly stimulate DCs to induce Ag-specific Th1/Tc1 responses, or modify the DC microenvironment to promote DC activation and their ability to polarize Th1/Tc1 responses. Recently, we have published (Chapter 2) that BMDCs express functional (full-length) NK1R, and agonistic signaling enhances DC longevity and their ability to promote cellular immunity.[268] However, the capability of signaling DCs with NK1R agonists to preferentially elicit Ag-specific Th1/Tc1 immune responses remains unclear.

In this study presented herein, we investigated the ability and mechanisms employed by signaling BMDCs with endogenous (SP and HK-1) and synthetic (SarSP) agonists for the NK1R to promote Th1/Tc1 responses against the model Ag ovalbumin (OVA). Through the utilization of various expression systems [adenoviral transduction of BMDCs and mice transgenic for diphtheria toxin receptor (DTR) under the CD11c promoter and knockout for IL-12p35], we assessed the role of cytokines IL-12p70 and IL-10, along with the contribution of endogenous DC populations, in modulating Ag-specific Th1/Tc1 responses induced by the adoptive transfer of NK1R-signaled BMDCs.

3.3 MATERIALS AND METHODS

3.3.1 Mice

Eight- to twelve-week old wild-type C57BL/6 (B6), B6.129S1-Il12a^{tm1Jm}/J (B6.IL-12p35$^{-/-KO}$), B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J (B6.CD11c-DTR), C57BL/6-Tg(TcraTcrb)1100Mjb/J (B6.OT-I) and B6.Tg(TcraTcrb)425Cbn/J (B6.OT-II) mice were purchased through The Jackson Laboratory. Mice were housed in the pathogen-free animal facility of the University of Pittsburgh and used according to institutional guidelines.

3.3.2 Generation of BMDCs

BMDCs were generated by culturing mouse BM-precursors isolated from the tibiae and femurs of B6, B6.IL-12p35$^{-/-KO}$ or B6.CD11c.DTR mice for 6d in complete medium supplemented with GM-CSF and IL-4 cytokines (both at 1,000 U/ml) (R&D systems), as previously described.[242,243] Total CD11c^{+} BMDCs were purified by histodenz gradient (16% wt/vol, purity of CD11c^{+} cells ≥85% as determined by FACS-analysis). The DC morphology was confirmed in cytospins of purified CD11c^{+} cells obtained, as previously described,[244] and stained with Hema-3 (Fisher).

3.3.3 Analysis of BMDC gene expression

The gene expression pattern of control BMDC groups was analyzed by probing isolated total RNA using the Affymetrix's Mouse Genome 430A v.2.0 arrays. Briefly, BMDCs ($3x10^6$) were cultured in complete media supplemented with or without SarSP (10^{-9} M, 18 h). After harvest, BMDCs were homogenized and isolated for RNA (Qiagen). Total RNA sample integrity and quality were tested using agarose gel electrophoresis and PCR using an S15 primer set (S15-F: TTCCGCAAGTTCACCTACC and S15-R: CGGGCCGGCCATGCTTTACG) to detect potential DNA contamination. Samples with positive PCR signal were re-digested to remove contaminating genomic DNA using DNase I enzyme. Only DNA contamination-free samples were used for the array-experiment. Fifteen mg of total RNA was used for each sample in one-cycle target labeling reaction according to the recommendations of Affymetrix. The hybridization (GeneChip Array Station) and scanning/data collection (GeneChip Scanner 3000) were done at the facilities of Genomics and Proteomics Core Laboratories of the University of Pittsburgh.

Raw data collected by Affymetrix GeneChip Command Console Software (AGCC) was imported to Affymetrix Expression Console software and processed. Data were sorted and better then $p<0.05$ data points were transferred into flat files. These data-files were processed by Microsoft Excel to build Microsoft Access databases for analysis.

3.3.4 Analysis of BMDC phenotype and cytokine production

For experiments analyzing cell-surface expression of DC maturation molecules, BMDCs were cultured in complete medium supplemented or not with SarSP (10^{-9} M, 18 h). DCs were collected and FcR-blocked with anti-CD16/32 mAb (BD-PharMingen) in 10% goat serum (Sigma) then stained with anti-mouse Alexa Flour 488-CD11c mAb in combination with PE-CD86, PE-I-A^b (MHC class II) or PE-CD40 mAbs (BD-PharMingen). Negative controls included cells stained with species-matched isotype IgG and non-stained cells. The cell-surface expression of CD11c, CD86, I-A^b and CD40 was quantified by FACS-analysis (FACSCalibur^{Tm}, Becton Dickinson).

To address BMDC cytokine production, BMDCs were cultured in the presence or absence of SarSP (10^{-9} M), LPS (500 ng/ml; Sigma) or αDC1 signaling-cocktail (IFN-γ, 20 μg/ml; polyI:C, 20 μg/ml; TNF-α 20 μg/ml and IL-1β, 20 μg/ml) (Sigma) for 18 h. We analyzed the secretion of IL-12p70 (ebioscience), IL-10 (BD-PharMingen) and TGF-β1 (BD-PharMingen) by ELISA, according to manufacturers' protocols, and quantified using an Emax microplate reader (Molecular Devices, Sunnyvale, CA).

3.3.5 OT-II $CD4^+$ T cell assays

Naive $CD4^+$ T cells were obtained from RBC-lysed spleens of transgenic OT-II mice, and purified by negative selection columns (R&D systems). BMDCs were pre-treated or not with SarSP, SP or HK-1 (10^{-9} M, 18 h) then selected groups were further cultured with OVA (1 mg/ml, 3 h). Afterwards, OVA-loaded BMDC groups were co-cultured with $CD4^+$ T cells at a

ratio of 1:20 (DCs-$CD4^+$ T cells) in 96-well round bottom plates for a period of 3 days. Control groups included BMDCs co-cultured with $CD4^+$ T cells without OVA exposure or $CD4^+$ T cells pulsed with OVA. Select DC-$CD4^+$ T cell co-cultures were supplemented with neutralizing antibodies targeting IL-12p40 (C17.8, 20 μg/ml; ebioscience), IL-12p35 (C18.2, 20 μg/ml; ebioscience), IL-23p19 (G23.8, 20 μg/ml; ebioscience), CD40L (MR-1, 10 μg/ml; ISC BioExpress) and/or IFNγ (20 μg/ml; BD-PharMingen).

For experiments analyzing $CD4^+$ T cell proliferation, BMDC groups were γ-irradiated (2000 Gy) prior to co-culture with isolated $CD4^+$ T cells. For the final 18 h, wells were pulsed with 1 μCi (0.037 MBq) [^{3}H] thymidine. The amount of radioisotope incorporated was determined using a β scintillation counter. Assays were performed in triplicate, and results are expressed as mean counts per minute ± 1 SD.

To assess cell cytokine production, supernatants were collected from non-irradiated co-cultures on day 3, and the secretion of IFN-γ (BD-PharMingen), IL-5 (ebioscience), IL-13 (ebioscience) and IL-12p70 (ebioscience) was analyzed by ELISA using an Emax microplate reader.

3.3.6 Induction of OVA-specific $CD4^+$ T cells in vivo

BMDCs were generated from B6 mice and were cultured with or without SarSP (10^{-9} M) for 18 h, and selected groups were further incubated with OVA (1 mg/ml, 2 h). BMDC groups were then extensively rinsed and adoptively transferred (footpad, s.c.; $1x10^6$ DCs in 50 μl per footpad) into B6 mice (n=3 animals per group) on day 0, and in some experiments, mice received an additional boost on day 7 (Figure 14C and 15C). Immunized mice receiving BMDCs without

OVA treatment and non-immunized mice (naïve) served as control groups. Mice were sacrificed 5 days after last immunization, and spleens were harvested and RBC-lysed to yield single-cell suspensions. $CD4^+$ T cells were purified by negative selection columns (R&D systems) and re-stimulated with naïve BMDCs pulsed with or without OVA (1 mg/ml, 2 h) at a 1:20 ratio (DCs-$CD4^+$ T cells) in 96-well round bottom plates. Supernatants were collected 3 days later, and the secretion of IFN-γ (BD-PharMingen), IL-5 (ebioscience) and IL-13 (ebioscience) was quantified by ELISA using an Emax microplate reader.

To quantify the frequency of cytokine-secreting T cells, $CD4^+$ T cells were co-cultured with naïve B6 BMDCs loaded with or without OVA, as described above, in 96-well ELISpot plates pre-coated with mAbs specific for IFN-γ (BD-PharMingen), IL-5 (ebiosciences) or IL-13 (ebiosciences) for 18 h. After culture, supernatants were discarded and plates were repeatedly washed and further incubated with biotin anti-IFNγ, IL-5 or IL-13 mAbs, followed by streptavidin-peroxidase inclusion, according to manufacturers' protocols. Plates were developed by adding the substrate 3-amino-9-ethylcarbazole (AEC) and spots were quantified with an ImmunoSpot automated counter (Cellular Technology).

For investigations addressing the role of IL-12p70 in promoting $CD4^+$ T cell responses in vivo, ELISA-based experiments were supplemented with additional groups utilizing BMDCs derived from B6.IL-12p35$^{-/-KO}$ mice and transferred into B6 and B6.IL-12p35$^{-/-KO}$ mice (n = 4 mice per group).

3.3.7 Induction of OVA-specific $CD8^+$ CTLs in vivo

$CD8^+$ CTL function induced by adoptive transfer of BMDCs was evaluated by performing an Ag-specific in vivo killing assay. BMDCs generated from B6 mice were pre-treated or not with SarSP (10^{-9} M), and select groups were further cultured with OVA (1 mg/ml, 2 h) and $OVA_{257-264}$ (SIINFEKL, Sigma) (100 ng/ml, 45 min). BMDC groups were extensively rinsed and adoptively transferred (footpad, s.c.; $1x10^6$ DCs in 50 µl per footpad) into B6 mice (n=4 animals per group). Immunized mice without OVA and SIINFEKL exposure and non-immunized mice served as control groups. Five days following immunization, the in vivo killing efficiency was assessed by using SIINFEKL-pulsed targets. Briefly, naïve B6 splenocytes pulsed with SIINFEKL (250 ng/ml, 45 min) and labeled with 5 µM CFSE ($CFSE^{High}$) were used as target cells, while non-pulsed B6 splenocytes labeled with 0.5 µM CFSE ($CFSE^{Low}$) were included as control cells. Target and control cells were mixed in a 1:1 ratio and transferred (tail vein, i.v.; $2x10^7$ cells in 200µl PBS/mouse) into immunized and non-immunized mice. Four hours later, splenocytes were collected from harvested and RBC-lysed spleens. The percentage of specific cell lysis (%SCL) was then calculated by using the formula: %SCL = 100 x [(1 – ($CFSE^{low}$:$CFSE^{high}$ of splenocytes from untreated mice)/($CFSE^{low}$:$CFSE^{high}$ of splenocytes from immunized mice))].

To quantify the abundance of cytokine secretion from $CD8^+$ T cells, the immunization scheme outlined above was followed with an additional boost 7 days after primary transfer. Five days after last immunization, splenic $CD8^+$ T cells were isolated from naïve and immunized mice. Briefly, $CD8^+$ T cells were purified by negative selection columns (R&D systems) and re-stimulated with naïve B6 BMDCs pulsed with or without OVA (1 mg/ml, 2 h) at a 1:20 ratio

(DCs-$CD8^+$ T cells) in 96-well round bottom plates for 3 days. After culture, supernatants were collected, and the secretion of IFN-γ (BD-PharMingen), IL-5 (ebioscience) and IL-13 (ebioscience) was quantified by ELISA using an Emax microplate reader.

For investigations addressing the role of IL-12p70 in promoting in vivo CTL responses, in vivo killing assays were supplemented with additional groups utilizing BMDCs derived from B6.IL-$12p35^{-/-KO}$ mice and transferred into B6 and B6.IL-$12p35^{-/-KO}$ mice (n = 4 mice per group).

3.3.8 Depletion of DC populations in vivo

To deplete DCs in vivo, B6 mice transgenic for DTR expression under the CD11c promoter (B6.CD11c-DTR) was utilized. BMDCs were generated from B6 or B6.CD11c-DTR mice and cultured with or without SarSP (10^{-9} M) for 18 h, followed by Ag loading with SIINFEKL (100 ng/ml, 45 min) and/or OVA (1 mg/ml, 2 h). To verify expression and functionality of the DTR, select B6.CD11c-DTR BMDC groups were cultured with diphtheria toxin (DT, Sigma) (10^{-7} M, 18 h) and apoptosis was quantified by FACS-analysis of PI inclusion. After extensive rinsing, B6 groups were adoptively transferred into B6.CD11c-DTR mice (n = 6 mice per group). Select groups of immunized B6.CD11c-DTR mice received i.p. injection of DT (4 ng/lb of body mass; flank) (sigma) or PBS (control) immediately prior to adoptive transfer of B6 BMDC groups. ELISpot and cytotoxicity assays were performed to assess the impact of abolishing endogenous DC populations in mediating acquired Th1 and Tc1 responses, respectively.

3.3.9 Adenoviral transduction of BMDCs

$E1^-$ $E3^-$ deleted rAds included (i) rAd-neo, lacking transgene or promoter (negative control) and (ii) rAd-mIL-10, encoding murine IL-10 cDNA under the control of the hCMV-MIE promoter [kindly provided by Paul Robbins (University of Pittsburgh) and expanded in our laboratory]. rAd preparation was performed as previously described,[243] and transduction was conducted at an MOI of 500 in serum-free RPMI media for 45 min. After infection, BMDCs were cultured in complete media with or without SarSP (10^{-9} M) for 18 h or longer. Efficiency of transduction was assessed by quantifying the secretion of IL-10 by ELISA (BD-PharMingen) using an Emax microplate reader (data not shown). For adoptive transfer into B6 mice (n = 4 mice per group), transduced BMDCs were treated with 16% histodenz density centrifugation to remove any dead cells. ELISpot and cytotoxicity assays were performed to assess the impact of over-expressing IL-10 in BMDC groups to mediate Th1 and Tc1 responses in vivo, respectively.

3.3.10 Statistical analysis

Statistical analysis was performed using the GraphPad Prism® 4 software (GraphPad software, San Diego, CA). Differences between more than two means ± 1SD were analyzed by a one-way ANOVA analysis followed by a Student Newman Keuls test. Comparisons between two different means ± 1SD were performed by a Student's "t" test. A "*p*" value <0.05 was considered significant.

3.4 RESULTS

3.4.1 Agonistic signaling via the NK1R enhances the immune-stimulatory function of BMDCs

We have previously reported that BMDCs express the conventional full-length NK1R, and agonistic signaling via the receptor enhances DC longevity and their ability to elicit cellular immune responses.[268] To analyze whether agonistic signaling via the NK1R enhances the immune-stimulatory profile of DCs, we initially investigated the ability of SarSP, a highly selective NK1R agonist, to promote DC maturation and modulate cytokines that favor (IL-12p70) and inhibit (IL-10 and TGF-β1) the induction of cellular immunity.

BMDCs cultured for 18 h in complete media were composed of mature and immature $CD11c^+$ DCs, as denoted by heterogeneous cell-surface expression (percent positive, left panels and mean fluorescent intensity, right panels) of CD86 with moderate and low detection of MHC class II ($I\text{-}A^b$) and CD40 molecules, respectively (Figure 13A). The expression of maturation molecules CD86, $I\text{-}A^b$ and CD40 was elevated on BMDCs when cultures were supplemented with SarSP (10^{-9} M), demonstrating enhancement of DC maturation (Figure 13A).

Supernatants from BMDC cultures were collected to determine the ability of SarSP to modulate secretion of the Th1/Tc1-biasing cytokine IL-12p70 and anti-inflammatory cytokines IL-10 and TGF-β1. After 18 h of culture, BMDCs secreted negligible amounts of IL-12p70 in comparison to high levels of IL-10 and TGF-β1 production (Figure 13C and D, respectively). In correlation with cDNA levels in BMDCs (Figure 13B), SarSP had no effect on IL-12p70 modulation, while IL-10 and TGF-β1 secretion was significant inhibited in BMDC cultures

(Figure 13C and D, respectively). BMDCs were capable of producing significant amounts of IL-12p70, as detected when cultures were supplemented with LPS and a DC1 signaling-cocktail. LPS and DC1 cocktail stimulation inhibited production of TGF-β1, but greatly up-regulated IL-10 production. Addition of SarSP to these BMDC cultures slightly elevated IL-12p70 production, while significantly reducing IL-10 and TGF-β1 secretion (Figure 13C and D, respectively).

Collectively, these results demonstrate that agonistic signaling of BMDCs via the NK1R favors the profile of immune-stimulatory DCs: elevated levels of co-stimulatory and MHC class II molecules and reduced secretion of anti-inflammatory cytokines.

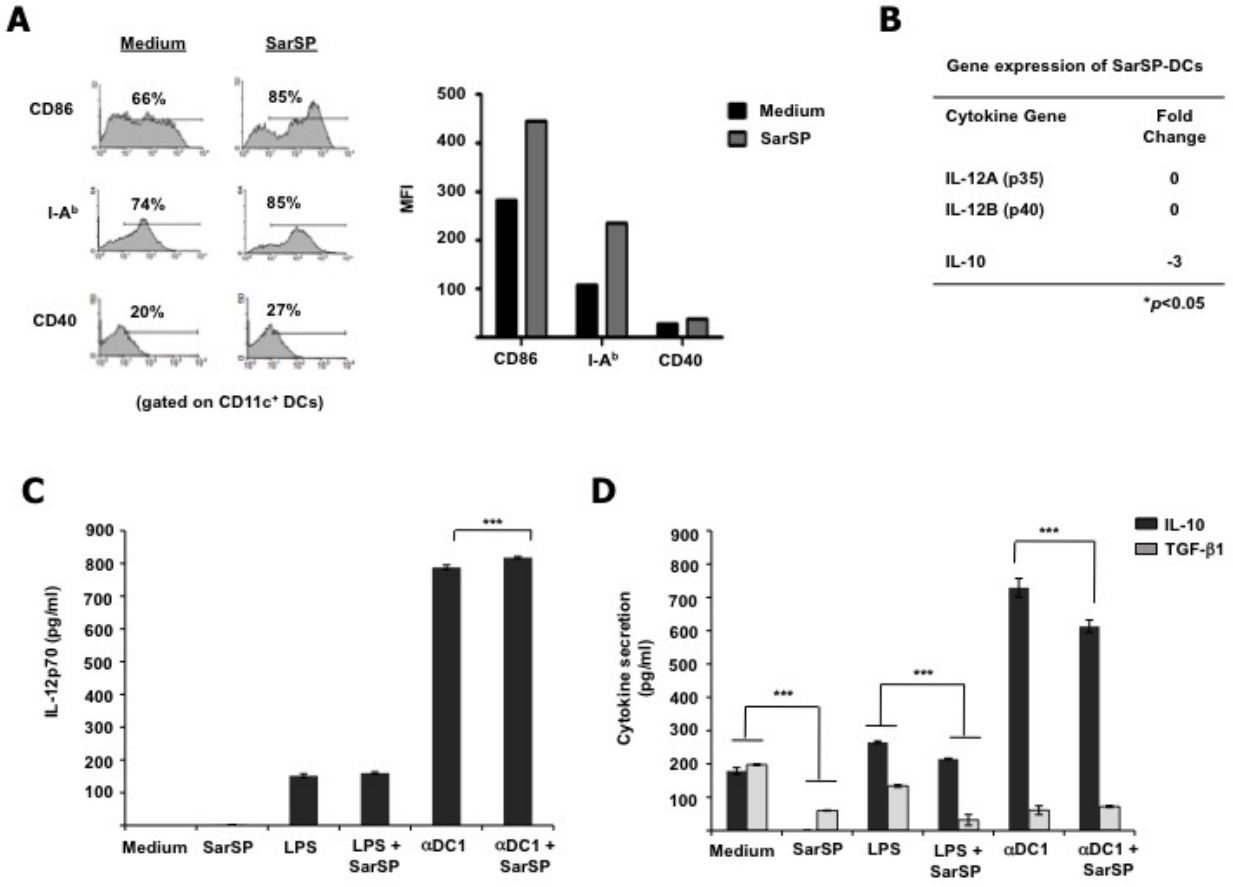


Figure 13: Agonistic signaling via the NK1R enhances BMDC expression of co-stimulatory molecules and reduces the secretion of anti-inflammatory cytokines.

Phenotype and cytokine analysis of BMDCs. (A) FACS-analysis illustrating the percent of cells (left panel) and mean fluorescent intensity (right panels) of $CD11c^+$ BMDCs cultured in the presence (SarSP-DCs) or absence (DCs, control) of SarSP (10^{-9} M) expressing co-stimulatory molecules CD86, $I\text{-}A^b$ and CD40. Data is representative of 4 independent experiments. (B) Modulation of genes encoding IL-12p35, IL-12p40 and IL-10 cDNA from SarSP-DCs in comparison to DCs (non-signaled, control). $p<0.05$. (C and D) ELISA-analysis of $CD11c^+$ BMDCs cultured with or without SarSP (10^{-9} M), LPS (600 ng/ml) and/or DC1 signaling-cocktail (IFN-γ, 20 μg/ml; polyI:C, 20 μg/ml; TNF-α 20 μg/ml and IL-1β, 20 μg/ml) secreting (C) IL-12p70, (D) IL-10 (dark bars) or TGF-β1 (light bars). (C-D) Means $\pm$ 1 SD of 3 independent experiments are displayed. ***$p<0.001$.

3.4.2 Natural and synthetic NK1R agonists activate BMDCs with $CD4^+$ Th1 stimulatory and biasing abilities in vitro and in vivo

Induction of cellular immunity requires the ability of immune-stimulatory DCs to generate Ag-specific type-1 T cell responses, particularly $CD4^+$ Th1 responses.[204] Due to observations demonstrating that SarSP enhances the immune-stimulatory features of BMDCs, we investigated the ability of signaling BMDCs via the NK1R to promote Ag-specific $CD4^+$ Th1 responses using the model Ag OVA (1 mg/ml). Initially, we assessed the ability of NK1R endogenous (SP and HK-1) and synthetic (SarSP) agonists (all at 10^{-9} M) to promote OVA-specific Th1 responses in vitro. OVA-loaded BMDCs were used as stimulators of naive $CD4^+$-purified OT-II T cells, which recognize the $OVA_{323-339}$ peptide when presented in the context of $I\text{-}A^b$ MHC class II molecules on the DC cell-surface. As shown in Figure 14A and B, respectively, BMDCs loaded with OVA and signaled with SP, HK-1 or SarSP significantly enhanced the proliferation and IFN-γ secretion of $CD4^+$ T cells in comparison to those stimulated with OVA-loaded BMDCs that were not stimulated with exogenous NK1R agonists (control). The enhancement was Ag-specific, as unloaded BMDCs were incapable of significantly inducing either $CD4^+$ T cell proliferation or IFN-γ secretion. In contrast to elevated secretion of the Th1 cytokine IFN-γ, NK1R agonists did not significantly modulate Th2 cytokines IL-5 and IL-13, demonstrating a preferential enhancement of the Th1 response (Figure 14B). Interestingly, SarSP signaling of OVA-loaded BMDCs elicited the highest proliferation and IFN-γ secretion of $CD4^+$ T cells in comparison to DC stimulation with NK1R physiological agonists, which could be attributed to greater stability of the synthetic agonist in culture or selective binding of the NK1R.

The relevance of our findings was further tested in vivo by adoptive transfer of BMDCs (DCs) vs. SarSP-signaled BMDCs (SarSP-DCs) loaded with or without OVA into B6 mice. Mice immunized with SarSP-DCs loaded with OVA enhanced the secretion of IFN-γ from re-stimulated OVA-specific $CD4^+$ T cells in comparison to those stimulated with control Ag-loaded DCs, while not inducing significant modulation in the production of Th2 cytokines IL-5 and IL-13 (Figure 14C). Together, these data demonstrate that BMDCs signaled with NK1R agonists promote robust Th1 responses in vitro and in vivo.

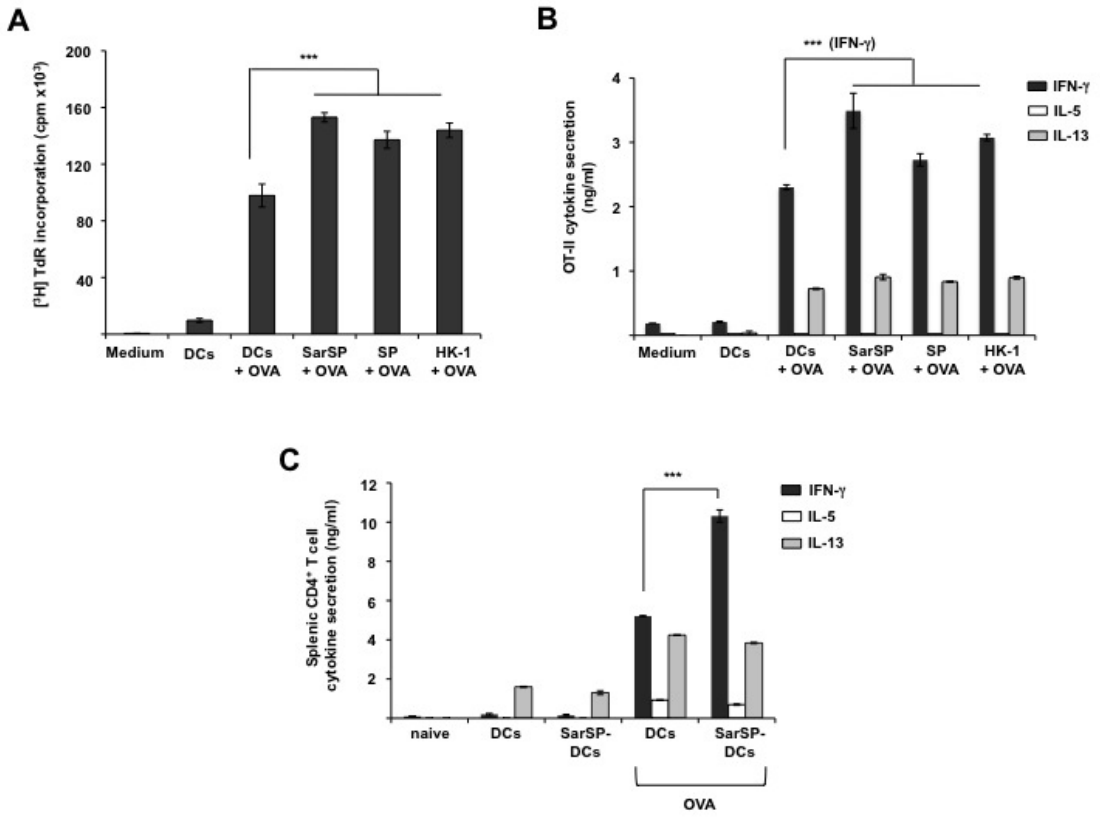


Figure 14: Agonistic signaling via the NK1R promotes BMDCs to enhance Th1 responses in vivo.

Immune-stimulatory function of BMDCs. (A and B) Bar-diagrams representing the (A) proliferation and (B) cytokine secretion [IFN-γ (black), IL-5 (white) and IL-13 (gray)] of $CD4^+$ T cells (OT-II, splenic) stimulated with $CD11c^+$ BMDCs loaded with or without OVA (1 mg/ml) and supplemented or not with SarSP, HK-1 or SP (all at 10^{-9} M). (C) Comparative analysis of splenic $CD4^+$ T cells secreting cytokines IFN-γ (black), IL-5 (white) and IL-13 (gray) after adoptive transfer of OVA-loaded (or not, control) $CD11c^+$ BMDCs signaled in the presence (SarSP-DCs) or absence (DCs) of SarSP. Control groups also include splenic $CD4^+$ T cells isolated from non-immunized (naïve) mice. Isolated $CD4^+$ T cells were re-stimulated by co-culture with $CD11c^+$ BMDCs loaded with OVA. (A-C) Means $\pm$ 1 SD of 4 independent experiments are displayed. ***p<0.001.

3.4.3 NK1R-signaled BMDCs enhance Th1 responses in vivo which are dependent on endogenous production of IL-12

Secretion of IL-12p70 is one of the main mechanisms known to promote Th1 responses in vitro and in vivo.[45] Thus, we investigated the role IL-12p70 secretion serves to induce Th1 responses mediated by signaling BMDCs via the NK1R. Given the importance of the DC-$CD4^+$ T cell synapse to potentiate and optimize IL-12p70 production,[216] we initially assessed the ability of BMDCs signaled with SarSP (10^{-9} M; SarSP-DCs) to promote IL-12p70 secretion in the context of OVA and in co-cultures with OT-II $CD4^+$ T cells. After OVA stimulation, SarSP-DCs did not produce significant amounts of IL-12p70 (Figure 15A). However, SarSP-DCs loaded with OVA were able to up-regulate IL-12p70 secretion in co-culture with $CD4^+$ T cells in comparison to co-culture with control OVA-loaded DCs. The enhanced IL-12p70 secretion in co-cultures of SarSP-DCs and $CD4^+$ T cells was abrogated due to neutralization of CD40 ligation (MR-1 mAb, 20 μg/ml), while inhibition of IFN-γ signaling (anti-IFN-γ, 20 μg/ml) did not significantly reduce IL-12p70 secretion (Figure 15A). Although the levels of IL-12p70 are low in OVA-specific DC-$CD4^+$ T cell co-cultures, we observe that neutralizing antibodies specifically targeting the p35 (anti-IL-12p35) and p40 (anti-IL-12p40) subunits of bioactive IL-12p70, but not the p19 subunit of IL-23 (anti-IL-23p19), impaired the enhanced Th1 response mediated by OVA-loaded SarSP-DCs (Figure 15B). These results suggest that SarSP-DCs promote Ag-specific Th1 responses in vitro by up-regulating IL-12p70 to a small extent during the DC-$CD4^+$ T cell synapse.

Due to the observations that (i) SarSP-DCs produce low levels of IL-12p70 and (ii) adoptively transferred DCs interact with local APC populations in vivo,[74] we investigated whether IL-12p70 secretion in vivo is necessary to mediate the ability of adoptively transferred

SarSP-DCs to enhance the Th1 response, and if so, determine whether IL-12p70 is contributed by transferred DCs or endogenous APCs. To address these questions, we performed a series of experiments utilizing $CD11c^+$ BMDCs derived from B6 (wild-type, WT) and B6.IL-$12p35^{-/-}$ ($p35^{-/-}$) mice, which were cultured in the absence (DCs) or presence (SarSP-DCs) of SarSP (10^{-9} M) and loaded with OVA (1 mg/ml) prior to adoptive transfer into B6 and B6.IL-$12p35^{-/-}$ mice. As we observed before, transfer of WT-SarSP-DCs enhanced the OVA-specific Th1 response in comparison to transferred WT-DCs into B6 mice (Figure 15C). The ability of SarSP-DCs to mount enhanced OVA-specific Th1 responses in vivo was almost completely abrogated if endogenous APCs could not produce bioactive IL-12p70, as indicated by a significant reduction in the amount of IFN-γ secreted from re-stimulated OVA-specific $CD4^+$ T cells (Figure 15C). In contrast, OVA-specific Th1 responses were only slightly diminished when $p35^{-/-}$-DCs were transferred into B6 mice, and $p35^{-/-}$-SarSP-DCs were still able to efficiently enhance the Th1 response (Figure 15C). These results demonstrate the importance of endogenous IL-12 in the Th1-biased observed under our experimental conditions.

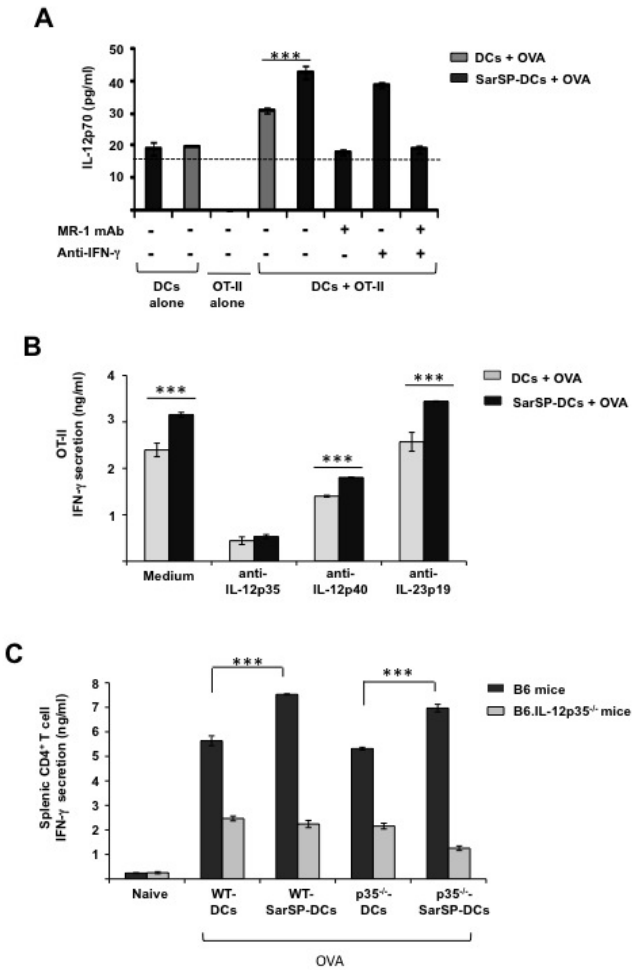


Figure 15: SarSP-DCs enhance Ag-specific Th1 responses in vivo which are dependent on endogenous production of IL-12.

(A) Bar-diagrams representing secretion of IL-12p70 in co-cultures of $CD11c^+$ BMDCs loaded with OVA (1 mg/ml) in the presence or absence of SarSP (10^{-9} M) and $CD4^+$ T cells (OT-II, splenic). Select co-cultures were supplemented or not with neutralizing antibodies targeting CD40 ligation (MR-1, 20 μg/ml) and/or IFN-γ (anti-IFN-γ, 20 μg/ml). (B) Comparative analysis of IFN-γ production in co-cultures of $CD4^+$ T cells (OT-II mice, splenic) and OVA-loaded $CD11c^+$ BMDCs signaled in the absence (DCs, light bars) or presence (SarSP-DCs, dark bars) of SarSP. Select co-cultures were supplemented with or without neutralizing antibodies specific to IL-12p35 (C18.2 20 μg/ml), IL-12p40 (C17.8, 20 μg/ml) and IL-23p19 (G23.8, 20 μg/ml). (C) Bar-diagrams illustrating IFN-γ secretion form OVA-specific $CD4^+$ T cells isolated from spleens of wild-type (B6, dark bars) or IL-12 deficient (B6.IL-12p35$^{-/-}$, light bars) mice that were non-immunized (naïve, control) or immunized with B6 (WT) or B6.IL-12p35$^{-/-}$ (p35$^{-/-}$) $CD11c^+$ BMDCs signaled in the absence (DCs) or presence (SarSP-DCs) of SarSP. Isolated $CD4^+$ T cells were re-stimulated by co-culture with B6 $CD11c^+$ BMDCs loaded with OVA. (A-C) Means $\pm$ 1 SD of 3 independent experiments are displayed. ***p<0.001.

3.4.4 NK1R-signaled BMDCs potentiate $CD8^+$ CTL/Tc1 responses in vivo which are mainly dependent on the production of IL-12

In addition to Ag-specific $CD4^+$ Th1 immune responses, cellular immune responses are characterized by induction of functional $CD8^+$ CTL responses that are biased to the Tc1 phenotype.[204,209] Initially, we investigated whether BMDCs signaled via the NK1R elicit OVA-specific cytotoxic responses in vivo. BMDCs were cultured in the absence (DCs) or presence (SarSP-DCs) of SarSP (10^{-9} M) then loaded with or without OVA (1 mg/ml) and SIINFEKL (SIINFEKL, 100 ng/ml) prior to adoptive transfer into B6 mice. In comparison to adoptively transferred control DCs, SarSP-DCs elicited higher Ag-specific cell killing of targets (Figure 16A). Non-immunized mice (naïve) and mice immunized with non-loaded DCs did not elicit OVA-specific cell killing, suggesting the involvement of Ag-specific effector CTLs. Effector $CD8^+$ T cells mediated by adoptive transfer of Ag-loaded SarSP-DCs induced higher secretion of IFN-γ with little modulation of Tc2 cytokines IL-5 and IL-13, suggesting the enhancement of a biased Tc1 response (Figure 16B).

Since adoptive transfer of SarSP-DCs favors a Tc1 response, we addressed the role of IL-12p70 contribution by transferred DCs and endogenous APCs. IL-12p70 production provided by both endogenous APCs and transferred DCs was required to elicit optimal Ag-specific CTL responses mediated by SarSP-DCs, as depicted by reduced cytotoxicity of targets in vivo upon adoptive transfer of $p35^{-/-}$-SarSP-DCs into B6 and B6.IL-$12p35^{-/-}$ mice (Figure 16C).

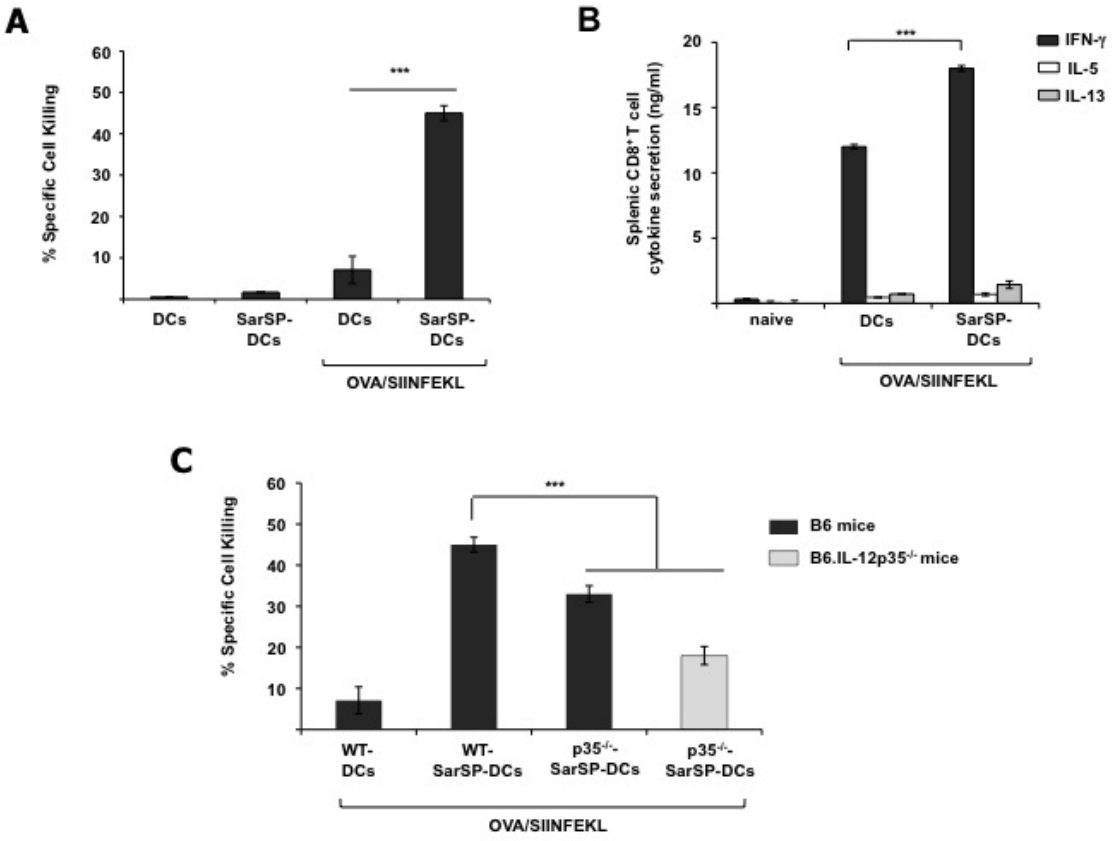


Figure 16: SarSP-DCs enhance Ag-specific CD8$^+$ CTL/Tc1 responses in vivo which are mainly dependent on the production of IL-12.

(A) Comparative analysis of in vivo cytotoxicity assays induced by adoptive transfer of CD11c$^+$ BMDCs cultured without (DCs) or with (SarSP-DCs) SarSP (10^{-9} M) then loaded with or without OVA (1 mg/ml) and SIINFEKL (250 μg/ml). (B) Bar-diagrams representing cytokine secretion [IFN-γ (black), IL-5 (white) and IL-13 (gray)] of CD8$^+$ T cells isolated from spleens of non-immunized mice (naïve) and immunized mice adoptively transferred with OVA/SIINFEKL-loaded CD11c$^+$ BMDCs cultured without (DCs) or with (SarSP-DCs) SarSP. Isolated CD8$^+$ T cells were re-stimulated by co-culture with CD11c$^+$ BMDCs loaded with OVA and SIINFEKL. (C) Comparative analysis of in vivo cytotoxicity assays induced by adoptive transfer of B6 (WT) or B6.IL-12p35$^{-/-}$ (p35$^{-/-}$) CD11c$^+$ BMDCs cultured in the absence (DCs) or presence (SarSP-DCs) of SarSP and loaded with OVA and SIINFEKL into B6 (dark bars) or B6.IL-12p35$^{-/-}$ (light bars) mice. (A-C) Means $\pm$ 1 SD of 3 independent experiments are displayed. ***p<0.001.

3.4.5 Endogenous DC populations are required to amplify Th1 and CTL responses mediated by adoptive transfer of NK1R-signaled BMDCs

Local DC populations likely contribute the requirement of endogenous IL-12p70, as specific endogenous DC populations are highly efficient in capturing Ag and producing IL-12p70.[45,95,96] Therefore, we investigated the role endogenous DCs play by abrogating their presence in vivo during adoptive transfer of BMDCs signaled without (DCs) or with (SarSP-DCs) SarSP to determine their impact on acquired OVA-specific Th1 and CTL responses. Utilizing mice transgenic for the DTR under the CD11c promoter, we are able to selectively deplete endogenous DC populations expressing DTR when DT is administered via i.p. injection.

BMDCs derived from DTR mice (DTR^+DCs) express similar levels of CD11c and maturation molecules CD86 and MHC class II molecules on their cell surface as wild-type BMDCs (data not shown), suggesting that the incorporation of DTR does not alter the generation or the immune-stimulatory phenotype of BMDCs. DTR^+DCs generated from DTR mice undergo significant apoptosis when DT (10^{-7} M) is supplemented to BMDC cultures for 18 h, and furthermore, we observe that addition of pro-survival SarSP was incapable of preventing cell-death induced by DT (Supplementary Figure 1; Appendix A).

When endogenous DC populations from recipient DTR mice were depleted during adoptive transfer of OVA-loaded DCs and SarSP-DCs, adoptively transferred BMDC groups were not able to induce robust Th1 and CTL responses in vivo (Figure 17A and B, respectively). Interestingly, killing endogenous DC populations had a greater impact on Th1/CTL responses mediated by OVA-loaded DCs, while OVA-loaded SarSP-DCs still were capable of mounting significantly higher Th1 and CTL responses. These results demonstrate that endogenous DC

populations play a crucial role in amplifying Th1/Tc1 responses induced by SarSP-DCs, and other mechanisms may be at hand to account for the ability for SarSP-DCs to stimulate enhanced Th1 and CTL/Tc1 immunity in vivo.

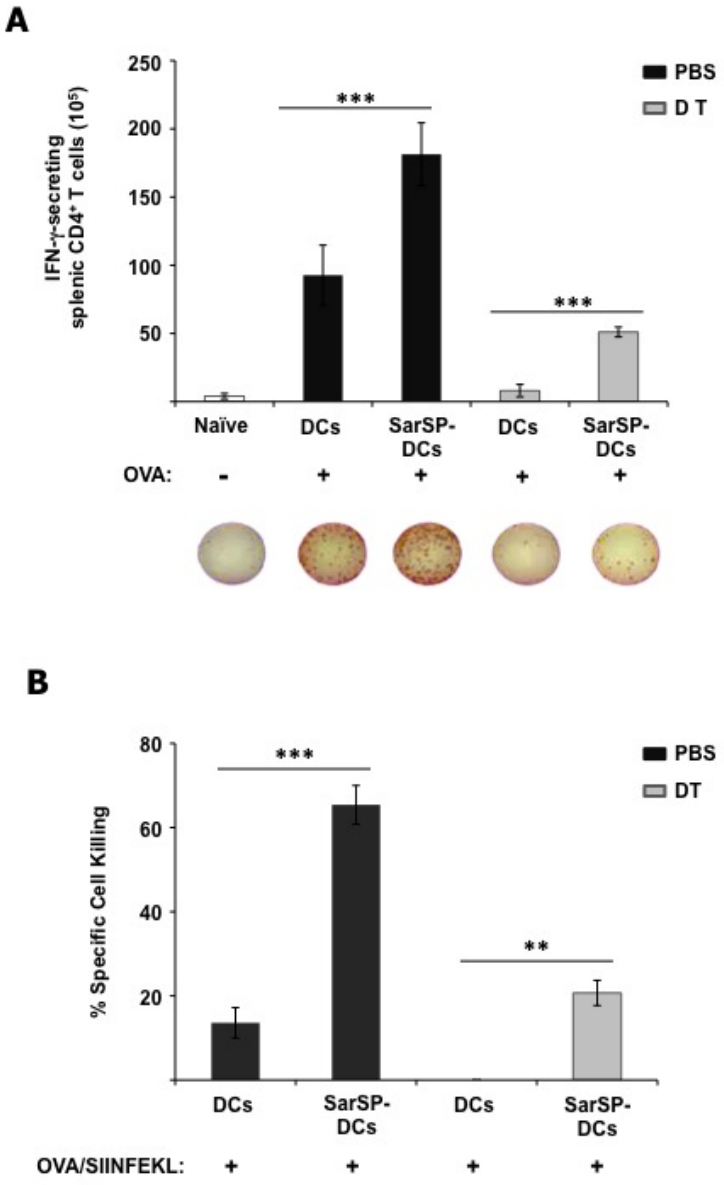


Figure 17: Survival of endogenous DC populations is crucial to mediate robust Ag-specific Th1 and CTL responses induced by adoptively transferred SarSP-DCs.

(A and B) Comparative analysis of (A) frequency of re-stimulated splenic $CD4^+$ T cells secreting IFN-γ and (B) in vivo cytotoxicity assays induced by adoptive transfer of DCs or SarSP-DCs loaded with (A) OVA or (B) OVA and SIINFEKL into B6 mice pre-treated (i.p.) with PBS (control, 0 h; dark bars) or DT (10^{-7} M, 0 h; light bars). (A-B) Means $\pm$ 1 SD of 3 independent experiments are displayed. ***p<0.001, **p<0.005.

3.4.6 NK1R signaling favors enhanced Th1 and Tc1 responses by inhibiting the secretion of IL-10 from adoptively transferred BMDCs

Due to the observations that SarSP significantly inhibits the secretion of IL-10 from BMDCs in vitro, and adoptive transfer of genetically-modified BMDCs expressing IL-10 promotes tolerance;[269,270] we investigated the impact of over-expressing IL-10 to abrogate the ability of SarSP-DCs to mount enhanced Th1 and CTL responses in vivo. BMDCs transduced with adenoviral vector encoding mIL-10 cDNA (500 MOI) secrete high levels of IL-10 (rAd_IL-10) and reduce expression of CD86 and MHC class II molecules in comparison to DCs transduced with an empty vector (rAd_neo, control), demonstrating functionality of rAd_IL-10.[271] Furthermore, we observe that SarSP-DCs transduced with rAd_IL-10 were no longer able to abrogate IL-10 secretion (data not shown).

Adoptive transfer of OVA- or OVA/SIINFEKL-loaded BMDCs transduced with rAd_IL-10 significantly reduced OVA-specific Th1 (Figure 18A) and CTL (Figure 18B) responses in vivo, respectively, in comparison to BMDCs transduced with rAd_neo. Furthermore, SarSP-DCs over-expressing IL-10 were no longer able to exhibit significant increases (p>0.05) in Th1 and CTL responses when compared to control Ag-loaded DCs (rAd_IL-10) (Figure 18A and B, respectively). Collectively, these results suggest that SarSP-DCs, when no longer capable of inhibiting IL-10, do not promote robust and enhanced Ag-specific Th1 and CTL/Tc1 immunity in vivo.

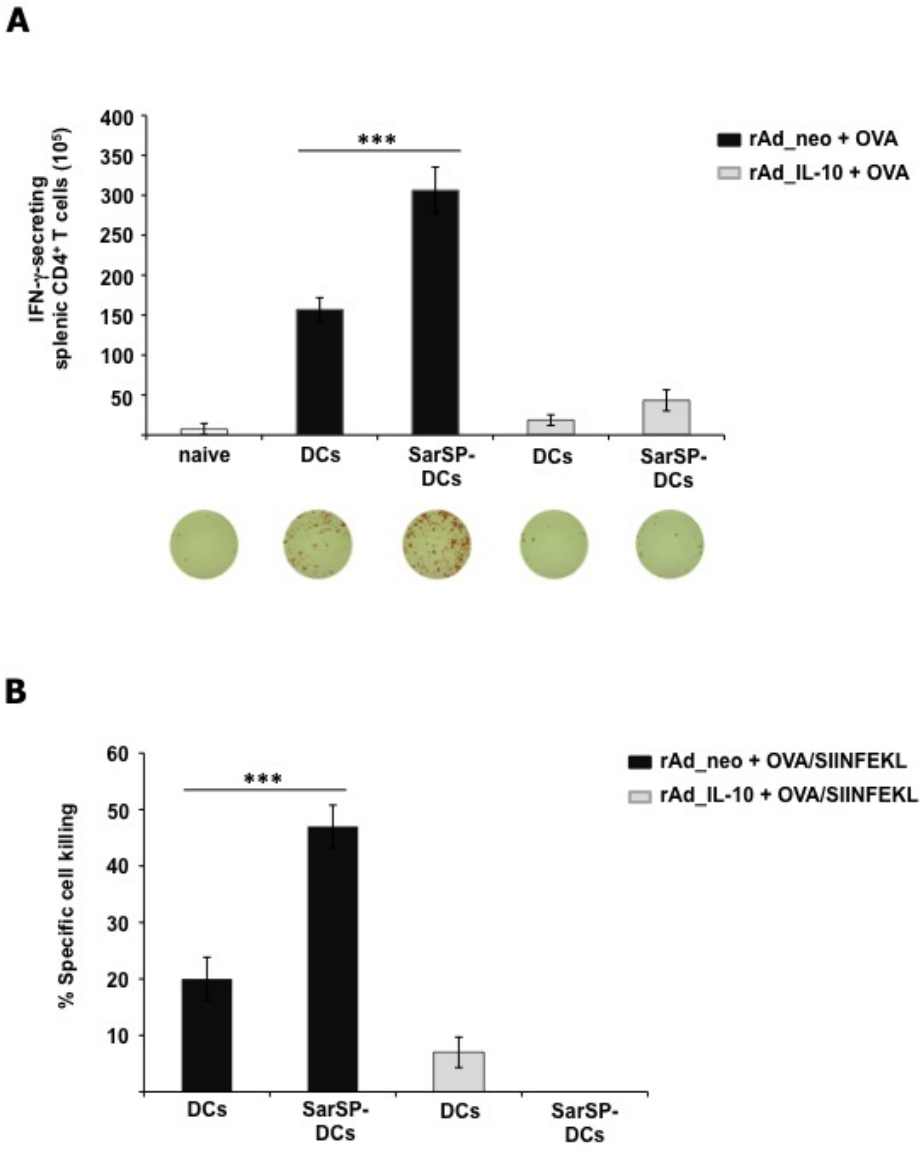


Figure 18: Over-expression of IL-10 inhibits the ability of SarSP-DCs to promote enhanced Ag-specific Th1 and CTL responses in vivo.

(A and B) Bar-diagrams illustrating the (A) frequency of re-stimulated splenic $CD4^+$ T cells secreting IFN-γ and (B) in vivo cytotoxicity assays induced by adoptive transfer of Ag-loaded [(A) OVA, (B) OVA and SIINFEKL] and transduced (rAd_neo, dark bars; rAd_IL-10, light bars) DCs or SarSP-DCs into B6 mice. (A-B) Means ± 1 SD of 3 independent experiments are displayed. ***p<0.001.

3.5 DISCUSSION

DCs are often used in immune-stimulatory therapies due to their ability to generate potent T cell immunity.[76] However, the mechanisms by which adoptively transferred DCs promote Th1/Tc1 responses in vivo have not been fully explored. Recent studies suggest that in addition to the survival and immune-stimulatory profile of adoptively transferred DCs, their ability to activate and cross-prime endogenous DCs is vital for optimal induction of Th1/Tc1 responses in vivo.[74]

Recently, it has been demonstrated that pro-inflammatory neuropeptides from the tachykinin family, including SP and HK-1, play crucial roles in favoring the induction and amplification of Th1 and Tc1 cells.[84,187,188] Due to the observations that DCs are potent inducers of naïve T cells and lie in close proximity to sources of tachykinins (i.e., nerve-fiber terminals and immune cells),[122,194,195] we hypothesized that agonistic binding of the NK1R favors immune-stimulatory function of DCs, which will result in efficient generation of Ag-specific Th1/Tc1 responses. Indeed, it has been reported that NK1R ligands induce nuclear translocation of the pro-inflammatory NF-κB transcription factor,[201] which is involved in the up-regulation of co-stimulatory and MHC class molecules and the Th1/Tc1-biasing IL-12p70 cytokine. However, the ability of NK1R ligands to promote immune-stimulatory features of DCs that are ideal for DC-based immune-therapies has not been investigated.

In the present work, using a model of ex vivo-generated BMDCs, we demonstrate that agonistic signaling via the NK1R enhances the maturation of BMDCs and inhibits the secretion of anti-inflammatory cytokines IL-10 and TGF-β1, without modulating IL-12p70 production significantly. Interestingly, NK1R agonists were able to slightly enhance IL-12p70 secretion mediated by pro-inflammatory mediators derived from peripheral and lymphoid tissues. Our

results illustrate that while typical Th1/Tc1-inducing factors, including TLR agonists and DC1 signaling-cocktail, produce paramount levels of IL-12p70; these stimulated BMDCs significantly enhance the secretion of IL-10 but not TGF-β1. It is well appreciated that high levels of IL-10 circumvent the induction of potent Th1/Tc1 cells, and DCs producing robust IL-10 may represent a major drawback in DC-based immune-stimulatory therapies.[272]

Due to the unique ability of NK1R ligands to down-regulate IL-10 and up-regulate IL-12p70 under certain pro-inflammatory conditions, we hypothesized that NK1R-signaled BMDCs may serve as beneficial adjuvants in biasing Th1/Tc1 responses in vivo. Adoptive transfer of NK1R-signaled BMDCs elicits enhanced Th1/Tc1 responses. Although exogenous IL-12p70 produced by our conditioned-DCs is negligible, endogenous IL-12p70 is required for the ability of NK1R-signaled BMDCs to elevate Th1/Tc1 responses in vivo, suggesting the activation and passage of Ag to endogenous APCs. In our experiments, endogenous IL-12p70 could be provided by DCs during T cell priming or during maintenance of the Th1 response by IFN-γ-activated macrophages in peripheral tissues. It is likely that IL-12p70 secretion is mainly attributed to DCs, as DC populations present in lymphoid tissues have been described to be potent contributors of IL-12p70 secretion and cross-priming.[4,45] Likewise, we observe that if endogenous DC populations were abrogated in our hosts, and consequently are incapable of cross-presenting Ag and producing IL-12p70, adoptively transferred NK1R-signaled BMDCs induce severely impaired Ag-specific Th1/Tc1 responses in vivo. Due to the observations that both DCs derived from migratory and lymphoid tissues capture and transfer Ag and are capable of directly priming and sustaining $CD4^+$ and $CD8^+$ T cell responses;[89,98] future studies are required to determine which endogenous DC populations are responsible for Th1/Tc1 responses mediated by adoptive transfer of NK1R-signaled BMDCs and their ability to produce IL-12p70.

While the ability and necessity of NK1R agonists to signal adoptively transferred BMDCs to secrete IL-12p70 in vivo is less clear, their ability to down-regulate IL-10 production, and subsequently, favor potent Ag-specific Th1/Tc1 responses is evident. We found that over-expressing IL-10 in BMDCs significantly reduces acquired Ag-specific Th1/Tc1 responses, and importantly, transfer of NK1R-signaled BMDCs is no longer able to engender increases in comparison to non-signaled BMDCs. It will be interesting to determine whether reduced IL-10 secretion allows for greater activation and presentation of Ag to endogenous DC populations, and additively, enhances IL-12p70-responsiveness in developing Th1 and Tc1 cells.

In conclusion, we provide evidence that signaling BMDCs via the NK1R with pro-inflammatory tachykinins enhances the immune-stimulatory function of DCs to promote Ag-specific Th1/Tc1 responses in vivo, which are dependent on their ability to inhibit IL-10 secretion and promote immune-stimulatory interactions with endogenous DC populations. Our data provide insight into the mechanisms by which adoptively transferred DCs promote Th1/Tc1 responses in vivo, and help to elucidate ideal parameters when designing adjuvants for DC-based immune-stimulatory therapies.

4.0 CHAPTER FOUR: SUMMARY

4.1 SIGNALING DENDRITIC CELLS VIA THE NK1R

Tachykinins are now appreciated to be secreted from both neuronal and non-neuronal sources in peripheral and lymphoid immune-competent organs.[123,158] As most immune cells express the NK1R and secrete NK1R agonists, tachykinins have been implicated in the modulation of cellular immunity.[159-162] DCs are essential for cellular immunity; however, the ability of tachykinins signaling via the NK1R to exert the immune-stimulatory function of DCs remains elusive. Our studies have allowed us to develop a model describing the ability and means by which NK1R agonists promote the T cell-stimulatory function of DCs (Figure 19).

DCs, comprised of both immature and mature cells, express the functional full-length NK1R variant on their cell-surface. DCs up-regulate the NK1R during inflammatory events, which often coincides with elevated secretion of SP, enabling DCs to be susceptible to further NK1R signaling. As endogenous (SP and HK-1) and synthetic (SarSP) agonists ligate the full-length NK1R, G proteins are able to interact with the C-terminus tail and intracellular loops of the receptor to mediate relevant immune function in DCs. For example, signaling DCs via the NK1R activates the PI3K-Akt intracellular pathway, leading to increased cell longevity. Additionally, NK1R signaling enhances the maturation and pro-inflammatory capacities of DCs, as determined by elevated expression of cell-surface molecules CD86, CD40 and I-A^b and diminished secretion of anti-inflammatory cytokines IL-10 and TGF-β1. NK1R-signaled DCs are capable of producing the Th1-biasing cytokine IL-12p70 during the DC-$CD4^+$ T cell synapse, although at low levels. In concert with enhanced cell survival and immune-stimulation mediated by NK1R signaling of DCs, these NK1R-signaled DCs are able to prime and generate enhanced Ag-specific $CD4^+$ Th1 and $CD8^+$ CTL/Tc1 responses.

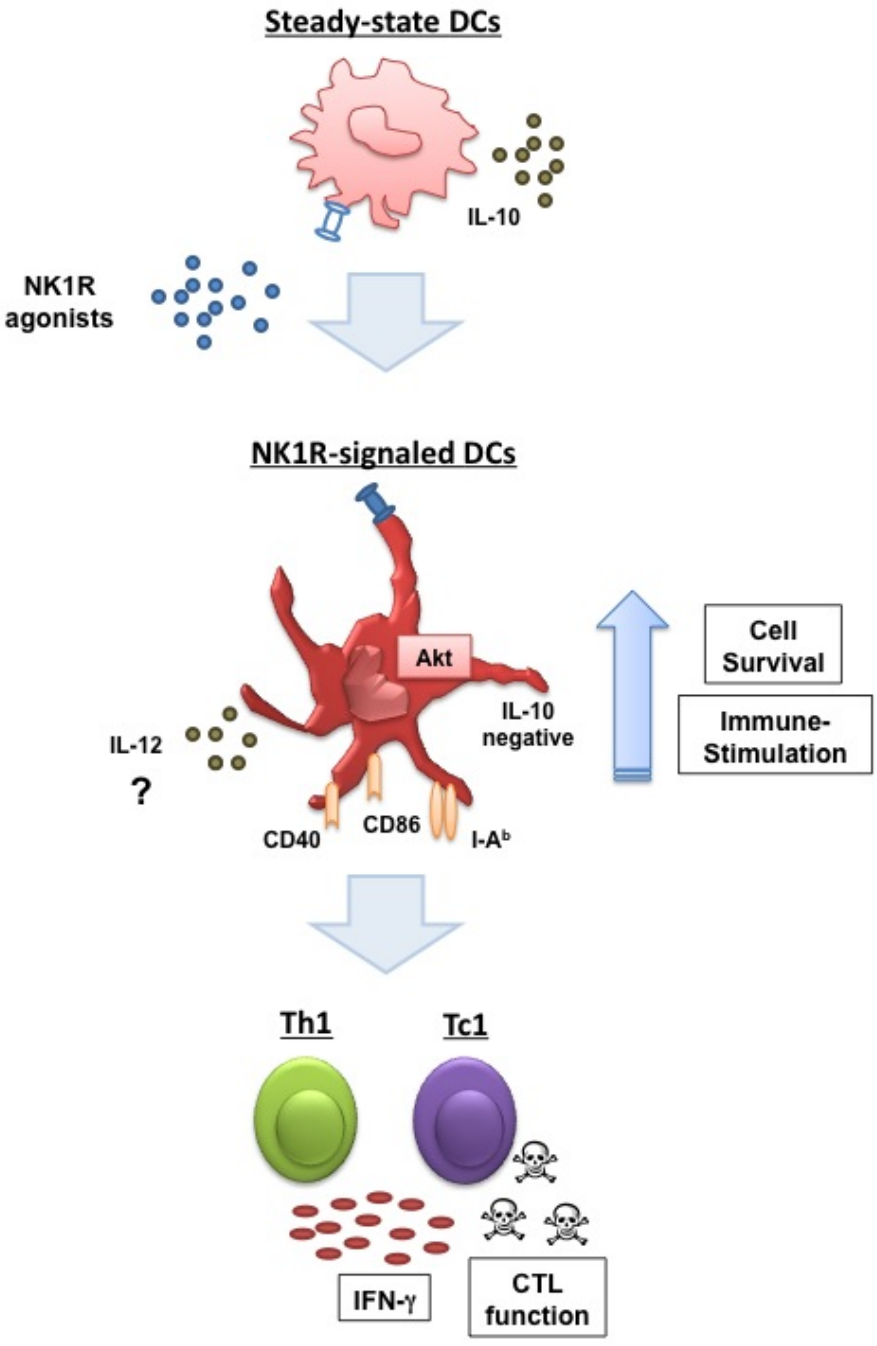


Figure 19: Proposed model for the role NK1R agonists mediate in modulating DC immune-stimulatory function.

4.2 INTERPLAY OF EXOGENOUS AND ENDOGENOUS DENDRITIC CELLS AND TYPE-1 T CELL IMMUNITY

Adoptive transfer experiments utilizing NK1R-signaled DCs illustrate the immune-relevance of our previous findings. NK1R-signaled DCs exhibit enhanced longevity and promote robust Ag-specific type-1 DTH. Mechanistic analysis demonstrates that endogenous DCs play essential roles in amplifying the ability of adoptively transferred NK1R-signaled DCs to induce robust Th1/Tc1 responses (Figure 20), which are crucial for the stimulation of effector cellular immunity.

After adoptive transfer, NK1R-signaled DCs are able to home into DLNs where they survive longer because they are far less susceptible to Ag-dependent and -independent clearance from DLNs. Our results show that the CD40 molecule, likely expressed by activated $CD4^+$ T cells, synergizes with NK1R signaling to promote DC survival and prolong the longevity of NK1R-signaled DCs in DLNs. The consequences of enhancing cell longevity of immune-stimulatory DCs could be attributed to enhanced duration of direct Ag presentation to specific T cells and/or (ii) greater spreading of Ag and interaction with endogenous DC populations. From our data, we conclude that the interaction between adoptively transferred DCs and endogenous DCs is crucial. Whereas the presence of endogenous DCs is necessary to amplify Th1/Tc1 responses, likely via IL-12p70 production; adoptively transferred DCs are still vital to control the microenvironment of the DC-T cell interaction as demonstrated by a crucial role of IL-10 secretion in modulating the outcome of Th1 and Tc1 responses. It could be postulated that adoptively transferred NK1R-signaled DCs migrate to DLNs and prime T cell responses, while spreading Ag to endogenous DC populations in lymphoid-tissues to further amplify the response.

The ability of NK1R-signaled DCs to inhibit IL-10 secretion warrants further investigation to determine the outcome on the immune-stimulatory function of endogenous DC populations. Furthermore, since Ag-loaded NK1R-signaled DCs survive longer, it will be interested to elucidate whether their enhanced survival, coupled with reduced IL-10 secretion, leads to more efficient activation and transfer of Ag to endogenous DC populations or direct priming of T cells in DLNs. Such experiments are currently being performed in our lab using the DTR model.

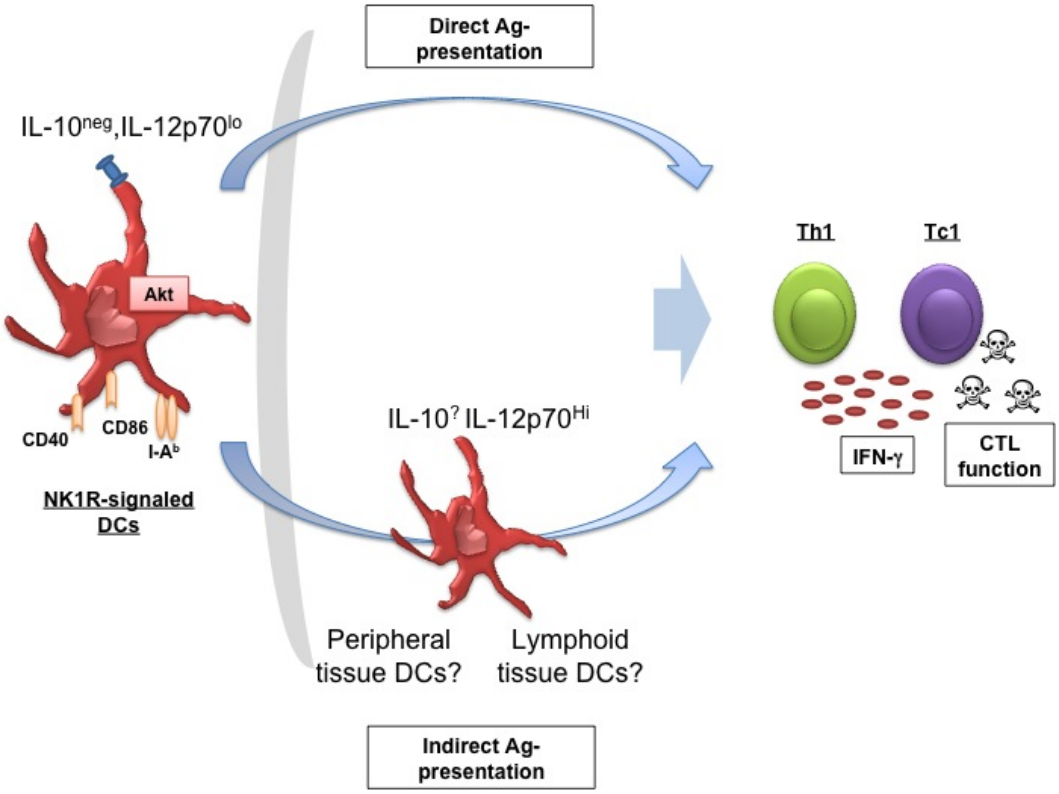


Figure 20: Possible mechanisms mediated by adoptive transfer of NK1R-signaled DCs to promote robust Th1/Tc1 responses in vivo.

4.3 CLINICAL IMPLICATIONS

The novel findings presented herein bring in accountability of pro-inflammatory neuropeptides to orchestrate cellular immune responses, particularly by eliciting immune-stimulatory DCs to promote robust type-1 T cell responses. Signaling DCs via the NK1R favors cell survival and potent Th1/Tc1 responses, demonstrating their potential application for immune-stimulatory therapies utilizing adoptive transfer of DCs or targeting DCs in vivo. Therefore, the results obtained are highly relevant to the fields of vaccination and immune-therapies for tumor, infectious diseases and chronic inflammatory and autoimmune disorders.

Likewise, we are currently investigating the ability of exogenous and endogenous NK1R agonists to signal DCs to promote anti-tumor immunity. Manske and colleagues have provided initial studies demonstrating the ability of exogenous NK1R agonists to induce anti-tumor immunity and prevent tumor growth in a murine model of melanoma; an effect that was mainly mediated by T cells.[273] In our unpublished findings, we observe that mice devoid of endogenous NK1R agonistic signaling (B6.NK1R$^{-/-KO}$ mice) are unable to elicit robust Th1 and CTL/Tc1 responses against tumor-associated Ag and inhibit tumor growth in comparison to wild-type mice (manuscript submitted). In this study, it is unknown whether endogenous NK1R agonists signal DCs to enhance Ag-specific type-1 T cell responses, or alternatively, directly ligate developing $CD4^+$ and $CD8^+$ T cells. If the former occurs, it would be interesting to specifically target NK1R agonists to desired endogenous DC populations in vivo to elicit optimal anti-tumor immunity. Since tumor cells express NK1R and agonistic signaling via the receptor induces tumor growth and survival,[274,275] applying NK1R agonists systemically could inhibit the ability of the immune system to eradicate established tumors. Thus, it may be important to administer

NK1R agonists away from the tumor micro-environment, while utilizing reagents to inhibit NK1R signaling directly on the tumor cells. Indeed, previous studies support the concept of employing NK1R antagonists or over-expressing tachykinin-degrading enzymes (i.e., NEP) in the tumor-microenvironment. NK1R antagonists have been shown to induce apoptosis of various tumor cell lines.[276-278] In most forms of cancer, NEP expression is usually reduced,[279] and subsequently, studies show that increasing NEP activity on tumor cells leads to the prevention of survival and proliferative effects induced by NK1R agonists.[280] In addition to application of NK1R agonists to DCs or NK1R inhibitory reagents in the tumor micro-environment, it may be possible to increase endogenous levels of NK1R agonists by promoting the production of Th1-inducing (IL-12p70) and Th1 (IFN-γ and IL-2) cytokines, while inhibiting anti-inflammatory cytokines (IL-10 and TGF-β1) and neuropeptides (NPY, POMC derivatives, adrenomedullin, somatostatin, cortistatin and urocortin).[130,234,281-283]

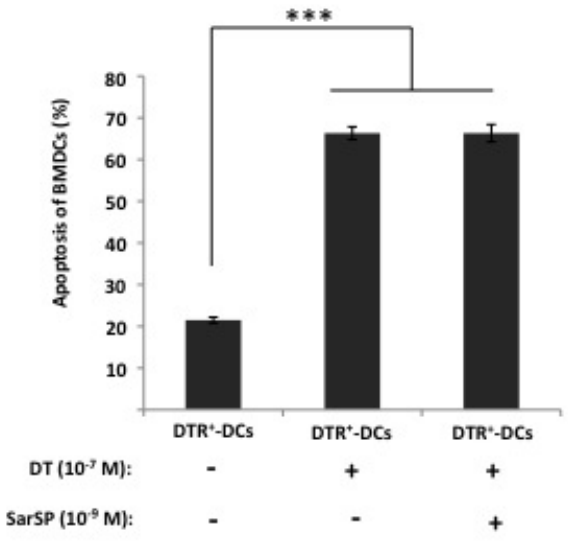
Alternatively, abrogation of NK1R signaling could be beneficial for immune-inhibitory therapies targeting chronic inflammatory and autoimmune disorders and T cell-mediated rejection in transplantation. We have begun studies pioneering the ability of the NK1R antagonist (L-733,600) to block human and murine allogeneic $CD4^+$ and $CD8^+$ T cell responses mediated by DCs (manuscript in preparation). It is important to note that the pathogenesis of various inflammatory and autoimmune disorders is associated with over-expression of NK1R and its agonists, leading to enhanced and sustained survival, chemotaxis and activation of immune cells.[186,284,285] In a model of Th1-type colitis in IL-$10^{-/-}$ mice, NK1R agonists promote chronic inflammation, and the use of NK1R antagonists are able to reverse the ongoing inflammation.[185] Moreover, evidence supports the notion that NK1R agonists contribute to inflammatory T cell responses in CNS autoimmunity, and abrogation of endogenous NK1R

signaling leads to impairment of disease serverity.[234,286,287] Further investigation is warranted to determine the ability of NK1R antagonists and tachykinins binding anti-inflammatory NK2R and NK3R to signal DCs in vivo for the purpose of inducing tolerance towards self-Ag in autoimmune disorders and allograft acceptance in transplantation.

4.4 FINAL STATEMENT

The studies discussed in this dissertation have allowed us to conclude that pro-inflammatory tachykinins signaling DCs via the NK1R induce cell survival and immune-stimulatory function, ultimately leading to the generation of potent type-1 T cell immunity in vivo. Due to the potential of DC-based vaccines for immune-therapies, further investigation is required to determine the efficacy of targeting exogenous and endogenous DCs via the NK1R to serve as adjuvants to promote immune-stimulation against tumors and intracellular infections.

APPENDIX A



Supplementary Figure 1: NK1R signaling of BMDCs expressing DTR are not rescued from DT-induced apoptosis.

Apoptosis of BMDCs generated from B6.CD11c.DTR mice as assessed by FACS-analysis of PI (50 µg/ml) inclusion. $CD11c^{+}DTR^{+}$DCs were cultured with or without SarSP (10^{-9} M) and/or DT (10^{-7}M) for 18 h. Means ($\pm$ SD) of 3 independent experiments are shown. ***$p<0.05$

APPENDIX B

In Vivo Signaling through the Neurokinin 1 Receptor Favors Transgene Expression by Langerhans Cells and Promotes the Generation of Th1- and Tc1-Biased Immune Responses

Alicia R. Mathers, Olga A. Tkacheva, Brian M. Janelsins, William J. Shufesky, Adrian E. Morelli, and Adriana T. Larregina.

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Abstract

The pro-inflammatory capacities of the skin and the presence of high numbers of resident dendritic cells (DCs) constitute an ideal microenvironment for successful immunizations. Regardless of the ability of DCs to respond to local inflammatory signals in an immune-stimulatory fashion, the immune functions of skin resident DCs remain controversial, and epidermal Langerhans cells (LCs) have been referred to recently as anti-inflammatory / pro-tolerogenic APCs. Substance P (SP) released by skin nerve fibers, is a potent pro-inflammatory neuropeptide that favors development of skin associated cellular immunity. SP exerts its pro-inflammatory functions by binding with high affinity to the neurokinin 1 receptor (NK1R). Here we tested whether signaling skin cells via the NK1R promotes humoral and cellular immunity during skin genetic immunizations. We utilized the gene gun to deliver transgenic (tg) Ag to the skin of C57BL/6 mice and the selective NK1R agonist [$Sar^{9}Met(O_2)^{11}$]-SP as a potential pro-inflammatory Th1-biasing adjuvant. Our strategy expressed tg Ag exclusively in the epidermis and induced a preferential migration of activated LCs to skin draining lymph nodes (sDLNs). Local administration of the NK1R agonist during skin genetic immunizations increased significantly the expression of tg Ag by a mechanism involving the translocation of NF-κB into the nuclei of cutaneous DCs homing to sDLNs. Importantly, our immunization approach resulted in Th1 and Tc1 bias of effector T cells that supported cellular and Ab-mediated immune responses. We demonstrate that signaling skin cells via the NK1R provides the adjuvant effect which favors the immunostimulatory functions of LCs.

Introduction

Efficient vaccine approaches for tumors and infectious diseases are designed to induce $CD8^+$ T cytotoxic (CTL) -1 (Tc1) lymphocytes that are able to eliminate intracellular pathogens and neoplasic cells, and $CD4^+$ T helper 1 (Th1) lymphocytes that support cellular immunity and CTL memory.[288-293]

The most efficient immunization methods to generate Th1 and Tc1 immunity rely on the direct administration of Ag to the skin, a highly immunogenic organ populated by dendritic cells (DCs), including epidermal Langerhans cells (LCs), dermal DCs (DDCs), and a pool of DC precursors.[80,81,294] It is currently believed that, as professional APCs, skin-resident DCs are extremely efficient at (i) sensing the presence of pro-inflammatory mediators in their microenvironment; (ii) acquiring/processing extracellular Ag; (iii) transporting Ag from the skin to the skin draining lymph nodes (sDLNs); and iv) priming and biasing the differentiation of naïve $CD4^+$ and $CD8^+$ T cells into effector cells.[1,80,295]

In addition to their role during the initiation of acquired immunity, the skin cells and nerve endings release pro-inflammatory mediators including cytokines, hormones, and neuropeptides, which influence the immunostimulatory function of resident DCs.[295,296] Pro-inflammatory neuropeptides from the innate immune system are released immediately following skin injury. Interestingly, epidermal LCs make physical contact with peripheral nerve δ fibers that secrete Substance P (SP) and Calcitonin Gene Related Peptide (CGRP).[297,298]

SP and the more recently described hemokinin-1 (HK-1) belong to the family of pro-inflammatory neuropeptides known as tachykinins. SP and HK-1 bind with high affinity the neurokinin 1 receptor (NK1R) and with low affinity the NK2R and NK3R.[110,134,299,300] Binding

of the NK1R on naïve $CD4^+$ T cells promotes proliferation, Th1 differentiation, and cellular immunity, whereas binding the NK2R inhibits the development of inflammatory responses.[110,160,197,301,302] In the skin, signaling through the NK1R with SP or NK1R agonists prevents cutaneous tolerance induced by UV-B light and favors delayed type hypersensitivity (DTH) reactions through the release of IL-12p70, a potent Th1-biasing cytokine.[160,237] Together, these observations highly suggest that signaling via the NK1R during the initiation of skin-mediated immune responses will favor development of Th1 effector immunity.

Due to the accessibility of the skin immune system and the ease of targeting the epidermis, genetic immunizations by the gene gun (GG) have been used as an appropriate vaccination method to promote the generation of CTL immunity directed to transgenic (tg) Ag.[303-306] However, there is increasing evidence that skin GG immunizations administered without Th1-biasing adjuvants generates Th2 effector cells, which are unable to sustain cellular immunity and CTL memory.[293,307-310] The absence of Th1 responses following skin GG immunizations can be ascribed to either: i) lacking of a Th1-biasing adjuvant effect; or ii) delivery of tg Ag almost exclusively to epidermal LCs, which have been described recently as tolerogenic / anti-inflammatory skin DCs.[311-314] Thus, for the purpose of skin genetic immunizations it would be relevant to determine whether: (i) administration of pro-inflammatory Th1-Tc1-biasing adjuvants provide LCs with the appropriate stimulus to favor effector cellular immunity during GG immunizations; or (ii) genetic immunizations should target exclusively DDCs, described as the most potent skin resident APCs.[315-317]

In the present work, we tested whether local administration of an NK1R agonist provides a Th1 adjuvant effect to favor the ability of skin DCs to induce Th1-Tc1 immunity during skin GG immunizations. We used the synthetic NK1R agonist [$Sar^9Met(O_2)^{11}$]-SP (NK1R agonist), a

highly stable SP derivative that binds exclusively to the NK1R but not to NK2R or NK3R. We show that, after GG immunization, the expression of tg Ag was limited to the epidermis and sDLNs, and that the expression of tg protein was significantly increased by simultaneous administration of the NK1R agonist. Mechanistic studies demonstrated that the NK1R agonist promoted acute inflammation in the skin, which correlated with rapid migration of epidermal LCs and their increased homing to sDLNs. Molecular analysis revealed that the NK1R agonist favored the activation of skin LCs as determined by the high expression of cell-surface I-A^b molecules and the translocation of NF-κB into the nuclei of GG transfected LCs homed to sDLNs. Importantly, our immunization approach stimulated efficient Ag specific humoral as well as Th1/Tc1-biased and CTL responses that supported effector cellular immunity as demonstrated in vivo by DTH assays.

Material and Methods

Mice

Female C57BL/6 (B6) mice were purchased from The Jackson Laboratories (Bar Harbor, Maine) and used between the ages of 6 and 12 weeks. Mice were housed under specific-pathogen-free conditions and treated according to the University of Pittsburgh's institutional animal care guidelines.

Genetic immunization

A He-powered (300 psi) GG was used to deliver naked plasmid DNA precipitated onto 1μm gold particles (Bio-Rad Laboratories, Hercules, CA) to the skin of B6 mice, as previously described.[306] The following plasmids encoding reporter proteins or the model Ag chicken OVA under control of the human IE-CMV promoter used in this study have been previously described:[244] i) pCMV-Luc encoding firefly luciferase (Luc); ii) pCMV-EGFP encoding the enhanced version of the green fluorescent protein (EGFP); and iii) pCMV-OVA-TR encoding the membrane-bound form of chicken OVA. The plasmid pNFκB-Luc encoding Luc downstream of 4 tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter was purchased from Clontech (Mountain View, CA). Genetic immunizations with the GG were performed by delivery of 2 overlapping shots on the abdominal (shaved) skin. Where indicated, mice were pre-treated with the synthetic NK1R agonist [$Sar^{9}Met(O_2)^{11}$]-SP (BACHEM Bioscience Inc., King of Prussia, PA) or the non-peptide NK1R antagonist L733060 (Tocris Bioscience, Ellisville, MO) both at 10 nmol/100 μl PBS/per dose, administered i.d. locally immediately before immunization.

Luciferase assays

Detection of Luc was performed by luminescence in samples of skin of identical size, excised from the area transfected with the GG, and sDLNs. Luc assays were performed using the Luc Assay System (Promega, Madison, WI), as previously described.[244,303] The luminescence was

assessed in triplicates in 96-well plates using the Lmax luminometer (Molecular Devices, Sunnyvale, CA). Results were expressed as Relative Luminic Units (RLU) per each sample analyzed.

Histological analysis of skin and lymph nodes

For histological analysis, skin samples and LNs were fixed in 4% formaldehyde/PBS solution. Twenty four h later tissues were processed and embedded for H&E staining. Skin and LN sections were analyzed by light microscopy using an Axiovert microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Preparation of epidermal sheets and single cell suspensions from epidermis and lymph nodes

Epidermal and dermal sheets were obtained by incubating the dorsal half of the ears (free of cartilage) in 0.02M EDTA/PBS pH 7.5, for 2 h at 37ºC. After incubation, the epidermis was dissected from the dermis with fine forceps under a microscopic examination. Unlike enzymatic digestions, EDTA treatment of the skin allows a clean separation of the epidermal/dermal junction and allows the purification of LCs without contamination with DDCs. To obtain epidermal single-cell suspensions, the epidermis was dissected as described and incubated with 0.25% trypsin / EDTA (Gibco, Grand Island, NY) for 15 min at 37ºC with continuous shaking. Trypsin activity was neutralized with FBS and single cell suspensions were obtained by vigorous pipetting (4°C) followed by filtration through a 70 μm cell-strainer (Falcon, Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ). Single cell suspensions from LNs were prepared by

teasing apart the tissue with 18-gauge needles followed by incubation in 1mg/ml collagenase (Sigma, St Louis, MO) and 0.02 mg/ml DNase I (Roche), 30 min at RT. Further cellular LN dissociation was accomplished by adding 1M EDTA during the last 5 min of incubation followed by filtration through a 70 μm cell-strainer (Falcon, Becton Dickinson).

Immunofluorescence staining of skin samples and lymph nodes

For assessment of LCs in epidermis, epidermal sheets were fixed in cold acetone (15 min at -20°C), washed in PBS, blocked with 10% normal goat serum in PBS, and incubated with biotin anti-DEC-205 mAb (clone NLDC-145, Cedarlane Laboratories Ltd. Hornby, Ontario, Canada) followed by Cy3-streptavidin (Jackson ImmunoResearch, West Grove, PA). Epidermal sheets were mounted on slides with the dermal side up for microscopic evaluation.

For labeling of frozen tissues, samples of skin and LNs were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap frozen in pre-chilled methyl-butane (Sigma) and stored at -80°C until use. Cryostat sections (8 μm) were mounted onto slides pre-treated with Vectabond (Vector Laboratories, Burlingame, CA), air-dried, and fixed in cold 96% ethanol (10 min). Tissue sections were blocked with 10% normal goat or donkey serum in PBS and the avidin/biotin blocking kit (Vector). For co-detection of CD11c and NK1R in skin, cross-sections of mouse ear skin were incubated with biotin anti-CD11c mAb (BD Biosciences PharMingen, San Diego, CA) and rabbit anti-mouse NK1R polyclonal Ab (Advance Targeting Systems, San Diego, CA) followed by Cy2 anti-rabbit IgG and Cy3-streptavidin. For co-detection of CD11c and langerin in LNs, sections were incubated with biotin anti-CD11c and alexa fluor 488 anti-langerin mAb (BD-PharMingen) followed by Cy3-streptavidin. For detection of T cells in the

skin, cross-sections were incubated with alexa fluor 488 anti-CD4 mAb (Caltag, Burlingame, CA) and biotin anti-CD8α mAb (eBioscience, San Diego, CA) followed by Cy3-streptavidin. For detection of macrophages in skin, sections were incubated with biotin anti-F4/80 mAb (Caltag) followed by Cy3-streptavidin. Nuclei were stained with 4'6-diamidino-2-phenylindole 2HCl (DAPI; Molecular Probes, Eugene, OR). To identify lymphatic vessels expressing the chemokine CCL21/SLC in LNs, sections of sDLNs were incubated with the lymphatic vessel specific anti-LYVE-1 mAb and with biotin anti-CCL21/SLC (R&D Systems, Minneapolis, MN) followed by Cy3 anti-goat IgG and Cy2-streptavidin (Jackson ImmunoResearch).

Flow cytometric analysis of single cell suspensions

LC-enriched epidermal cell suspensions were blocked with 10% normal donkey serum and incubated (30 min, 4°C) with PE anti-CD11c mAb in combination with goat anti-mouse NK1R polyclonal Ab, recognizing the N-terminus epitope of the human and mouse NK1R (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) followed by FITC conjugated donkey anti-goat IgG, $F(ab')_2$ fragment (Jackson ImmunoResearch). Negative controls included cells incubated with goat serum followed by FITC anti-goat IgG $F(ab')_2$ fragment. LN cells were labeled with combinations of allophycocyanin anti-CD11c, PE anti-IA^b, FITC anti-CD11b mAbs, and alexa fluor 488 anti-langerin (CD207) polyclonal Ab. All Abs were purchased from BD-Biosciences PharMingen with the exception of langerin (e-Bioscience). Appropriate fluorochome-conjugated species and isotype-matched irrelevant mAbs were used as negative controls. After staining, cells were fixed with 2% paraformaldehyde and analyzed with a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA).

ELISA and ELISPOT assays

Analysis of OVA specific total IgG was performed by ELISA and the Th1 and Tc1 bias of OVA-specific $CD4^+$ and $CD8^+$ T cells were assessed by ELISPOT assays. For these experiments mice were GG immunized on the abdominal skin with pCMV-OVA-TR (1 priming + 2 boosting doses, 7 d apart) in the presence (or not, control) of the NK1R agonist.

For ELISA, 96 well EIA/RIA flat bottom plates (Costar, Corning, NY) were coated with 100 μg/ml OVA in a 0.05M carbonate-bicarbonate buffer (Sigma) (overnight at 4°C). Then, plates were washed in PBS supplemented with 0.05% Tween-20 (Sigma), blocked with 3% BSA (wt/vol) (2 h at RT) and 100 μl of serially-diluted serum samples or reference serum (diluted in 10% FBS) were added to the wells and incubated overnight at 4°C. The plates were then rinsed and incubated (1 h at RT) with 0.04 μg/ml of biotin goat anti-mouse IgG (Caltag) in 10% FBS/PBS. Plates were washed and 100 μl of an Avidin-HRP (BD-Pharingen) solution diluted in 10% FBS/PBS was added to the wells (30 min at RT). After rinsing, the plates were developed with the TMB system (Sigma). The reaction was stopped with 0.5M H_2SO_4 and read at 450 nm on the Emax microplate reader (Molecular Devices).

For ELISPOT assays, splenic $CD4^+$ and $CD8^+$ T cells (positively selected by magnetic sorting, Miltenyi Biotec, Auburn, CA) from B6 mice non-immunized (control) or GG-immunized with pCMV-OVA-TR (in the presence or absence of NK1R agonist) were used as responder cells. As stimulators, we used bone marrow (BM)-derived DCs generated in vitro as previously described.[244] Day 6 BMDCs were pulsed (2 h, 37ºC) with either (i) 100 ng/ml of the H-$2K^b$-restricted $OVA_{257-264}$ peptide (SIINFEKL); (ii) 10 μg/ml of the IA^b-restricted $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR); or left untreated (control). BMDCs were then γ-irradiated

and co-cultured with 2 x 10^5 $CD4^+$ or $CD8^+$ responder T cells, respectively (1:5 stimulator : responder cell ratio) in 96-well ELISPOT plates pre-coated with anti-IFN-γ or anti-IL-5 mAb (BD Biosciences). ELISPOT plates were cultured for 36 h at 37ºC followed by incubation with biotin anti-IFN-γ or biotin anti-IL-5 mAb, streptavidin-peroxidase and the substrate 3-amino-9-ethylcarbazole. Spots were quantified with an ImmunoSpot™ automated counter (Cellular Technology Ltd., Cleveland, OH).

In vivo killing assay

B6 mice were GG immunized (abdominal skin) with pCMV-OVA-TR (1 priming + 1 or 2 boosting doses, 7 d apart) in the presence (or not, control) of the NK1R agonist. Non-immunized mice were used as controls. Target B6 splenocytes were pulsed with SIINFEKL (0.25 μg/ml, 45 min, 37ºC) and labeled with 5 μM CFSE ($CFSE^{high}$). Control (non-pulsed) B6 splenocytes were labeled with 0.5 μM CFSE ($CFSE^{low}$). Five days following the last boosting dose, target cells ($CFSE^{high}$ SIINFEKL-pulsed B6 splenocytes) and control cells ($CFSE^{low}$ non-pulsed B6 splenocytes) were injected i.v. (2×10^7 cells in 300 μl PBS per mouse, 1:1 target : control cell ratio) into pre-immunized and control (untreated) mice. Four h later, the relative percentage of splenic $CFSE^{high}$ and $CFSE^{low}$ cells was assessed by flow cytometry. The percentage of specific cell lysis (%SCL) was then calculated by using the formula: %SCL= 100 × [1-($CFSE^{low}$: $CFSE^{high}$ of splenocytes from untreated mice) / ($CFSE^{low}$: $CFSE^{high}$ of splenocytes from immunized mice)].[43]

DTH assay

B6 mice were sensitized with the pCMV-OVA-TR delivered with the GG to the abdominal skin (1 priming + 2 boosting doses, 7 d apart). Sensitization was performed in the presence (or not, control) of the NK1R agonist or the non-peptide NK1R antagonist L733060 (Tocris Bioscience), both used at 10 nmol/100μl PBS/ per dose and administered i.d. locally immediately before immunization. Three d following the last immunization, mice were challenged with the pCMV-OVA-TR delivered with the GG to the dorsal surface of the right ear. Controls included mice sensitized with pCMV-Luc (irrelevant gene) and non-sensitized mice but GG transfected in the ear with pCMV-OVA-TR 24h before ear measurement. The thickness of the right (challenged) and left ear pinna (control) was measured with a digital caliper (Mitutoyo Corp, Aurora, IL) at different time points. The severity of the DTH response was assessed by the swelling of the challenged ear (right) compared with the thickness of the control ear (left) in sensitized animals and was expressed as percentage increase in ear thickness based on the formula: [(thickness of challenged ear - thickness of control ear) / thickness of control ear x 100]. In some experiments, mice were injected with the anti-CD154 blocking mAb MR1 (a single 250μg dose, BIO Express, West Lebanon, NH) administered i.p. at the time of immunizations.

Statistical analysis

Results from multiple different groups were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Comparison of 2 groups was performed by a 2-tailed Student's T-test. A p value of < 0.05 was considered statistically significant.

Results

Immunization using the GG restricts expression of tg Ag to epidermis and sDLNs

The individual contributions of LCs and DDCs to the outcome of cutaneous immunity remain controversial. Recent studies indicate that DDCs and lymph node-resident CD8$\alpha\alpha^+$ DCs are the main APCs that trigger T cell immunity following skin Ag insult, whereas epidermal LCs play tolerogenic / anti-inflammatory roles.[88,312-315,189,318-320] Therefore, we analyzed whether tg Ags encoded by plasmid DNA (pDNA) and GG-delivered to the skin (ear) of mice were expressed in the epidermis and/or in the dermis wherein LCs and DDCs reside, respectively. The anatomical localization of the 1μm gold particles in the skin was investigated immediately after treatment with the GG. This strategy allowed us to analyze the original distribution of the particles in the skin before mobilization of epidermal LCs containing gold particles, through the dermal lymphatic vessels. Histological analysis of cross-sections of skin demonstrated the presence of gold particles mainly in the epidermis, with very few beads at the epidermal-dermal junction however, no particles were found in the dermis (Fig. 1A).

Next, we assessed the expression of the reporter protein EGFP in epidermis and dermis following delivery of pCMV-EGFP by the GG. Twenty four h after transfection, the expression of EGFP was detected exclusively in the epidermis (Figs. 1B-D). This result was further confirmed by quantification of the reporter protein Luc in the whole skin, as well as in separated epidermal and dermal layers of mouse skin transfected by the GG with the pCMV-Luc or with a control plasmid encoding no transgene (pBackbone). High levels of Luc were detected in the cell lysates prepared from whole skin (Fig. 1E) as well as in those obtained from epidermal sheets (Fig. 1F). However, the levels of Luc in the dermis remained low and were similar to those detected in dermis from control mice transfected with pBackbone (Fig. 1F).

Our laboratory and others have shown that treatment of the skin with the GG results in a rapid migration of cutaneous DCs to the sDLNs.[303,305,321] As a consequence, reporter proteins encoded by pDNA delivered by the GG were detected in the sDLNs as soon as 24 h after cutaneous immunization.[305] However, it is unknown for how long these tg proteins are being expressed in the skin and sDLNs, a relevant aspect for the purpose of genetic immunization. Thus, we delivered pCMV-Luc to abdominal skin of mice and we analyzed the levels of expression of tg Luc in skin and inguinal (local) sDLN cells up to 7d following cutaneous immunization by the GG. The levels and length of expression of Luc observed in the skin (Fig. 1G) correlated with the levels and length of Luc expression in the sDLNs (Fig.1H). The highest expression of Luc in the sDLNs was observed 24 h after immunization and decreased but was still detectable up to 4 d (Fig. 1H). Luc expression in cervical sDLNs situated distant from the area of gene delivery remained low and was similar to background levels observed in sDLNs

after transfection of the skin with pBackbone (negative control). Together these results demonstrate that tg Ags delivered to the skin by the GG are expressed up to 3-4 d by cells of the epidermis and by migratory cells that traffic from the epidermis to sDLNs.

LCs and DDCs constitutively express NK1R

Our goal was to test whether the NK1R agonist SarSP enhances the ability of skin DCs to promote the differentiation of Th1 and Tc1 cells following vaccination by the GG, therefore, we analyzed the expression of NK1R in skin cells from untreated mice. By double-immunofluorescence microscopy of cross-sections of skin and using CD11c as a murine DC specific marker, we demonstrated that the NK1R was constitutively expressed by CD11c positive epidermal LCs and DDCs, as well as by CD11c negative cells including keratinocytes, and some cells located in the dermis (likely, mast cells) (Fig. 2A-F). Expression of NK1R on the surface of CD11c positive LCs was further confirmed by flow cytometric analysis of epidermal cell suspensions enriched in LCs (Fig. 2G).

Signaling via NK1R triggers skin acute inflammation and enhances transgene expression

It is our hypothesis that signaling via the NK1R at the time of GG immunization triggers acute inflammation in the skin and therefore provides the appropriate "danger signal" to promote the activation and migration of epidermal LCs transporting tg Ags to the sDLNs. Therefore, we assessed the damage caused to the skin by the NK1R agonist or by the GG in combination (or not, control) with the NK1R agonist administered by local i.d. injection immediately before

immunization. The NK1R agonist alone induced a moderate acute inflammatory infiltrate composed mainly of polymorphonuclear granulocytes (PMNs) in the dermis of treated mice (Fig 3B). Whereas, fewer PMNs were observed infiltrating the epidermis and in the papillary dermis of mice treated with GG alone compared to untreated skin (Fig 3A and 3C). By contrast, immunization with the GG combined with the NK1R agonist triggered a severe and extensive PMN infiltrate in the epidermis and dermis of mouse skin (Fig. 3D).

Next, we tested whether administration of the NK1R agonist affects the level of expression of tg Ag in skin and sDLNs induced by the GG. To address this question, mice were transfected with pCMV-Luc in the skin (abdomen) with the GG in the presence (or not, control) of the NK1R agonist. Injection of the NK1R agonist in combination with the GG increased significantly the expression of Luc in the skin compared to the skin of mice treated with the GG alone (Fig. 3E), as determined by luminometry in cell lysates obtained from identical sized whole skin samples. Importantly, treatment with the GG and the NK1R agonist resulted in a 6-fold increase of Luc expression in the sDLNs compared to the expression observed in sDLNs obtained from mice treated with the GG alone (Fig. 3F). Luc expression in the sDLNs induced by the GG alone diminished significantly after local administration (i.d.) of the NK1R antagonist L733060. This latter result indicates that the GG exerts, to some extent, an intrinsic NK1R agonistic activity, likely through local release of endogenous SP in the skin.

Signaling via NK1R favors homing of activated LCs and expression of tg proteins in sDLNs

GG-transfection of the skin triggers activation and migration of LCs to sDLNs.[303,305,306] In response to DC-activation signals, LCs translocate NF-κB into the nuclei, a phenomenon that triggers LC maturation and enhances the expression of those transgenes controlled by hIE-CMV promoter.[243,244,322] Therefore, the augmented expression of tg proteins in the sDLNs that follows skin GG-transfection with the NK1R agonist may be ascribed to a higher number of activated LCs expressing transgenes and mobilized to the sDLNs. Thus, we analyzed i) the mobilization of epidermal LCs to sDLNs; and ii) the expression of tg Luc in sDLNs dependent on NF-κB translocation by migrating LCs that follows skin GG immunization in the presence of the NK1R agonist.

First, we assessed whether the NK1R agonist decreases the number of LCs resident in the epidermis 24 h after i.d. injection in the presence or absence of GG transfections of the skin (Fig.4A-E). The NK1R agonist alone induced a significant decrease in the number of LCs in epidermal sheets compared to control skin samples ($p<0.001$) (Fig 4A and 4B). GG transfections alone triggered a highly significant reduction of the density of LCs (> 90%, from 1,350±90 to 110±30 and LCs /mm^2 of skin, $p<0.0001$) (Fig. 4C). As expected, this pronounced mobilization of epidermal LCs could not be further augmented by GG plus NK1R agonist treatments (Fig. 4C-E).

Nevertheless, the fact that 90% of LCs diminished from the epidermis following GG treatments does not necessarily imply that all these cells will home efficiently to the sDLNs. Indeed, it has been shown that after GG or i.d. administration of DC-based vaccines only a small percentage of cutaneous DCs reach the local sDLNs.[321,323] The NK1R agonist is a pro-

inflammatory neuropeptide that even when administered locally (i.d.), might elicit functional changes in the skin and sDLNs, which may enhance the "danger-induced" chemotaxis of skin LCs, a phenomenon that is highly relevant for the outcome of skin genetic immunizations. We hypothesized that the intense inflammation induced in the skin following GG immunizations in the presence of NK1R agonist might augment the number of lymphatic endothelial cells expressing the chemokines known to attract LCs to the sDLNs. Therefore, we analyzed the presence of lymphatic vessels (identified by the specific lymphatic endothelial cell marker LYVE) expressing the DC chemoattractant CCL21/SLC under our experimental conditions. Twenty four h following GG immunizations, we observed an expansion of sub-capsular LYVE-1^+ lymphatic vessels co-expressing CCL21/SLC compared to sDLNs isolated from naïve mice (Fig. 4F). Importantly, administration of NK1R agonist together with the GG resulted in a significantly increased expansion of lymphatic vessels expressing CCL21/SLC throughout the whole parenchyma of the sDLNs (Fig. 4F). These results highly suggest that the combination of GG and the NK1R agonist promotes a more efficient homing of skin LCs to sDLNs by increasing the number of lymphatic endothelial cells expressing CCL21/SLC.

To test this hypothesis we further addressed the homing of LCs to sDLNs under our experimental conditions. First, we analyzed the inflammatory changes induced by the GG and the NK1R agonist in sDLNs. As shown in Figure 5; 24 h after skin treatment with the GG or the NK1R agonist we observed sinus hyperplasia limited to the subcapsular and paracortical areas of sDLNs (Fig. 5B and 5C). Following the combination of the GG with the NK1R agonist the sinus hyperplasia replaced a high percentage of the normal histological architecture observed in sDLNs of untreated mice (Fig. 5A and 5D). These changes were characterized by infiltration of the subcapsular and paracortical areas of the sDLNs with cells having abundant pale cytoplasm,

some of them with indented peripheral nuclei and dendritic membrane processes (Fig. 5B and 5C). Importantly, after GG treatment alone or in combination with NK1R agonist several cells with DC morphology contained 1μm gold beads, a further indication that these cells were mobilized from the skin (Fig 5C and 5D, insets).

A phenotypic analysis performed in sections of sDLNs, 24 and 48 h after treatments, demonstrated that the GG or the NK1R agonist by themselves increased significantly the population of DCs co-expressing CD11c (red) and langerin [CD207, (green)] situated in the subcapsular and in the paracortical areas of sDLNs compared to the population of CD11c and langerin double-positive cells present in the sDLNs of untreated animals (Fig. 5 E-H). A combination of GG and NK1R agonist further increased the number of CD11c and langerin double-positive cells in sDLNs compared to NK1R agonist or GG treatments administered alone (Fig. 5H). Few $CD11c^+$ DCs expressing langerin were detected in control inguinal LNs of non-immunized mice or in mesenteric LNs (non-sDLNs) of mice immunized in the abdomen with GG plus the NK1R agonist (data not shown).

To further determine whether treatment with the NK1R agonist facilitates the homing of LCs and/ or DDCs to sDLNs, we quantified by flow cytometry the percentage of both migratory skin DC subsets, LCs [$CD11c^+CD11b^+$ MHC-II $(IA^b)^{high}$, $langerin^+$] and DDCs ($CD11c^+$ $CD11b^+$ $MHC\text{-}II^{high}$ $langerin^-$) present in the sDLNs 24, 48, and 96 h following skin treatments. Based on previous publications the following markers were used to identify LCs from DDCs and other DC populations homing to sDLNs:[315,311] i) surface CD11c (a pan DC marker in mice); ii) surface CD11b (expressed by myeloid DCs and not by $CD8\alpha\alpha^+$ DCs); iii) surface $MHC\text{-}II^{high}$ (DC activation marker highly expressed by skin derived DCs), and iv) intracellular langerin [CD207 (expressed by LCs and not by DDCs)]. Skin migratory LCs, expressing CD11c, CD11b, MHC-

II^{high} and langerin, were the main skin DC subset that increased in the sDLNs after GG immunization in the presence of the NK1R agonist (Fig.5I). Langerin negative skin migratory DDCs expressing CD11c, CD11b, and MHC-II ^{high} were increased following GG treatments alone but reduced following GG plus NK1R agonist treatments. The high increase of LCs homing to sDLNs was observed 24 h after skin treatment and decreased gradually thereafter (48 and 96 h follow up, data not shown). Additionally, a population of langerin^{+} CD11b^{-} DCs were detected in the sDLNs (Fig. 5I), which was not affected by any treatment group. These cells likely represent the population of CD8αα^{+}langerin^{+} LN resident DCs previously described as blood-derived langerin^{+} DCs.[315] Together these results indicate that the GG in combination with i.d. injections of the NK1R agonist preferentially favors the LN-homing of activated LCs, which are the only skin DC-subset expressing tg Ag under our experimental conditions.

To further confirm that the enhanced expression of transgenes observed was dependent on the activation state of LN-homing LCs, which have translocated NF-κB into their nuclei, we GG- transfected the skin (abdomen) of mice with the plasmid pNFκB-Luc encoding Luc under control of a promoter containing upstream 4 tandem copies of the NF-κB consensus sequence. Mice were sacrificed 24 h later and Luc assays were performed on sDLN cells. GG transfections of mouse skin i.d. injected with the NK1R agonist resulted in a 9-fold increase in NF-κB promoter activity in sDLNs, compared to Luc expression in sDLNs of control mice transfected with GG alone (Fig. 5J). This latter result further confirms that the addition of NK1R agonist to GG immunizations enhances transgene expression in sDLNs by favoring the arrival of LCs from skin transfected sites. These LCs were highly activated and consequently have translocated efficiently NF-κB into their cell nuclei.

Signaling via NK1R enhances OVA-specific IgG production and secretion of IFN-γ by T cells stimulated by cutaneous GG immunizations

Our results indicate that genetic immunizations of the skin with the GG in combination with the NK1R agonist (i) targets epidermal LCs; (ii) increases the homing of epidermal LCs to the sDLNs; and (iii) enhances the capability of migrating LCs to express tg proteins driven by promoters with NF-κB responsive elements (i.e. CMVp). An important function of an adaptive immune response is the production of Ag-specific Igs particularly relevant for the purpose of anti-viral vaccines. Thus, we analyzed whether the NK1R agonist favors high levels of Ag specific IgG. For this purpose mice were immunized with the GG on the abdominal skin (1 priming + 2 boosting doses, 7d apart) with pCMV-OVA-TR in the presence (or not, control) of the NK1R agonist. The titer of OVA-specific IgG was assessed in mouse serum 5 d following the last immunization dose. GG immunizations alone increased the production of OVA-specific IgG (detected at a dilution of 1:1,000) (Fig. 6A and 6B), whereas the administration of the NK1R agonist during GG immunizations significantly increased the production of OVA-specific IgG (detected at a dilution of 1:16,000) (Fig. 6A and 6B).

To further analyze the adjuvant effects of the NK1R agonist we investigated its ability to promote development of Th1- and Tc1-biased immunity following GG immunizations. Five d after the second boost, the frequency of purified splenic $CD4^+$ and $CD8^+$ T cells secreting IFN-γ or IL-5 in response to re-stimulation with syngeneic bone-marrow DCs pulsed with either IA^b-$OVA_{323-339}$ or H-$2K^b$-$OVA_{257-254}$ restricted peptides, was measured by ELISPOT assays. The NK1R agonist significantly increased the number of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells when compared to T cells from mice immunized with GG alone (Fig. 6C and 6D). Secretion of

IL-5 remained at background levels in all groups (Fig. 6C and 6D). As controls, splenic $CD4^+$ and $CD8^+$ T cells from untreated mice or mice immunized with the irrelevant pCMV-Luc did not secrete detectable levels of IFN-γ or IL-5 in response to re-stimulation (data not shown). Additionally, we tested whether the NK1R agonist augmented the capability of skin GG immunizations to promote the development of $CD8^+$ effector T cells with cytotoxic function by in vivo killing assays. B6 mice were GG-immunized on the abdominal skin with pCMV-OVA-TR as aforementioned, in the presence (or not, control) of the NK1R agonist. Five d following the last immunization, target cells ($CFSE^{high}$ SIINFEKL-pulsed B6 splenocytes) and control cells ($CFSE^{low}$ non-pulsed B6 splenocytes) were adoptively transferred (i.v.) into immunized mice. Four h later the relative percentage of splenic $CFSE^{high}$ and $CFSE^{low}$ cells was assessed by flow cytometry. The percentage of specific cell lysis (%SCL) was then calculated by using the formula: $\%SCL = 100 \times [1-(CFSE^{low} : CFSE^{high}$ of splenocytes from untreated mice) / ($CFSE^{low}$: $CFSE^{high}$ of splenocytes from immunized mice)].

Induction of robust CTL responses by genetic immunizations delivered with the GG require an aggressive immunization scheme consisting of one priming dose followed by two or more boosting doses.[306] Using such an immunization protocol, under our experimental conditions, the GG induced a robust CTL function in vivo (as high as 79% of Ag-specific cell killing), which was augmented by combining GG with the NK1R agonist (as high as 92% of specific cell killing) (not shown). To further determine whether the NK1R agonist adjuvant activity favors robust CTL responses during a suboptimal GG immunization protocol we analyzed the Ag-specific CTL function induced in B6 mice immunized by one priming and one boosting dose in the presence or absence of NK1R agonist. Under these suboptimal

immunization conditions the NK1R agonist induced a significantly higher CTL response compared to GG immunizations alone (Fig. 6E) demonstrating that the NK1R agonist promotes both Th1 and CTL-Tc1 immune responses.

Signaling via NK1R favors skin effector cellular immune responses

The potential of the NK1R agonist to promote differentiation of effector T cells under our experimental conditions was investigated by DTH assays. Mice were immunized on the abdomen with pCMV-OVA-TR delivered by GG immunizations in the presence (or not, control) of the NK1R agonist. Three d after the last immunization, DTH responses were elicited by GG delivery of pCMV-OVA-TR to the ear pinna. The effector cellular immune response was assessed by measuring the increase of ear thickness 24, 48, and 72 h following elicitation. Negative controls included mice immunized with pCMV-Luc (irrelevant gene) and animals not sensitized but elicited with pCMV-OVA-TR 24 h before measuring of ear thickness. Immunization with the GG in the presence of the NK1R agonist increased significantly the intensity of the DTH reaction compared to GG immunizations alone (Fig. 7A). Interestingly, the DTH response elicited by the GG was almost completely abrogated by the NK1R antagonist L733060 administered i.d., indicating that in the absence of exogenous [$Sar^{9}Met(O_2)^{11}$]-SP, immunization of the skin with the GG stimulates the release of low levels of endogenous NK1R agonists (likely SP) (Fig. 7A). Likewise, blockade of CD40-CD40L interaction by the neutralizing Ab CD154 (MR1), injected i.p. before immunizations, abrogated completely the induction of DTH responses (not shown). This result indicates the relevance of $CD4^{+}$Th cells for the effector cellular immunity induced by our immunization approach.

The characterization and severity of the cellular immune infiltrate of DTH responses were assessed in ear skin sections obtained 72 h after elicitation. Histological analysis demonstrated a dramatic increase in the thickness of the epidermis and dermis in those animals immunized with GG in the presence of NK1R agonist compared to GG alone or control groups (Figs. 7B-E). Increase of skin thickness correlated with the presence of severe cellular infiltrates composed of mononuclear cells localized mainly in the epidermis where the tg Ag was expressed (Fig. 7E). Compared to control mice, GG alone induced significant mononuclear inflammatory infiltrate, which localized mainly in the dermis (Fig. 7B-D). Phenotypic analysis by immunofluorescent microscopy demonstrated that the inflammatory infiltrate observed with GG in the presence or absence of the NK1R agonist was a Th1 mediated DTH response, composed mainly of $CD4^+$ and $CD8^+$ T cells and macrophages (Fig. 7F-K). Importantly, quantification of the inflammatory infiltrate demonstrated a significantly higher number of $CD8^+$ T cells and macrophages in the skin of mice treated with GG and NK1R agonist compared to GG alone (Fig. 7L).

Discussion

Rational vaccine designs must induce long-lasting Th1- and Tc1-biased immunity, both critical for eradication of intracellular pathogens and tumor cells. Skin genetic immunizations have been proven to elicit potent T cell immune responses characterized by high levels of transgene expression combined with rapid activation and mobilization of cutaneous DCs. For genetic immunizations in vivo, delivery of pDNA is preferred over recombinant vectors, which provide the immune system with additional immune-dominant epitopes.[244,323-325] Using the GG as a

method to deliver pDNA to the skin results in high efficient transfection of epidermal cells.[303,305,306,326] Importantly, our laboratory and others have demonstrated that cutaneous immunizations with the GG are followed by CTL responses that depend on the activation and migration of directly transfected DCs as well as of DCs that have acquired tg proteins from neighboring cells. Nevertheless, regardless of the ability of the GG to promote CTL differentiation, the critical development of a Th1-biased immune response necessary to support cellular immunity and CTL memory after GG immunization is, to some extent, lacking.[307,309,310,327]

In the present work, we have investigated two of the main reasons by which cutaneous immunizations with the GG fail to induce efficient cellular immunity: i) the possibility that the GG transfects mostly epidermal LCs that, unlike the DDCs, have been recently described as APCs with regulatory function;[88,315,317,323] and ii) the lack of or insufficient Th1-biasing adjuvant effect.

Currently, the individual contributions of epidermal LCs and DDCs to the outcome of skin related immune responses are controversial. Although, several studies have described LCs as potent APCs,[328-333] recent observations propose a rather tolerogenic role for LCs. Utilizing pathogenic infections or LC-depleted tg mice recent studies implicate LCs as having an immune suppressive role and they point toward DDCs and CD8$\alpha\alpha^+$ blood-derived DCs as the main inducers of efficient antiviral, allogeneic, and hypersensitivity immune responses.[88,315,318-320,323]

Different from these observations, our results demonstrate that GG-immunization delivers pDNA to the epidermis and support the idea that epidermal LCs, instead of DDCs, are the main skin DCs transfected by the GG. Epidermal LCs loaded with tg Ag migrated rapidly to the sDLNs, as shown by the presence of DCs containing gold particles and by the expression of

the intact tg proteins in the inguinal LNs of mice GG-immunized on the abdomen 24 h earlier. The possibility that the tg protein expression observed in sDLNs may have been caused by free naked DNA or secreted tg protein mobilizing from the skin via lymph is very unlikely. The GG delivers pDNA either into the cytoplasm/nucleus of cells or in the extracellular compartment. pDNA delivered to the extracellular compartment is highly unstable and becomes degraded by tissue DNAses soon after being released.[334,335] Under our experimental conditions the possibility that tg protein secreted by transfected keratinocytes could have reached the sDLNs has been excluded since tg Luc remains intracellular and cervical sDLNs distant from the skin transfected area did not express tg proteins.

Our results demonstrated that, following a rather aggressive protocol of GG immunizations, targeting exclusively the epidermis induced a robust CTL response accompanied by a weak Th1 biasing of $CD4^+$ T cells, an indication that under our experimental conditions LCs are immunostimulatory rather than tolerogenic APCs. Thus, the second question addressed was whether a potent pro-inflammatory Th1-biasing mediator administered during GG immunizations enhances the ability of LCs to generate/amplify Ag-specific $CD4^+$ Th1 responder cells and the CTL function of $CD8^+$ Tc1 cells**.** We chose the pro-inflammatory neuropeptide NK1R agonist SarSP as a Th1-Tc1 biasing adjuvant based on the fact that signaling T cells via the NK1R favors Th1-biased responses and local injection of NK1R agonist prevents the tolerogenic effects exerted by irradiation of the skin with UV-B light.[237] In addition, the NK1R agonist mimics the endogenous secretion of SP during the initial stages of acute inflammation of the skin.[106,336] Likewise, our results demonstrated that the NK1R agonist increased the ability of the GG to trigger acute inflammation of the skin at the site of immunization and favored a more effective homing of activated epidermal LCs to sDLNs.

Although the pro-inflammatory effects that follow cutaneous administration of the NK1R agonist can be attributed to the secretion of mediators by mast cells and keratinocytes as previously described,[337,338] other reports[201] have shown that murine DCs in secondary lymphoid organs and commercially available human DCs express NK1R. Importantly, our data demonstrate for the first time, constitutive expression of the NK1R by murine LCs indicating that signaling LCs directly via the NK1R may affect their APC function and ability to express transgenes. Accordingly, neuropeptides such as SP and CGRP, secreted by cutaneous δ fibers, exert important immune-modulation of LCs[296] and previous reports[237,339] have shown that the NK1R agonist down-regulates the migratory function of LCs. However, our results clearly indicate that the NK1R agonist triggers a massive and rapid LC mobilization out of the epidermis. These discrepancies can be ascribed to the use of different experimental models and time points analyzed. Nevertheless, in our studies we should not overlook that besides epidermal LCs, keratinocytes and skin-resident leukocytes (i.e. mast cells, DDCs) might also be direct targets of the NK1R agonist. In this scenario, the beneficial effects of the NK1R agonist on GG immunizations might be the result of the release of inflammatory mediators by by-stander cells combined to the direct signaling of LCs during the initiation of the acute skin inflammatory response.

The observation that local administration of the NK1R antagonist L733060 decreased the ability of the GG immunization to induce the expression of transgenes in skin and sDLNs and to promote a potent DTH response against the tg Ags indicates that the GG treatment by itself releases endogenous NK1R agonists, likely SP. However, the levels of endogenous NK1R agonist released by GG immunizations alone were not sufficient to trigger the inflammatory changes observed in the skin and sDLNs after administration of the NK1R agonist alone or in

combination with the GG. Accordingly, administration of exogenous NK1R agonist induced the highest expression of transgenes driven by transpromoters containing NF-κB responsive elements (i.e. CMV promoter) by migrating LCs.[244,322] This phenomenon was due to a combination of the following factors: (i) a high level of activation of LCs, which have translocated NF-κB into their nuclei and (ii) an efficient homing of LCs to sDLNs. The rapid homing of LCs to the sDLNs observed here, is in contrast to previous reports, describing a slow migration of LCs even 96 h after treatment of the skin with an irritant and the sensitizer tetramethylrhodamine-5-(and 6)-isothiocyanate.[315] In this regard, we have previously shown that the treatment of the skin with the GG induces a rapid and massive mobilization of human epidermal LCs that begins as soon as 3 h following GG treatment.[303] Accordingly, Porgador et al.[305] have demonstrated homing of directly transfected DCs expressing tg reporter proteins in sDLNs 24 h following GG immunization of mouse skin. Moreover, the present work shows that the pro-inflammatory effects of the NK1R agonist combined with the GG induced immunologically relevant changes in sDLNs as demonstrated by significant expansion of lymphatic vessels expressing CCL21/SLC and by extensive sinus hyperplasia. Thus, we can conclude that the rapid mobilization of LCs from the epidermis to sDLNs observed in our study was caused by a combination of factors including the damage triggered by the GG in the skin and the pro-inflammatory effects exerted by the NK1R agonist in the skin and sDLNs. In agreement with our data, a recent report[340] has demonstrated a higher DC mobilization from the periphery into the sDLNs following inflammatory expansion of lymphatic vessels after skin treatments with keyhole limpet haemocyanin in the presence of CFA.

The NK1R agonist neuropeptide [$Sar^{9}Met(O_2)^{11}$]-SP meets the requirements for a Th1-Tc1-biasing adjuvant while also favoring the elicitation of Ab responses. Our data demonstrates that GG immunizations induced the secretion of Ag-specific IgG as well as cellular immunity mediated by $CD8^+$ and $CD4^+$ T cells. Importantly, these immunological responses were greatly increased by the presence of NK1R agonist as demonstrated by the significantly higher number of IFN-γ secreting Ag-specific $CD4^+$ Th1 and $CD8^+$ Tc1 cells. Moreover, the NK1R agonist was able to trigger strong CTL function of $CD8^+$ T cells in vivo following a suboptimal GG immunization protocol. Importantly, the adjuvant effect of the NK1R agonist further tested by DTH assays demonstrated that local administration of the NK1R agonist during sensitization favored the generation of Th1 effector immunity, an effect confirmed by the intense cutaneous infiltrate of $CD8^+$ T cells and macrophages during the elicitation phase.

To our knowledge this is the first study that has tested the ability of an NK1R agonist to enhance the generation of Ag-specific humoral and cellular Th1 and Tc1 immune responses elicited by GG immunizations of the skin. The synergistic effect of the NK1R agonist on cutaneous GG immunization was mediated, at least in part, by inducing inflammatory changes in the skin and sDLNs. The use of the synthetic NK1Ra such as SarSP seems to represent a safe adjuvant to promote Th1-Tc1 mediated cell immunity Our results strongly support the concept that under the appropriate pro-inflammatory environment, epidermal LCs are capable of eliciting potent T cell immune responses, which can be exploited for vaccine development.

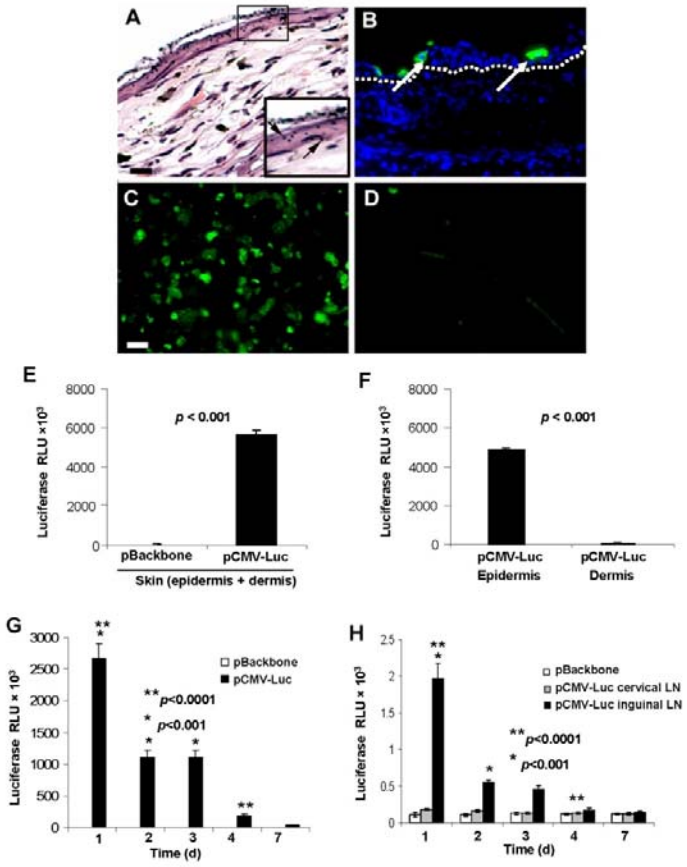


Figure 1. Immunization with the GG limits the expression of tg Ag to the epidermis and the sDLNs. (A) Cross-section of skin (ear), obtained immediately after GG treatment, showing 1μm gold particles located in the epidermis. Inset: detail of gold particles (arrows) within the epidermis. (**B)** Cross-sections of skin (ear) showing the expression of tg EGFP (green) by epidermal cells (arrows), 24 h after GG delivery of pCMV-EGFP. Dotted line in **B** indicates the epidermal-dermal junction. **(C and D)** Epidermal **(C)** and dermal **(D)** sheets obtained 24 h after GG delivery of pCMV-EGFP demonstrates the expression of EGFP (green) by epidermal cells. **(E)** Quantification of tg Luc in samples of skin (ear) GG-transfected with pCMV-Luc 24 h earlier. The plasmid pBackbone (encoding no transgene) was used as control. **(F)** Assessment of tg Luc in epidermal and dermal sheets dissected from skin (ear) 24 h after GG transfection with pCMV-Luc. Means ±1 SD of RLU of triplicates are illustrated. Data are representative of 3 independent experiments. **(G and H)** Kinetics of tg Luc expression by cells from the epidermis (abdomen, G) and sDLNs (H) following GG delivery of pCMV-Luc. LNs analyzed included sDLNs from transfected area (inguinal, black), control sDLNs from non-transfected areas (cervical, gray), and control sDLNs from skin areas transfected with pBackbone (white). Means ±1 SD of RLU of triplicates are shown. Data are representative of 5 independent experiments. **(A)** H&E staining, (**B-D)** immunofluorescence. Magnification: 200X. Inset: 1000X.

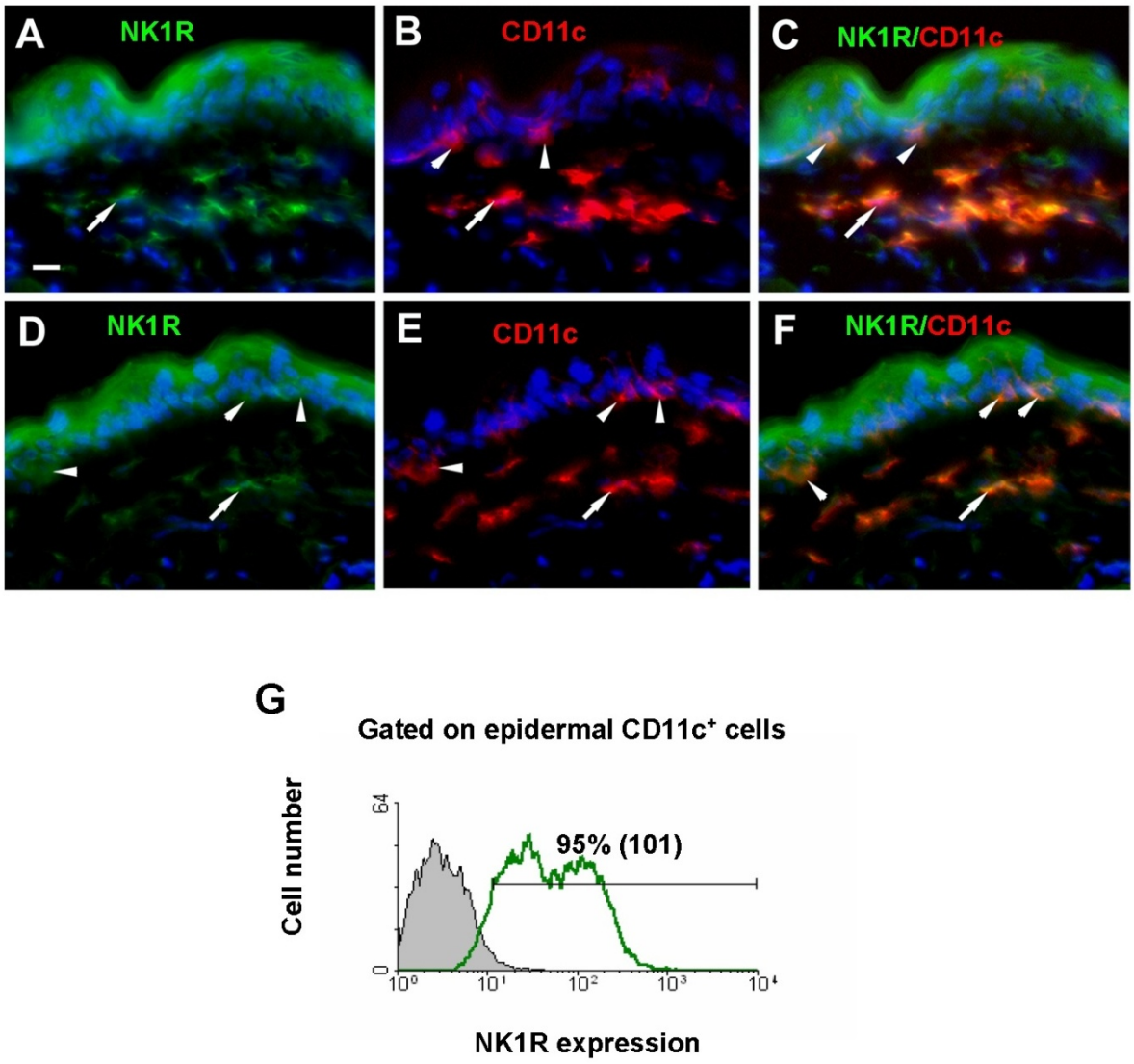
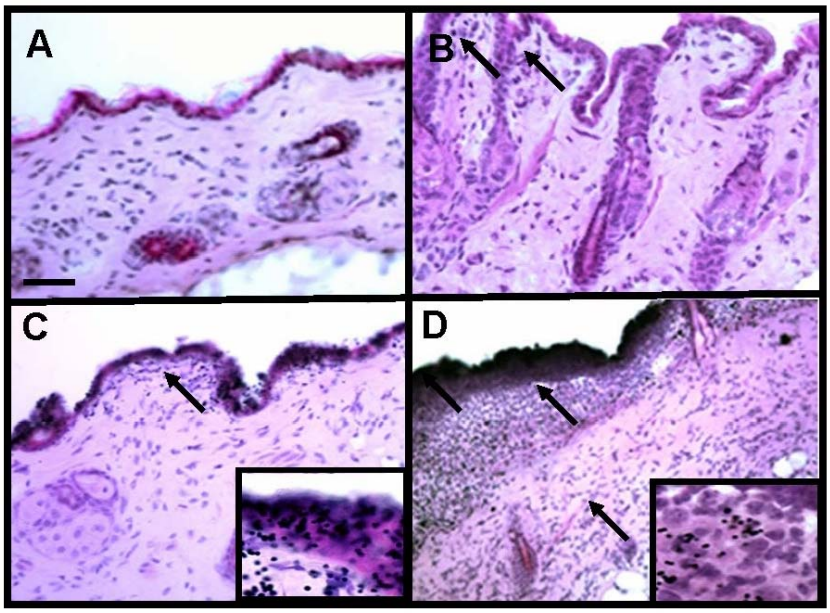


Figure 2. Skin DCs constitutively express the NK1R. Panels **(A-C)** and **(D-F)** are cross-sections of skin (ear) from two independent experiments showing the expression of the NK1R (green) by keratinocytes, epidermal LCs (arrowheads), and DDCs (arrows) (the latter two identified by their expression of CD11c in red). In **(C and F)** the yellow fluorescence is due to the overlap of red (CD11c) and green (NK1R). Cell nuclei were counterstained with DAPI (blue). Immunofluorescence, magnification: 200X. **(G)** The green histogram demonstrates the expression of NK1R by freshly-isolated LCs gated on CD11c expression. The grey histogram corresponds to negative control cells. The numbers in the histogram represents the percentage of NK1R positive LCs and the mean fluorescent intensity (between parenthesis). Data are representative of 2 independent experiments.



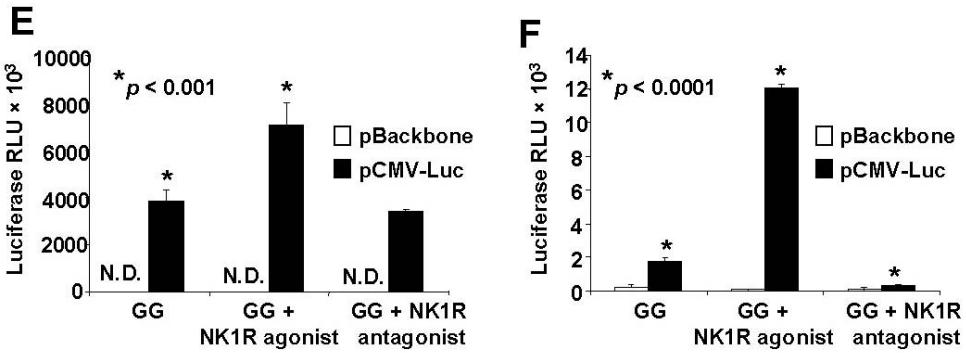


Figure 3. GG immunizations with the NK1R agonist triggers skin acute inflammation and increases the expression of tg Ag. (A-D) Cross-sections of skin (abdomen) obtained from mice untreated **(A)** and experimental animals 24 h after administration (i.d.) of the NK1R agonist **(B)**, immunization with GG **(C),** or treatment with GG plus NK1R agonist **(D)**. The arrows indicate the localization of the acute cellular infiltrate composed mainly of PMN cells. The insets detail the characteristics of the acute inflammatory infiltrate. H&E staining, magnification: 200X. Insets: 1000X. **(E and F)** Expression of tg Luc in the skin **(E)** and sDLNs **(F)** 24 h following GG delivery of pCMV-Luc in the presence (or not, control) of the NK1R agonist or the NK1R antagonist. Data are representative of 4 independent experiments. **(E and F)** Results are expressed as mean RLU ± 1 SD of the fold-increase compared to background levels obtained from non-immunized control mice. N.D.: Non-detected.

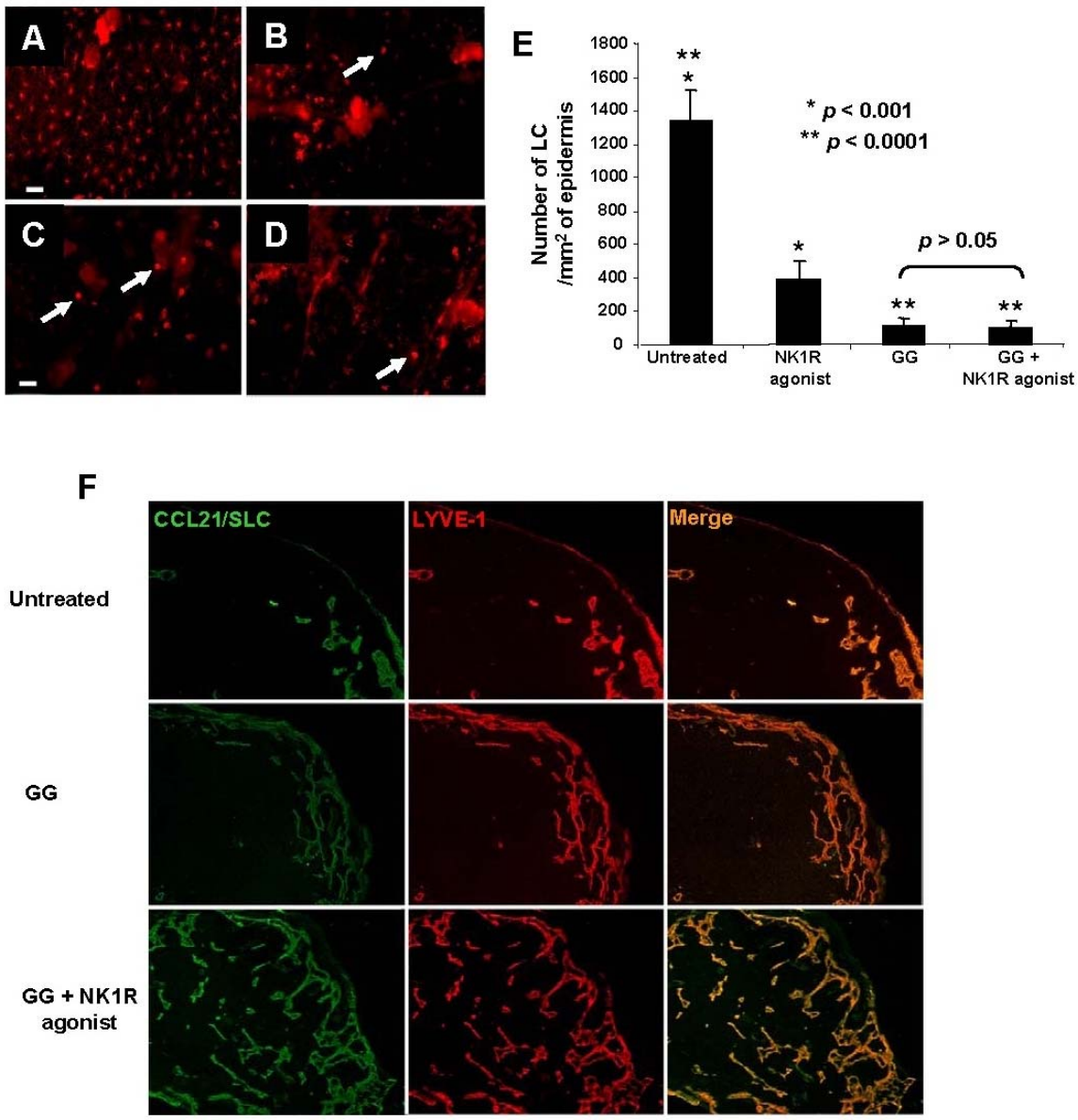


Figure 4. Effects of skin genetic immunizations with the GG and the NK1R agonist on migration of epidermal LCs. (A-D) Distribution of DEC-205$^+$ LCs (in red) in epidermal sheets (ear) obtained from non-immunized control mice **(A)** and from animals treated (24 h prior) with the NK1R agonist (i.d.) **(B),** GG (pCMV-Luc) **(C)**, or GG plus NK1R agonist **(D)**. The few LCs remaining in the epidermis after treatment became round-shaped (arrows), a feature indicative of LC migration. Immunofluorescence, magnification: 200X. **(E)** Quantification of the density of DEC-205$^+$ LCs in epidermal sheets (ear) 24 h post-treatment. Data represents the means ±1 SD from 3 independent experiments performed. **(F)** Comparison of the expression of LYVE-1 (red) and the chemokine CCL21/SLC (green) in the sDLNs 24 h following the indicated treatments. Immunofluorescence, magnification: 100X.

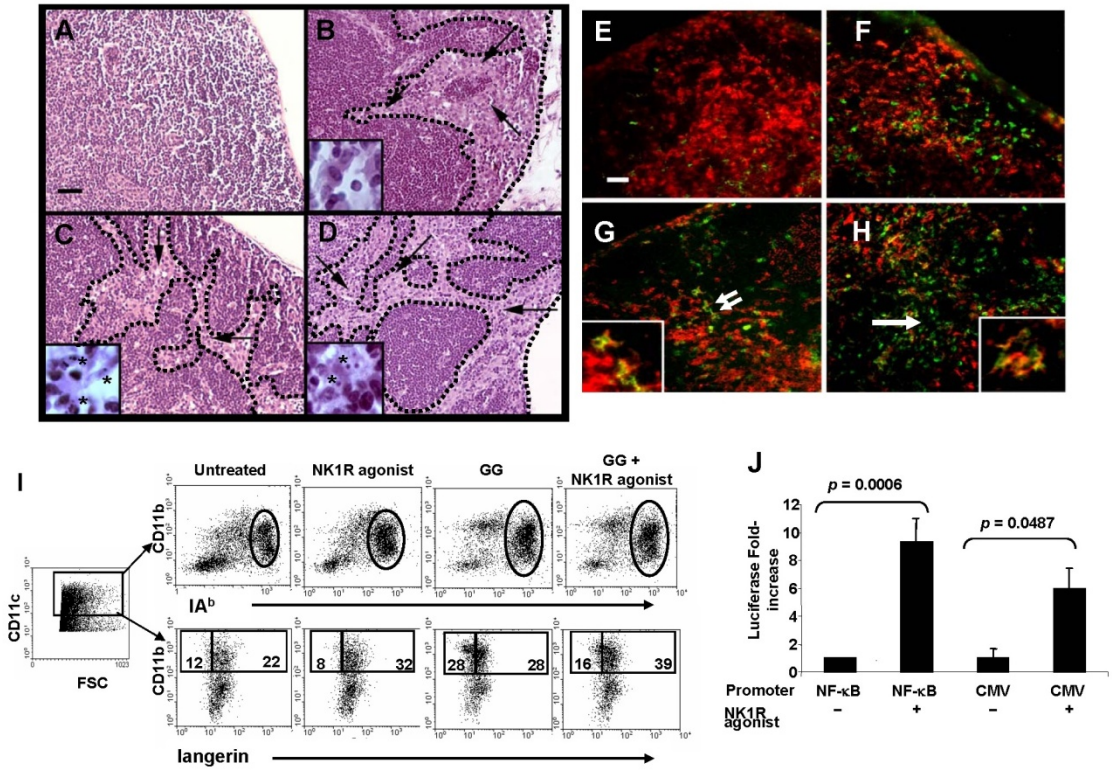


Figure 5. The NK1R agonist enhances the abilities of the GG to promote sDLN inflammation and homing of activated LCs. (A-D) Structure of sDLNs (inguinal) excised from non-treated control mice **(A)** and from animals treated (24 h prior) with: the NK1R agonist (i.d.) **(B)**, GG (pCMV-Luc) **(C)**, or GG plus NK1R agonist **(D)**. sDLNs from treated mice show sinus hyperplasia characterized by the presence of cells with abundant pale cytoplasm (some with morphological features of DCs indicated by arrows and detailed in the insets) located mainly within the subcortical and paracortical areas (dotted lines). Additionally, 1 μm gold beads were detected in the cytoplasm of DCs 24h after GG treatments alone or in combination with the NK1R agonist (illustrated in insets of **C** and **D**, and indicated by asterisks)**.** H&E stain. Magnification: 400X. Insets:1000X. **(E-H)** Identification of the population of LCs co-expressing langerin [CD207 (green)] and CD11c (red) (arrows) in the paracortical areas of sDLNs (inguinal) excised from non-immunized control mice **(E)** and from animals treated (24 h prior) with NK1R agonist (i.d.) **(F)**, GG (pCMV-Luc) **(G)**, or GG plus NK1R agonist **(H)**. Insets show $CD11c^+$ $langerin^+$ LCs at higher magnification. Immunofluorescence; magnification: 200X. Insets:1000X. **(I)** Quantification by flow cytometry of $CD11c^+$ $CD11b^+$ MHC-II $(IA^b)^{high}$ $langerin^+$ epidermal LCs and $CD11c^+$ $CD11b^+$ MHC-II $(IA^b)^{high}$ $langerin^-$ DDCs in the sDLNs (inguinal) of non-immunized control mice and animals treated (24 h prior) with NK1R agonist (i.d.), GG (pCMV-Luc), or GG plus NK1R agonist. Data are representative of 2 independent experiments. **(J)** Comparative analysis of tg Luc expression in sDLNs, 24 h after GG delivery of pNFκB-Luc or pCMV-Luc in the presence (or not, control) of the NK1R agonist. Means± 1SD of the fold-increase of RLU compared to background levels are illustrated. Three independent experiments were performed.

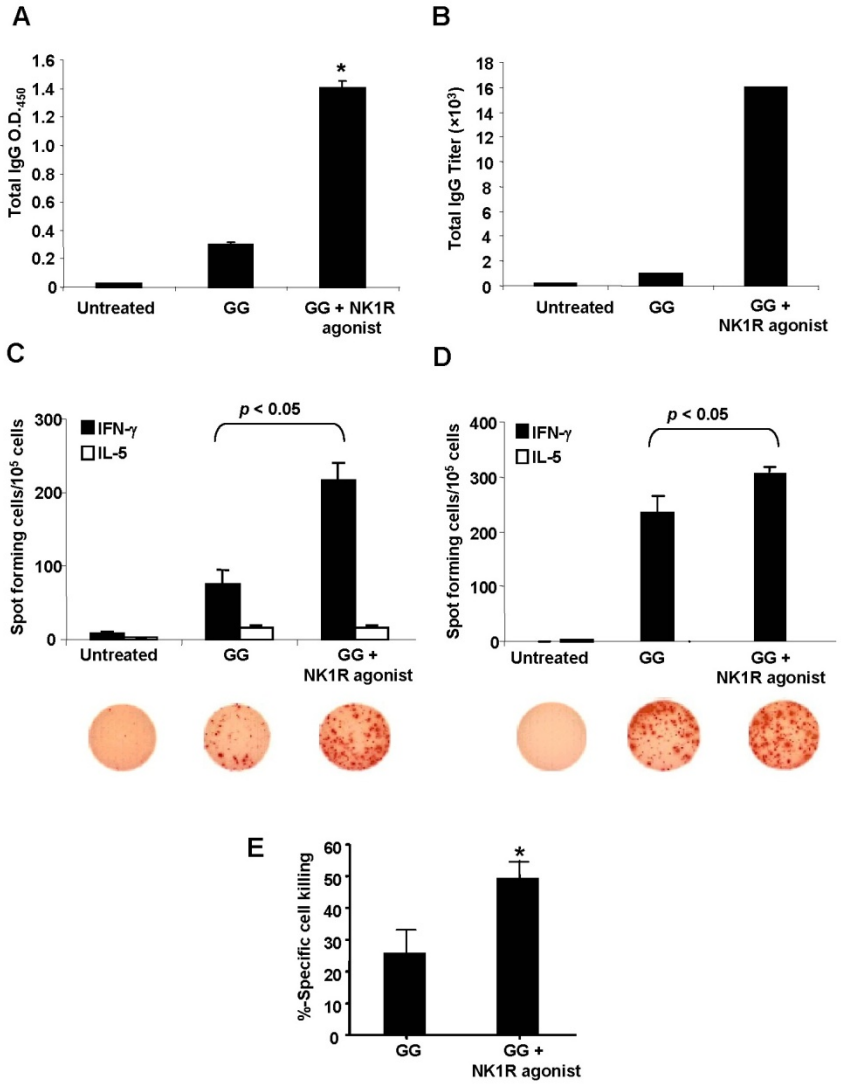


Figure 6. Skin GG immunizations in the presence of NK1R signaling enhances IFN-γ secretion by Ag-specific T cells. (A and B) Detection by ELISA of total OVA-specific serum IgG. **(A)** $O.D._{450}$ at a 1/2,000 serum dilution and **(B)** OVA-specific serum IgG titer at an $O.D._{450}$ of 0.2. **(C and D)** Detection by ELISPOT assays of IFN-γ and IL-5 secreted by $CD4^+$ **(C)** and $CD8^+$ **(D)** T cells isolated from spleens of B6 mice left untreated (control) or GG immunized with pCMV-OVA-TR in the presence (or not, control) of NK1R agonist. Results are expressed as the means of spot forming cells ±1 SD of triplicates. Data are representative of 3 experiments. Insets are representative images of the IFN-γ ELISPOT wells. **(E)** Ag-specific cytotoxic in vivo killing assays performed in B6 mice pre-immunized with GG on the abdominal skin with pCMV-OVA-TR and one boosting in the presence (or not, control) of the NK1R agonist. Means ± 1 SD of the specific cell lysis determined by the percentage of splenic cells, $CFSE^{hi}$ and $CFSE^{lo}$, analyzed by flow cytometry. * indicates a $p < 0.05$ compared to GG immunizations alone.

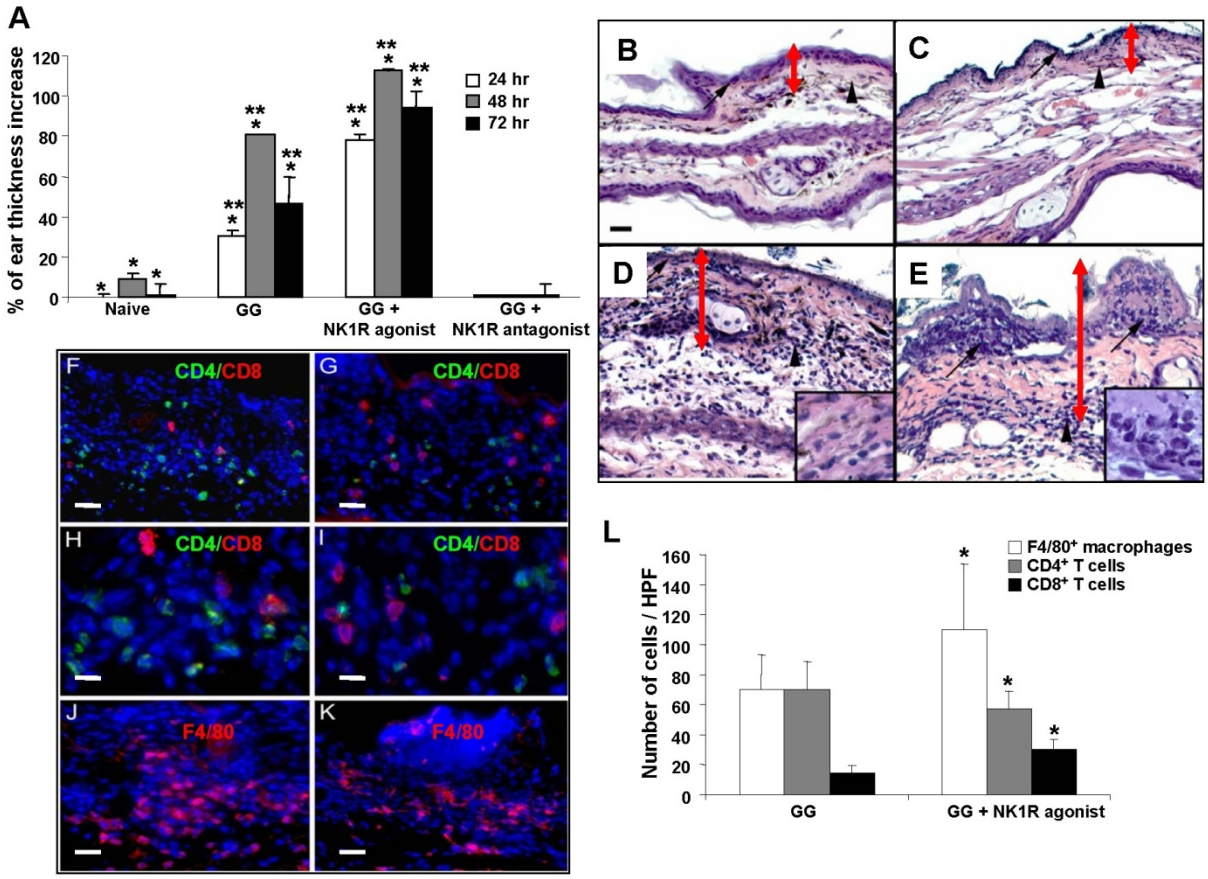


Figure 7. Administration of the NK1R agonist increases the ability of the GG to promote T cell responses. (A) Illustrates the ear thickness of mice immunized with the GG in the presence or not of the NK1R agonist and analyzed following the elicitation of DTH assays. Data are representative of 3 independent experiments. * indicates a $p < 0.05$ compared to the non-sensitized animals (Naïve). ** indicates $p < 0.05$ compared to GG immunizations alone at each time point. (**B-E**) Analysis of the cellular infiltrate in the skin (right ear) from non-sensitized control mice **(B)**, control animals sensitized with the irrelevant pBackbone **(C)**, and mice sensitized with the pCMV-OVA-TR alone **(D)** or GG in the presence of the NK1R agonist **(E)**. The skin was analyzed 72 h after sensitization. The mononuclear cell infiltrate is detailed in the insets. Epidermis is indicated by arrows and dermis by arrowheads. Red arrows indicate the thickening of the skin (excluding the hypodermis). H&E staining, magnification: 200X. Insets, 1000X. **(F-K)** Cellular infiltrate composed of $CD4^+$ and $CD8^+$ T cells **(F-I)** and $F4/80^+$ macrophages **(J-K)** in the presence **(G, I, K)** or absence **(F, H, and J)** of NK1R agonist. Immunofluorescence, magnifications: F, G, J, and K: 200X; H and I: 400X. **(L).** Quantification of the cellular infiltrate in tissue sections by microscopy is expressed as means ±1 SD of cell numbers from at least 20 high power fields (HPF, magnification: 400X).

APPENDIX C

PUBLICATIONS

Mathers AR, Tkacheva OA, **Janelsins BM**, Shufesky WJ, Morelli AE, Larregina AT. In vivo signaling through the neurokinin 1 receptor favors transgene expression by Langerhans cells and promotes the generation of Th1- and Tc1-biased immune responses. J Immunol. 2007;178:7006-7017.

Janelsins BM, Mathers AR, Tkacheva OA, Erdos G, Shufesky WJ, Morelli AE, Larregina AT. Pro-inflammatory tachykinins that signal through the neurokinin 1 receptor promote survival of dendritic cells and potent cellular immunity. Blood. 2009;113.3017-26.

Mathers AR, **Janelsins BM**, Rubin JP, Tkacheva OA, Shufesky WJ, Watkins SC, Morelli AE, Larregina AT. Differential capability of human cutaneous dendritic cell subsets to initiate Th17 responses. J Immunol. 2009;182:921-33.

Mathers AR, Tkacheva OA, **Janelsins BM**, Shufesky WJ, Morelli AE, Larregina AT. Agonistic signaling via the neurokinin-1 receptor is necessary for anti-melanoma immunity elicited by cutaneous genetic immunzations. *Submitted to Cancer Res.*

Janelsins BM, Tkacheva OA, Mathers AM, Erdos G, Shufesky WJ, Morelli AE, Larregina AT. Pro-inflammatory tachykinins that signal via the neurokinin 1 receptor induces immune-stimulatory DCs capable of promoting Ag-specific $CD4^+$ Th1 and $CD8^+$ CTL/Tc1 immune responses in vivo. *In preparation.*

Morelli AE, **Janelsins BM**, Shufesky WJ, Tkacheva OA, Larregina AT. The absence of the neurokinin 1 receptor inhibits allogeneic T cell responses by inducing apoptosis of effector $CD4^+$ and $CD8^+$ T cell responses. *In preparation.*

Morelli AE, Tkacheva OA, **Janelsins BM**, Rubin JP, Mathers AR, Falo LD, Larregina AT. Role of $CD34^+$ cells resident in human dermis. *In preparation.*

Morelli AE, Tkacheva OA, **Janelsins BM**, Rubin JP, Mathers AR, Falo LD, Larregina AT. Migratory abilities of $CD14^+$ cells resident in human dermis. *In preparation*.

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