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The Influence of Immunization Route on the Adjuvant Effect of alpha-Galactosylceramide

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Potent vaccine formulations ideally include adjuvants to activate innate immune responses and enhance antigen-specific adaptive immunity. The synthetic glycolipid alpha-Galactosylceramide (α-GalCer) effectively activates the innate immune mediating NKT cells to produce cytokines and activate downstream immune cells, resulting in development of humoral and cell mediated immune responses to co-administered antigens. While a single intravenous immunization of α-GalCer strongly activates NKT cells, multiple doses by this route are well documented to induce anergy in NKT cells. Anergy is defined as the deficiency in NKT proliferation and cytokine production, including IL-4 and IFNγ. However, our studies have shown that two doses of α-GalCer administered intranasally by the intranasal route leads to reactivation of NKT cells and improved adaptive immune responses after each subsequent dose. I therefore investigated the role of multiple routes of immunization in activation of NKT cells, i.e. anergy versus repeated activation. Specifically, I hypothesized that the differential capacity of NKT cells to produce IFNγ, as a result of route of immunization with α-GalCer, influences the induction of adaptive immune responses to co-administered antigen. Our experimental design utilizes the observation that intranasal immunization primarily induces immune responses in the lungs while intravenous immunization induces responses in the liver.

Using intracellular cytokine staining for IFNγ production and Elispot analyses for determining NKT and T cell activation, respectively, it was determined that administering two consecutive intravenous doses resulted in anergy to NKT cells (no IFNγ production) in the liver and lack of adaptive immunity while second immunization by the intranasal route overcame anergy in the lung. The outcome in the other tissues analyzed was mixed and could be the result of tissue microenvironment among others possible reasons. When intranasal dosing preceded systemic, NKT cells were reactivated to produce IFNγ and induced positive adaptive immune responses in the responding lung tissue. These results indicate that the mechanism by which mucosal and systemic immunization routes activate NKT cells may differ in that there is a differential tissue-specific effect induced by each route. Future studies are necessary to determine the reason for these tissue-specific effects and how they relate to NKT cell activation.

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LIST OF ABBREVIATIONS

1D	one dose
2D	two doses
α-GalCer	α-Galactosylceramide
aGC	α-Galactosylceramide
APC	antigen presenting cell
APC	allophycocyanin
B6	C57 black 6 mouse
BTLA	B and T lymphocyte attenuator
CCL22	(C-C motif) chemokine ligand 22
CCR4	(C-C motif) chemokine receptor 4
CTLA4	cytotoxic T-lymphocyte antigen 4
CXCL16	(C-X-C motif) chemokine ligand 16
CXCR6	(C-X-C motif) chemokine receptor 6
CT	cholera toxin
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethyl sulfoxide
DN32.D2	NKT cell hybridoma
E6/E7	HPV "early" genes
Egr-2	early growth response gene 2
FasL	ligand to apoptosis factor Fas
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
Gb4	globoside
HIV	human immunodeficiency virus
HPV	human papillomavirus
IFNγ	interferon gamma
ID2	DNA-binding protein inhibitor

IL 12	interleukin 12
IL-13	interleukin 13
IL18	interleukin 18
IN	intranasal
IP	intraperitoneal
IV	intravenous
LFA1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
MALT	mucosal-associated lymphoid tissue
MdLN	mediastinal lymph node
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cells
NK	natural killer cell
NKG2D	activating receptor on NK cells
NKT	natural killer T cell
OCH	alpha-galactosylceramide analog
Ova	chicken ovalbumin protein
PAMP	pathogen-associated molecular patterns
PB	pacific blue
PBS	phosphate buffered saline
PD-1	programmed death 1
PD-L	programmed death ligand
PE	R-phycoerythrin
PECy7	phycoerythrin conjugate
PIM2/4	proviral integrations of Moloney virus 2/4
PMA	phorbol myristate acetate
Qa-1b	a mouse MHC class I molecule
qPCR	quantitative polymerase chain reaction
SFC	spot forming colony
TCR	T-cell receptor

LIST OF ABBREVIATIONS CONTINUED

IgA	immunoglobulin A
iGB3	isoglobotrihexosylceramide
IgG	immunoglobulin G
IL-4	interleukin 4

TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 MUCOSA AND ADJUVANTS

1.1.1 Mucosal Immunity

The mucosal membranes make up an area of several hundred square meters in adult humans (1). A substantial number of infectious agents, estimated around 70%, enter the body via mucosal surfaces including the digestive, respiratory, and genitourinary tracts (2, 3). Unlike systemic immunization, mucosal vaccination has the ability to activate multiple arms of the immune system in order to combat pathogens that cannot be contained by antibody responses alone; they may be ideal for inducing immune responses to protect against pathogens entering via mucosal portals. Mucosal vaccines offer additional advantages such as avoiding the use of needles that carry the risk of possible spread of blood-borne pathogens, and also ease of use facilitating both better compliance and administration by non-medically trained personnel (4). The mucosal associated lymphoid tissues function to protect the mucosa from invasion by microbial pathogens, prevent the uptake of harmless antigens such as those from commensal bacteria and non-degraded food proteins, and to induce tolerance against commensal bacteria and soluble antigen (1). Due to constant assault by environmental antigens, mucosal tissues exert tolerance as the natural response to most invading foreign pathogens (4). Tolerance at mucosal tissues prevents the generation of inflammatory responses to harmless antigens like those found in the air or in food. Tolerance is mediated by the mucosal immune system that is made up of lymphoid and non-lymphoid cells, and effector mechanisms mediated by antibodies, T cells, and cytokines (2). In response to infection, the mucosal immune system activates innate and adaptive immune responses to provide protection. Organized lymphoid tissues, including the tonsils and adenoids, and Peyer's patches of the intestinal tract, are found in areas where pathogens are most likely to enter the body, and they house the machinery to allow B cell isotype switching from IgM to IgA, the major class of immunoglobulin in mucosal secretions (2, 5). . The presence of these organized lymphoid tissues allows for the differentiation of follicle-associated epithelium (FAE) containing microfold "M" cells that deliver antigen samples to local underlying dendritic cells (DCs) and lymphocytes by transcytosis (3, 5, 6). This creates a high density of cells that can phagocytize pathogens directly at their port of entry to ultimately limit systemic infection. In tissues lacking M cells, such as the genital mucosa, DCs migrate directly to the epithelial spaces to sample foreign

antigen where they can act locally or migrate through the lymphatic system to draining lymph nodes and even other organized lymphoid tissues such as distant lymph nodes and the spleen (7-9). The primary type I mucosae effector sites, the lung and gut, are protected by immunoglobulin IgA and utilize both mucosal DCs and M cells to pick up and present antigen within mucosal-associated lymphoid tissue (MALT) (8, 10, 11). In contrast, the tissues of the type II mucosae, the mouth, esophagus, and cornea, are primarily protected by IgG and lack MALT. They must rely on migration of activated antigen-presenting DCs to activate naïve lymphocytes in the peripheral mucosa-draining lymph nodes (12).

After antigen presentation, innate immunity goes on to activate adaptive immune responses. Innate immunity is the body's first line of defense against foreign pathogens. Made up of anatomical barriers, the complement system, and inflammatory cells, the innate immune system is designed to act quickly and indiscriminately to neutralize foreign invaders. Innate immunity relies on non-specific surface pattern recognition receptors (PRRs) on epithelial cells, such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), retinoic acid (RA)-inducible gene I-like helicases, and C-type lectins (13, 14). The PRRs recognize pathogen-associated molecular patterns (PAMPs), which represent conserved characteristics of bacteria and viruses such as peptidoglycan or double-stranded RNA, and thus mount an immune response (2, 15, 16). Such PAMPs have been identified in many of the mucosal surfaces, for example the NOD1 receptor in the lung and its role in asthma (17), TLR7 in the human intestine, and TLR4 in the genital mucosa (1). It is the innate system which is responsible for activating APCs such as DCs by sending out chemokines and cytokines in response to PRRs interacting with PAMPs; the APCs then go on to link the two systems and present antigen to the adaptive effector cells. The B and T cell effectors of adaptive immunity express antigen-specific receptors which, when activated, trigger expansion and memory to repeated antigen exposure. Many studies have described the existence of the common mucosal immune system (CMIS), in which antigen-specific lymphocytes can home not only to the site of pathogen presentation but also to other distant mucosal effector sites (13, 18). This effect depends on mucosal compartmentalization, "the process of antigen uptake, migration, and processing by local APCs and magnitude and phenotype of T cells or predominant antibody isotype(s) induced in relationship to the route of immunization and mucosal site(s) analyzed" (13). For example, IgA secreting B cells in the mucosa express CCR10, the receptor for CCL28 expressed by epithelial cells in various mucosal tissues including intestines and the respiratory tract (5, 19). Thus, the responding

cells can migrate to CCL28-expressing tissues, explaining the far reaching responses of the common mucosal immune system. In general, these compartmentalized immune responses include innate, serum IgG and serum IgA, and cytotoxic T lymphocyte (CTL) responses in the local tissue that confer protection against mucosal pathogens. Mucosal vaccines approved for use in humans to date include those targeting poliovirus (20), influenza virus (21), *Salmonella typhi* (22), and *V. cholera* (22, 23). While systemic vaccine delivery involves immunizing with a known amount of antigen and measuring responses in the blood,administering mucosal vaccines is complicated by the fact that antigen uptake cannot be measured, and mucosal responses are more difficult to detect. Thus, improvement in the breadth and caliber of mucosal vaccines is necessary.

1.1.2 Overcoming the mucosal barrier

Within the mucosal tissues, mucous acts as a barrier to pathogen entry and movement, thus doubling as a potential barrier to vaccine delivery as well. Various methods are being developed to overcome these disadvantages, including engineering vaccine vehicles to take advantage of surface charges on mucous to better adhere to or penetrate it (2, 24, 25). Another strategy, developed by Ralph Steinman's group, focuses on targeting vaccines directly to the dendritic cells using DC-specific receptors expressed on the surface (2, 26-28). Various cell types can be targeted using natural ligands or even antibodies to their receptors (29). Timing of antigen and adjuvant delivery is also important. While co-delivery of adjuvant with antigen has been shown to enhance CD8+ T cells responses (2, 29-31), delivering these components separately leads to enhanced antibody titers (2, 32). Interestingly, it has also been shown that co-localization of antigen and TLR ligand adjuvant within the same phagosome is necessary to induce DC maturation and thus antigen presentation (2, 33). A study using the vaginal route to deliver HIV antigen revealed that repeated immunization without the use of adjuvant induced antibody responses (2, 34). This, however, is in contrast to the general perception that in the absence of an adjuvant, a state of tolerance is induced rather that immunity. Tolerance can be induced by constant exposure to low doses of antigen (T regulatory cells) or a sudden exposure to high levels of antigen (via mechanisms such as anergy and deletion), necessitating in depth kinetics studies for mucosal adjuvants. The ideal mucosal vaccine should encompass several characteristics including the ability to protect antigen from degradation, to direct antigen to

the appropriate cells to maximize uptake by APCs, and to preferentially assist the uptake of antigen and adjuvant by the same cells that are augmenting immune responses (1).

1.1.3 Mucosal vaccination strategies

Many delivery strategies for mucosal vaccines have been studied to date. Live-attenuated viruses have been used as vaccines against influenza and rotavirus. These types of vaccines are composed of live bacteria or viruses that have been weakened, or attenuated, with respect to infectivity, but retain the immunogenicity of the parent strain. Attenuation can be performed by multiple passages through host cultures or even by selected gene deletion (2). Attenuation can be a challenge as can be optimizing the stability of these vaccines (4). Subunit vaccines are non-living whole-cell vaccines or pathogen-specific proteins. Their utility is limited by the need to protect the protein from degradation in the mucosa as well as requiring the use of adjuvant, for which there are few approved for use in humans (2). Virus-like particles (VLPs) are composed of virus-derived immunogens that assemble into three-dimensional structures similar to the capsids of the native virus. They self-assemble after being recombinantly expressed in yeast, insect, or even mammalian cells. Examples include the Hepatitis B and Human papillomavirus vaccines (2, 35). Unfortunately, there are several disadvantage to using VLPs such as the need for large scale production, purification from the expression system, and the requirement for use of adjuvants (2). Nanoparticle carriers are engineered to overcome the limitations presented by the mucosal barriers. They can be made of both organic and inorganic materials and can either encase the antigen or have it adsorbed to the surface (1, 36). They are ideal for delivering biologics such as peptides and nucleic acids, and can help with proper orientation of antigen and adjuvants (2, 37). Interestingly, a nanoparticle formulated α-GalCer was able to avoid anergy induction by targeting the appropriate APC (38). However, it is interesting to note that studies conflict on whether nanoparticles increase serum IgG or it remains unchanged (1). There are also few studies that were able to show an increase in mucosal IgA in response to nanoparticle formulations used for vaccination as compared to antigen alone (1, 39). All the same, delivery of antigen via particle theoretically leads to better recognition and stronger immune responses than with soluble antigens, which may require the help of adhesion augmenting substances such as chitosan to attach to the mucosal membrane (4, 40). Chitosan is thought to interact with protein kinase C to open tight junctions between cells and allow for better drug transport (1, 41). Chitosan has also been

shown to increase the adjuvanticity of cholera toxin (CT) subunit B in mice (1, 42). Liposomes have been shown to increase absorption of antigen in the nasal cavity (1, 43). While liposomes can contain many copies of antigen within and protect the antigen from degradation, lipid-based delivery systems are also costly and formulations may be unstable when stored. Examples of liposomal formulations include immune stimulating complexes (ISCOMs), virosomes, and proteosomes, among others (1). Virosomes and proteosomes have shown promising results in clinical studies (44, 45).

1.1.4 Mucosal routes of immunization

Aside from differences in formulation, several studies in the literature have investigated the efficacy of using various routes for vaccine delivery. It is believed that protective immunity against mucosal pathogens can be effective if immune responses can be tailored to operate at the anatomic location of invasion. The strongest responses occur near the site of vaccination, where cells activated by vaccines, infection, or inflammation can upregulate the expression of addressins on epithelial cells and homing molecules on T cells (5, 46, 47). Oral vaccination is ideal for simplicity's sake. Oral vaccines target antigen to the gut associated lymphoid tissue (GALT), where specialized cells known as microfold cells, or M cells, are able to transport a broad range of materials by transcytosis (1, 48). It is well known that oral immunization is able to induce humoral and cellular responses both at the GALT and also in other mucosal compartments (1, 49). However, the GALT also plays a significant role in inducing tolerance to antigens presented via the oral route. Thus, oral immunization requires the use of higher doses of antigen as well as carriers or adjuvants, driving up the cost. One alternative is the use of transgenic plants as production centers and vehicles for mucosal vaccines, thus providing a cheaper format for production resulting in an edible vaccine which can protect antigen from degradation (1, 50, 51). Other examples of mucosal vaccination routes include the sublingual route, which enables antigen entry directly into the bloodstream to avoid degradation or tolerance induction (1). This route is currently only used in humans for allergy therapy, but studies in mice have shown that this route allows for broadly reaching mucosal and systemic immune responses, including antigen-specific T cells in the cervical lymph nodes and antibodies to VLP in the serum and genital tissues that provide protection against genital challenge with the specific pathogen (1, 52-54). One thing to keep in mind is that mice have keratinized sublingual tissues while humans do not, and this must be taken into consideration as it may play a role in differential

uptake of antigen (1). Vaginal delivery is an important potential vaccination strategy as it allows for strong immune responses to be induced at the site of vaccination, and because many STDs are transmitted via this route, it is necessary for the protective immune responses to be active at this site. In this tissue mainly, but also at other mucosal surfaces, degradation-resistant dimeric serum IgA is made, which might be important in blocking infection at this site (1, 55, 56). It is noted that sIgA acts to trap antigen in the mucous to block pathogens from directly contacting the mucosal surfaces (5, 57). It may also sterically hinder pathogens from attaching to cells (5, 58). Vaccination strategies at this site not only aim to induce immune responses, but may actually aim to block antigen entry at the site. For example, estradiol has been shown to both prevent CD8+ T cell priming as well as antigen loading onto vaginal APCs (1, 59, 60). However, this route of vaccination is not only impractical, but is also prone to be influenced by hormonal changes during female menstrual cycles, which affect the levels of IgG and IgA secretions (1, 5, 56, 61). Intranasal delivery is also of interest given the ease of application and the requirement of less antigen and adjuvant than the oral route. The intranasal surfaces are small and lack enzymes that can cause breakdown of antigens allowing for lower doses to be effective, thus making the intranasal route ideal for mucosal vaccination. Intranasal administration has been shown to induce mucosal IgA in multiple tissues including salivary glands, the respiratory tract, genital tract, and the intestines (56, 62, 63), as well as CTLs in the female genital tract (64). In humans, studies showed broadly disseminated immunity at multiple systemic and mucosal tissues after intranasal vaccination against diphtheria, tetanus, and influenza (1, 65, 66). Similar to the oral route, the intranasal route also takes advantage of the transcytosing properties of M cells in the nasal associated lymphoid tissue (NALT). The NALT in mice is a smooth dome containing M cells, while human NALT has crypts containing M cells which can create germinal centers similar to those in the GALT, a phenomenon not seen in mice (1, 67, 68). As evidence of its widespread reach, intranasal immunization primarily induces immune responses in the lung and respiratory tracts, but can also reach sites such as the gastric and genital mucosa (4). It has also been shown to induce antibody responses in the genital tract (69). In fact, some intranasal vaccines, such as FluMist™ (MedImmune Vaccines Inc.), are approved by the FDA for use in patients. The ideal intranasal immunization will protect the antigen from degradation long enough to allow its interaction with the proper cells, stimulate both innate and adaptive humoral and cell-mediated responses, and, if necessary, include adjuvants that protect the natural function of the nose and avoid reactogenicity and toxicity (1). Optimal protection against pathogenic invasion will

likely require involvement of both systemic and mucosal immune responses, and thus may require a combination of systemic and mucosal vaccinations delivered as prime-boost combinations. It has been shown that mucosal immunization can prime lymphocytes by inducing expression of homing receptors targeting both systemic and mucosal tissues (5, 19). However, “parenteral priming might not prime the immune system for subsequent mucosal vaccination” (70).

1.2 ADJUVANTS

1.2.1 What is an adjuvant?

An adjuvant is defined as a substance that can enhance, prolong, or accelerate immune responses to co-administered antigen. Adjuvants are important to include in mucosal vaccines as they improve immunogenicity and help overcome the natural barriers against immune response presented by the mucosa. Adjuvants act by harnessing innate immunity to induce effector rather than regulatory T cell responses. Responses must be potent, but not so potent as to become toxic. Currently, the only adjuvants approved for use in humans include aluminum salts (alum), such as aluminum phosphate and aluminum hydroxide, and certain squalene oil emulsions (namely AS03) used in the H5N1 influenza vaccine (71-73). Both of these adjuvants are aimed at enhancing systemic immunity and are ineffective at producing protective responses at mucosal sites. In addition, a mixture of aluminum hydroxide and monophospholipid (MPL) A, an LPS analogue, make up AS04 adjuvant used in hepatitis B virus and human papilloma virus vaccines (71, 74). Alum adjuvant works by inducing an influx of innate immune cells, including neutrophils, DCs, and NK cells, at the site of injection (75-78). It is also thought to act as an antigen depot, slowly releasing antigen to the environment (71). Alum adjuvant induces release of endogenous danger signals, including release of uric acid which degrades RNA and DNA in addition to releasing host dsDNA which is recognized by TLR9 (76, 79). With respect to other agents with known adjuvant functions, the underlying mechanisms of action are not well understood, adding another road block to their use in the development of vaccine formulations. Additionally, while mucosal immunization can induce both systemic and mucosal immunity, most antigens are poor immunogens and can even induce tolerance, thus highlighting the importance of adjuvant discovery (80). Adjuvants can be classified into two subgroups, those that interact with innate immune players to enhance adaptive

responses, and those that facilitate delivery of antigen in order to harness the CMIS. Adjuvants are thought to primarily function by creating an inflammatory environment that recruits important innate immune players, such as those with antigen presenting functions, to the site of interest (2). They can further act by causing the release of cytokines and activating APCs to allow for improved antigen presentation. Adjuvants such as cholera enterotoxin (CT) are known to confer protective immunity but have not been connected to an innate immune mediator (2).

1.2.2 Danger model

In the classical immunological model of self/non-self-discrimination, in which encountering foreign antigen initiates an immune response, the need for adjuvants in vaccine formulations could not be explained. In 1994, in partnership with Fuchs, Matzinger published a paper detailing their Danger model of immune activation (81). This theory claims that an immune response is not induced by foreign antigen itself, but by alarm signals sent out by the body tissues after they incur damage caused by the carrier of the antigen, for example, cells which are damaged or send out distress signals, or cells that die by necrosis or abnormal induced cell death (82). These distress signals activate the APCs (and thus the immune system), not the presence of foreign antigen, which may or may not be harmful (83). Similarly, abnormal reactions of the body that cause injury to cells can activate the immune system, even though these are self-cells. Examples of endogenous danger signals, or adjuvants, include IFNα, heat shock proteins, necrotic cell death, activation of NF-kB, and blood clotting factors (82). Heat shock proteins, or HSPs, are evolutionarily conserved proteins that play a role in protein folding and transport, and are upregulated when cells experience stress, for example at high temperatures. They are capable of presenting antigens to APCs by modulating PAMP-induced stimulation of immune responses (84, 85). However, multiple groups have argued that HSPs in fact counteract inflammatory responses (85-88). Death by necrosis, unlike normal apoptotic cell death, leads to a degradation of the cell membrane and release of intracellular DAMPs, damage associated molecular patterns (89). Necrotic cells have been shown to activate DCs and release HSPs (82, 90).

1.2.3 Mucosal adjuvants

Commonly studied exogenous adjuvants include TLR agonists, mutant bacterial enterotoxins, and viral vectors. For example, *Salmonella* and reovirus are known to target M

cells, and work is being done to attenuate them to act as targeted antigen delivery vehicles (91-93). TLRs activate innate immunity to produce inflammatory cytokines and APC costimulatory molecules (93). Unmethylated CpG dinucleotides like those found in bacteria have been used as adjuvants to activate TLR9 and induce Th1 immune responses (94). Components of bacterial cell walls such as LPS stimulate antigen presenting cells through TLR4 (95). Bacterial endotoxins such as cholera toxin (CT) have been mutated to remove toxicity, but maintain adjuvanticity to induce humoral and cell-mediated immune responses (96, 97). For example, our group has studied a mutated cholera toxin in which ADP-ribosylating activity and toxicity was removed (98). CT-2*, as it is called, has been shown to induce T helper (Th) and CTL responses after intranasal immunization with antigen, as well as Th1 responses in both mucosal and systemic compartments, while avoiding tolerance induction (99, 100). These adjuvants fall under the subgroup of adjuvants that interact directly with the innate immune system to enhance immunity. The latter subgroup encompassing carrier adjuvants, including ISCOMs, liposomes, chitosan, and viral vectors, has been previously discussed.

1.3 α-GALACTOSYLCERAMIDE AND INVARIANT NKT CELLS

1.3.1 α-GalCer Structure

First discovered by the Koezuka group at the Kirin Brewing Company in Japan in 1993, alpha-galactosylceramide (α-GalCer) is a glycosphingolipid originally synthesized based on agelasphins isolated from the sea sponge *Agelas mauritanius* when it was screened for the presence of antitumor compounds against liver metastases (101, 102). Most glycosphingolipids in mammals are beta-linked. The main difference between these mammalian ceramides and α-GalCer is that α-GalCer lacks the double bond between sphingosine carbons 4 and 5 and has an extra hydroxyl group on carbon 4 (Figure 1) (103). Additionally, naturally occurring ceramides contain unsaturated bonds, whereas α-GalCer is fully saturated (104). Studies showing that small molecule attachments to the C6 of the galactose ring do not interfere with α-GalCer activity (105) have allowed attachment of small fluorophores at this site to accommodate trafficking studies (106).

Figure 1. Structure of α-Galactosylceramide (α-GalCer).

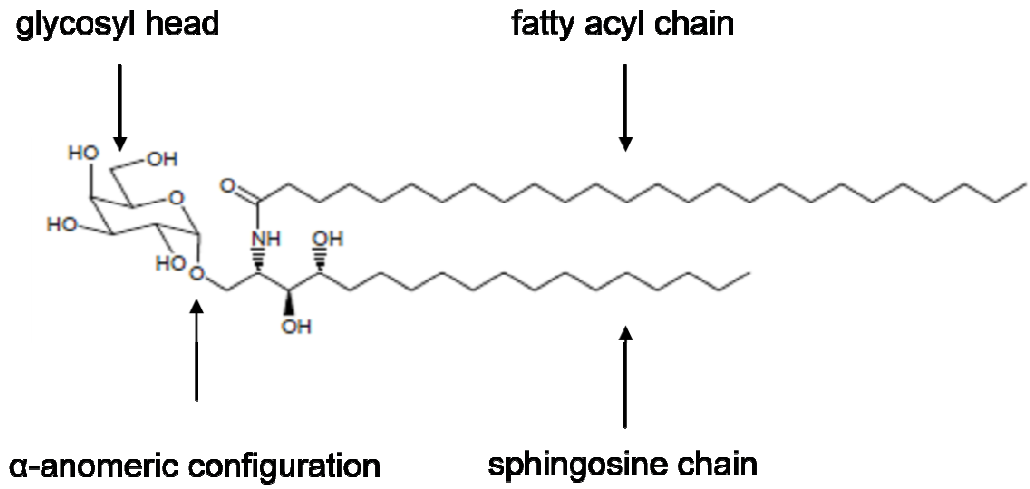


Figure 1. α-GalCer is a glycosphingolipid that consists of a sphingosine base, fatty acyl chain and an α-anomeric sugar moiety. The CD1d binds the lipid portion and the NKT TCR binds the glycosyl head. This figure is adapted from α-GalCer Data Sheet from Funakoshi/DiagnoCine (Hackensack, NJ).

1.3.2 α-GalCer modulates NKT cell activity

α-GalCer potently activates the innate immune-mediating invariant natural killer T cells (NKT) which play a role in controlling adaptive immunity (107-109). NKT cells were originally identified by 3 independent groups (110). In 1986, Taniguchi and co-workers discovered that 90% of their independent T cell suppressor hybridomas expressed Vα14 and Jα281 genes (111, 112). Furthermore, in various mouse strains with different MHC haplotypes, they found that these Vα14+ cells were fewer in number in β-2-microglobulin deficient mice, which don't express MHC class I or its related molecules, indicating a monomorphic MHC class I-like receptor (113, 114). They additionally showed that these Vα14+ cells were able to develop in extrathymic lymphoid tissues, like bone marrow and liver, but not in the spleen (115). In the second study in 1987, Fowlkes and Budd found a CD4-CD8- thymocyte population that expressed Vβ8 only (116, 117), which was later found to also express NK1.1, CD5, and CD44 (118, 119) as well as both Th1 and Th2 cytokines (120, 121). Finally, the third study carried out by Bendelac's group in 1994 established a hybridoma line of Vβ8+CD5highCD44+ cells which were found to express mRNA for Vα14 (122). This group also found that these hybridomas recognized the MHC I-like monomorphic CD1d molecule (123, 124). Thus the invariant NKT cell population was identified.

As previously mentioned, invariant NKT cells are termed as such because they express a single form of Vα TCR, in mice Vα14-Jα18, and in humans Vα24-Jα18, as well as NK cell markers such as NK1.1, and are even able to perform killing functions similar to NK cells (110, 125-129). As some CD8+ T cells have been found to express NK cell markers and expression levels of NK markers vary in NKT cells (130, 131), NKT cells are now restricted to those cells whose TCRs respond to CD1d. In fact, even NK1.1 negative cells are considered to be NKT cells as long as they are CD1d restricted (128). CD1d- dependent NKT cells expressing invariant TCR can be found mainly in the liver as well as the omentum lining the abdomen in humans (132), while CD1d-independent NKT cells expressing a variety of TCRs are found in the spleen and bone marrow (133). CD1d-independent NKT cells are activated by DCs responding to bacterial components via TLRs, as well as directly through exposure to IL-12 and IL-18 (134, 135). Invariant NKT cells are functionally diverse based on their anatomic locations (136-138). Depending on the tissue in which the NKT cells are located, they can be regulated by different factors; for example, liver NKT cell numbers depend on CXCR6, LFA-1, and transcriptional regulator ID2 (139-144), whereas NKTs in the thymus and spleen are not dependent on these factors (139). Interestingly, NKT

cells are highly evolutionarily conserved as evidenced by the fact that mouse cells can recognize human CD1d+antigen, and vice versa (139, 145-147). CD1d preferentially presents lipid, binding to the saturated hydrocarbon chains in the galactose ring of α-GalCer (148). Most NKT antigens contain a lipid tail that binds CD1d and a sugar head that binds to the NKT TCR (147, 148). Furthermore, the stability of the TCR:α-GalCer:CD1d complex is determined by the length of the phytosphingosine chain (147, 149). CD11c+ DCs interact with the NKT cells through antigen presentation and CD40-CD40L interaction to activate the NKT cells (109, 150). When activated, NKT cells produce large amounts of IFNγ and IL-4, a phenomenon known as a cytokine storm. This storm can influence responses against tumors and even autoantigens, and is responsible for the role of NKT cells in inducing adaptive immune responses (109, 151, 152). Additionally, NKT cells play a role in innate immunity as they are equipped with cytotoxic capabilities from perforin, granzyme, tumor necrosis factor (TNF)-α, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) (147, 151, 153, 154). These NKT cells have been shown to play roles both in controlling and suppressing anti-tumor immune responses, as well as contributing to asthma but protecting against autoimmune disease (155). The NKTs playing a protective role have been shown to be mainly CD4- classical invariant, or type I, while suppressive NKTs are mainly CD4+, or type II. Alternatively, others have shown that α-GalCer is able to activate CD4+ NKT cells which activate NK cells and produce cytokines (150). The rapid response by these cells has been attributed to the existence of pre-formed mRNAs for various cytokines including IFNγ, IL-4, and IL-13 (139, 156-160). Classical type I (invariant NKT) cells are known to make IL-4 within two hours of α-GalCer administration, and IFNγ within 6 hours; these are the cells that play a role in asthma in both mice, and less so, in humans (155, 161-163). Type II NKT cells express a more diverse array of TCRs and do not respond to α-GalCer, while responding to other lipids presented on CD1d. They were found first in MHCII deficient mice in the CD4+ population and also in a tumor negative for MHC I or II (155, 164, 165). These type II NKT cells are much less well understood, and functions identified include a suppressive role in EAE (166) and diabetes mellitus in NOD mice (167).

1.3.3 NKT cells and anti-tumor immunity

NKT cells were first implicated in immunity when the same group that discovered α-GalCer found that injection of the compound afforded protection against tumor development and metastasis (168-170). This protection was linked to IFNγ production and NK cell activation (Figure 2) but not to perforin from the NKT cells, leading to the conclusion that NKT cells act as mediators of the immune response rather than direct killers (171-173). The cells were identified to be CD4- and CD8-, consistent with reports in humans showing that double negative NKT cells are prone to produce Th1 cytokines (e.g. IFNγ) (136, 137, 155, 174). Dendritic cells were soon tied to the evolving mechanistic picture of NKT cells when it was shown that pulsing DCs with α-GalCer led to better protective responses by NKT cells (175) and that after interaction with NKT cells, the mature DCs produced IL-12 and IL-15 and enhanced T cell responses (31). Thus, DCs loaded with α-GalCer were examined for their ability to fight tumor progression in both mice (176) and humans (177-182). Studies have gone further to expand NKT cells in vitro with α-GalCer-pulsed DCs or with α-GalCer and IL-2 in clinical trials (183, 184). However, these studies did not lead to conclusive results of beneficial effects using these treatment methods. In contrast to the protective effect conferred by NKT cells, it was discovered that NKT cells can also play an immune suppressive role in tumorigenesis and autoimmunity. Two tumor models testing NKT cell-mediated responses in CD1d knock-out mice revealed that tumors did not recur or metastasize in the absence of NKT cells; this tolerogenic effect of NKT cells was linked to their production of IL-13 rather than IL-4 (185-187). Further studies utilizing α-GalCer and sulfatide (166) to selectively activate NKT type I and type II cells, respectively, revealed that indeed NKT type I cells activated by α-GalCer and even the lesser agonist OCH, which skews to Th2 responses (188), confer protection while activation of type II NKT cells suppress, an effect abrogated by depletion of CD4+ T cells (189). Furthermore, activation of NKT cells in vitro with both α-GalCer and sulfatide simultaneously revealed a decrease in proliferation normally induced by α-GalCer (189). In mouse models, administration of α-GalCer followed shortly by sulfatide resulted in reduction or complete elimination of protective effect (189). These studies revealed a new level of immune regulation by NKT cells in tumorigenesis, and may play a role in the adaptive responses induced by activation of NKT cells.

1.3.4 NKT cells, costimulation, and anergy

Conventional T cells are activated by a series of signal inputs, starting with signal I which encompasses the interaction of MHC-antigen complex with the TCR, and signal 2, or the costimulatory signal, when CD28 interacts with B7.1 or B7.2 on pAPCs (147, 190). In the absence of signal 2, signal 1 will induce anergy in T cells. In the presence of both signals, T cells go through changes to promote survival and growth. In contrast, negative costimulatory molecules exist including cytotoxic T lymphocyte-associated antigen (CTLA)-4, programmed death (PD)-1, and B and T lymphocyte attenuator (BTLA) (191). NKT cells also express costimulatory molecules, but they exist in a pre-activated state, even in germ-free mice (192). Given the presence of pre-formed mRNAs, it is possible that NKT cells have a lower activation threshold. In addition, upon activation, NKT cells proliferate and quickly produce cytokines, but this is followed by cell death and a phenotypic state known as anergy (193). Anergic NKT cells do not respond to re-exposure to α-GalCer by proliferating or producing IFNγ, but they do retain some ability to produce IL-4. The involvement of costimulatory signals in activation and anergy of NKT cells remains to be elucidated. However, given its vital role in T cell activation, CD28 has been studied extensively in NKT cells. CD28 and B7.1/B7.2 (CD80 and CD86, respectively) knockout mice both experience a decrease in NKT cell numbers of greater than 50% (194-196). CD28 is also important in the maturation process as measured by expression of surface markers such as NK1.1, CD69, and intracellular IFNγ (194, 195), whereas overexpression studies have revealed defective NKT cell development (194). Finally, blocking of B7.2 (CD86) decreased IFNγ production in α-GalCer-stimulated splenocytes, more so than blocking IL-4 and B7.1, indicating that this interaction may play a role in skewing NKT cells toward Th1 responses (197). PD-1 acts as a coinhibitory receptor in T cells (198) and is associated with a phenotype of functional exhaustion. PD-1 has been shown to be dispensable for NKT cell development in both single and double knockout mice for its two ligands, PD-L1 and PD-L2. However, PD-1 is upregulated after a single treatment of α-GalCer and remains so in anergic NKT cells. It has been shown that blocking the PD-1/PD-L(1 or 2) interaction avoids anergy, but that blocking PD-1/PD-L1 does not reverse established anergy in vivo (199). The involvement of PD-1 in anergy induction appears to be exclusive to α-GalCer-induced anergy, as anergy was induced by heat inactivated *Escherichia coli* or sulfatide regardless of PD-1/PD-L1 interaction blockade (199). Additionally, in a melanoma challenge model, where multiple doses of α-GalCer exacerbated metastasis as compared to one dose and even vehicle, co-administration of α-GalCer with anti-PD-L(1 and 2) enhanced anti-metastatic effects (199).

When activated, NKT cells express CD40L (200, 201) which interacts with CD40 on DCs, activating them to produce IL-12 (150, 201). IL-12 stimulates NKT cells to produce IFNγ, though it has been shown that IL-12 is dispensable for IL-4 production by α-GalCer-activated NKT cells (150, 200, 202). This may be why DC-NKT interaction skews to Th1 response. In fact, in CD40 knockout mice, α-GalCer no longer exhibits anti-metastatic properties (200). Each of these costimulatory factors points to an additional method to target α-GalCer treatments for improved immune responses.

Figure 2. Natural killer T cells interact with many different cell types.

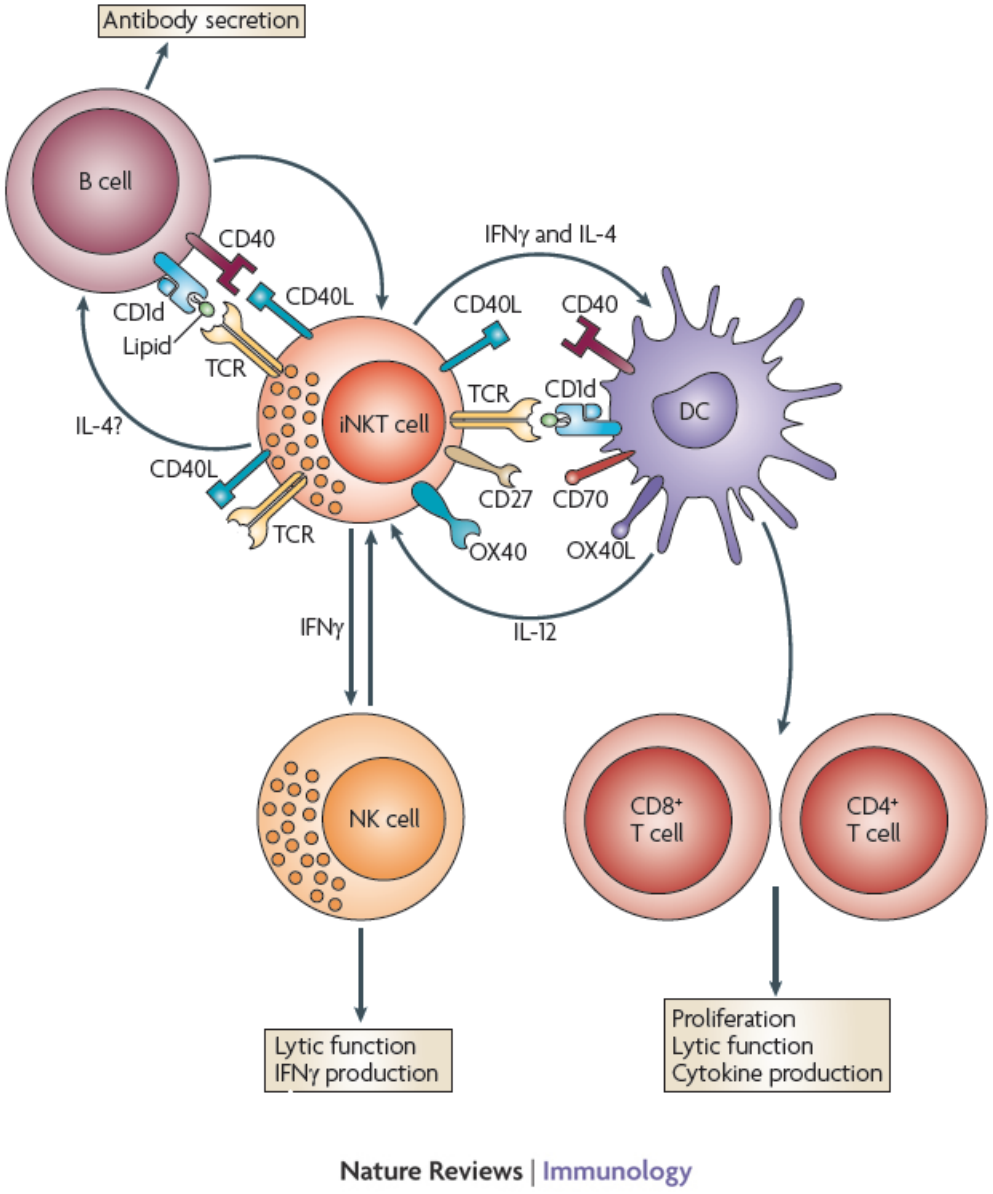


Figure 2. Invariant natural killer T (iNKT) cells interact with other cell types such as NK cells and T cells to modulate immune activity. These interactions are reciprocal between the APC and iNKT cell. Signaling utilizes the CD1d-glycolipid-TCR interaction, costimulation through the interactions of CD40, CD27, and OX40 with their ligands, and cytokine release. This figure is adapted from Figure 1, Cerundolo V. et al, Nat Rev Immunol 2009. (203)

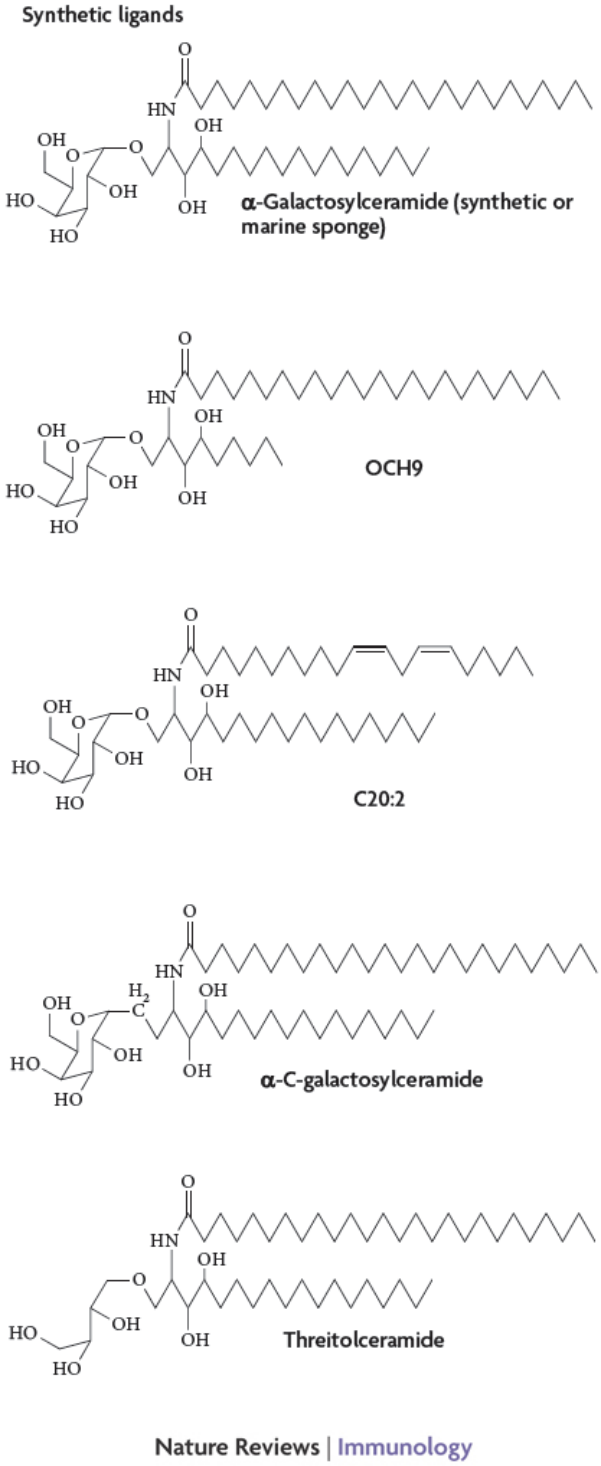
1.3.5 α-GalCer Analogues

Structural, kinetic, and functional studies are being conducted to identify NKT cell agonists that possess the optimal qualities of an NKT cell-activating vaccine adjuvant. The structure of α-GalCer is composed of a hydrophilic carbohydrate head connected via alpha-linkage to the hydrophobic ceramide or lipid portion (Figure 3). The ceramide portion is made up of a long fatty acyl chain that is 26 carbons long as well as an 18-carbon long sphingosine chain (110). Studies have concluded that the stability of the lipid-CD1d complex is affected by the length of the acyl and sphingosine chains, that saturation of the alkyl chains plays a role in lipid dissociation from CD1d, and that the length of the lipid chain modulates the TCR binding to the lipid-CD1d complex (149, 203). For example, unsaturated acyl chains form a kink which helps binding to CD1d and allows presentation by non-professional APCs and skewing towards Th2 responses (203-206). Functional studies have shown that a shortened length of either the acyl or sphingosine chains leads to a reduction in NKT cell proliferation (110, 145). Specifically, treatment with the α-GalCer analogue OCH9, with its shortened 9-carbon long sphingosine chain (Figure 3), resulted in more IL-4 secretion than α-GalCer and successfully treated EAE (188, 203, 207), (188, 208). In further studies, OCH was shown to be beneficial in treating other autoimmune diseases such as collagen-induced arthritis (209), and type I diabetes (210). Teyton's group subsequently showed that OCH is primarily presented by B cells (208, 211). Substituting the acyl chain of α-GalCer C26:0 (26 carbons, 0 unsaturated) with the shortened and di-unsaturated C20:2 acyl chain induced Th2 polarized immune responses with less IFNγ and NKT cell proliferation (93). Other important functional moieties include the 2'-hydroxyl of the sugar head and the 3'-hydroxyl of the sphingosine chain, as changes at these locations using α-mannosylceramide and α-GalCer lacking the 3'-hydroxyl did not activate NKT cells (110, 145, 203). Three hydrogen bonds at the junction of the carbohydrate head and ceramide tail "anchor" α-GalCer into the binding groove of CD1d (212). Another analogue, α-C-GalCer, has a CH2 methylene instead of O at the 1' position (Figure 3) and was found to produce more prolonged responses and higher IFNγ production, as much as 1000 times higher than α-GalCer, in mouse models of malaria and metastatic melanoma, perhaps by protecting the CD1d-lipid complex from enzymatic degradation (203, 208, 213). Threitolceramide (ThrCer), an analogue of α-GalCer lacking a sugar head and containing non-glycosidic linkages between the sugar head and ceramide portions (Figure 3), stably binds CD1d and is capable of forming the three hydrogen bonds formed by α-GalCer and CD1d (203, 214). It

has been shown that ThrCer can activate antigen-specific B and T cell immune responses similarly to α-GalCer, but does not induce NKT cell-dependent lysis of DCs, perhaps due to weaker binding to the TCR (214). Additionally, it was shown that while after 7 days, a second injection of ThrCer did not induce IFNγ production by NKT cells, this ability had recovered by day 14, indicating that induction of anergy is in some way determined based on either the affinity of the antigen for CD1d, or the on and off rates of the CD1d-glycolipid complex with the NKT TCR.

Figure 3. Synthetic α-GalCer analogues.

α-GalCer analogues with varying lengths of the sphingosine chain, degrees of saturation, anomeric linkages, and carbohydrate heads. Figure is adapted from Figure 2, Cerundolo V. *et al, Nat Rev Immunol* 2009. (203)



In addition to analogue studies, scientists have been searching for endogenous antigens of NKT cells, without which NKT cells would not exist. A study by Stanic et al revealed that a cell line lacking beta-D-glucosylceramide synthase could not stimulate NKT cells, while mice lacking beta-galactosylceramide have intact and functional NKT populations (163, 208). Another study by Zhou et al showed an NKT cell deficiency in mice lacking beta-hexosaminidase B, which is responsible for cleaving globosides to isoglobosides, specifically Gb4 to iGb3 including intermediate steps (105). They then showed that iGb3 stimulated NKT cells, but none of the precursors did so, thus revealing an endogenous antigen potentially playing a role in NKT positive selection. This stimulation is different from that caused by LPS, in which DCs are activated to release IL-12, which then transactivates NKT cells (208, 215, 216). Other exogenous antigens have been elucidated. For example, studies by Fischer et al revealed that the PIM4 glycolipid from mycobacteria can modestly stimulate NKT cells, while PIM2 which has two fewer sugars does not, revealing that the NKT TCR can recognize sugars distal from the antigen portion bound to CD1d (217).

1.3.6 Repeated activation of NKT cells

In 2011, our report in the European Journal of Immunology presented evidence that, in contrast to systemic immunization, two doses of α-GalCer admixed with Ova antigen via the intranasal route allow for repeated activation of NKT cells in the lung and lung-draining lymph nodes as well as induction of Ova-specific T cell responses (218). These studies explore the potential mechanism behind our 2009 publication in which multiple doses of α-GalCer by the intranasal or oral routes induced systemic and mucosal immune responses. In the previous study, we characterized the NKT cell response as a mediator of the observed adaptive immune responses reported previously. We observed that a single dose of intranasal α-GalCer resulted in a peak of IFNγ production in the lungs of mice 1 day post-immunization, which corresponded with an expansion of the NKT cell population that peaked at 5 days post-immunization. A second intranasal dose of α-GalCer again resulted in a peak of IFNγ one day post-secondary immunization and corresponded to a second expansion of the NKT cell population which peaked 5 days after the second dose. Results from each consecutive dose corresponded with an increase in CD86 expression by DCs above Ova alone control 1 day after each dose was delivered. Additionally, two doses of α-GalCer were shown to be necessary to induce significant antigen-specific CTL responses as

well as antigen-specific IFNγ-producing CD8+ T cells. To study whether the tissue of origin plays a role in the lack of NKT cell anergy induction, multiple tissues including spleen, lung, and lung draining lymph nodes were co-cultured with the NKT cell clone DN32.D3 and subsequent IL-2 production was measured. Strong activation of the clones when co-cultured with cells from the lung and mediastinal lymph node indicated that these tissues are the site of α-GalCer presentation after intranasal immunization. To characterize the cellular phenotype of the APC responsible for α-GalCer presentation in the lung, we performed bead isolation of CD11c+ and B220+ cell populations after intranasal immunization with α-GalCer, and isolated cells were co-cultured with the NKT cells clones. Only CD11c+ cells from mice immunized with α-GalCer induced IL-2 production by the NKT cell clones. Additionally, we examined the expression of PD-1, as upregulation of this marker for functional exhaustion has been associated with NKT cell anergy induced by systemic immunization with α-GalCer. There was no increase in PD-1 expression after a single intranasal dose of α-GalCer as compared to Ova alone, in contrast to a sharp increase in PD-1 expression after a single dose of intravenous α-GalCer. These results indicated a difference in functional competence of NKT cells between systemic and mucosal α-GalCer delivery, while also highlighting the necessity for multiple intranasal doses of α-GalCer to induce cellular immune responses. These studies served as the backbone of the studies in this thesis in which we explore the effects of combining intranasal and intravenous immunization routes on the induction of NKT cell anergy and subsequent adaptive immune responses.

CHAPTER 2: STATEMENT OF OBJECTIVES

Adjuvants are an indispensable part of vaccine formulations as they act to enhance adaptive immune responses to co-administered antigens. This is particularly important for vaccinations that target the tolerogenic mucosal tissues, which serve as the main portal of entry into the body for many pathogens. However, there are no mucosal adjuvants approved by the FDA. Adjuvants possess the capacity to bridge innate and adaptive immunity through activation of innate immune mediating cells such as invariant natural killer T cells (NKT). Alpha-Galactosylceramide (α-GalCer) is a synthetic glycolipid that selectively activates NKT cells, making it an attractive candidate to serve as an adjuvant in peptide and protein vaccines. We previously reported that α-GalCer delivered by the intranasal route is able to reactivate NKT cells to produce IFNγ and downstream adaptive responses after multiple immunizations, which contrasts with the trend seen after systemic immunization in which a single administration of α-GalCer results in anergy of responding NKT cells. **In the present investigation, I tested the hypothesis that the differential capacity of NKT cells to produce IFNγ in response to α-GalCer immunization, as based on the route of immunization, influences the adjuvant effect of α-GalCer and, thus, the induction of adaptive responses.** I obtained evidence that significant production of IFNγ production after each immunization may be dispensable for inducing adaptive immune responses. In addition, I found that multiple intranasal immunizations beyond two doses may in fact be inducing NKT cell anergy, but at a slower rate than the systemic counterpart. The following specific aims were designed to examine the central hypothesis that IFNγ production by NKT cells plays an important role in harnessing the adjuvant effects of α-GalCer.

Specific Aims

Aim 1: Determine the influence of combining routes on activation of NKT cells. Mice were immunized by the intranasal or intravenous routes with a combination of α-GalCer and ovalbumin (Ova) protein. Mice were immunized by one of the four following regimens: IV-IV or IV-IN, IN-IN-IN or IN-IV-IN. Adaptive immunity was measured using the IFNγ Elispot assay to enumerate antigen-specific IFNγ-producing cells in various tissues.

Aim 2: Determine the role of NKT cell reactivation and subsequent DC activation in enhancing adaptive immunity. Cells from mice following the above immunization schemes

were fluorescently stained to enumerate and determine activation levels of the NKT cell and dendritic cell (DC) populations using flow cytometry. Additionally, another group of mice was delivered four sequential doses of α-GalCer intranasally to determine if anergy is onset via this route after multiple immunizations. Adaptive responses were measured using IFNγ Elispot, and sequential intranasal immunizations with α-GalCer were further tested using a mouse model of HPV, utilizing α-GalCer and HPV peptides corresponding to conserved regions of the E6 and E7 HPV-16 oncoproteins found in cervical neoplasia.

CHAPTER 3: MATERIALS AND METHODS

3.1 ANIMALS

Female C57BL/6 mice 6-10 weeks of age were purchased from the National Cancer Institute (Bethesda, MD) and maintained in a specific pathogen-free environment at The University of Texas M.D. Anderson Cancer Center. All procedures were carried out in accordance with institutionally approved protocols. The animal facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and studies were conducted according to National Institute of Health Guidelines on the Care and Use of Laboratory Animals.

3.2 CELL LINES AND CELL CULTURES

The TC-1 tumor cells are primary lung epithelial cells of C57BL/6 mouse origin that were transfected to express the E6 and E7 oncogenes of HPV-16 as described earlier (219) and were a kind gift from Dr. T.-C. Wu, Johns Hopkins Medical Institution, Baltimore, MD. The TC-1 cells were grown in RPMI supplemented with 10% heat-inactivated FBS, 400 ug/ml of G418. The EG7·Ova cells are EL-4 thymoma cells transfected with chicken ovalbumin (220) and were a kind gift from Dr. Chen, Dept. of Immunology, UT MD Anderson Cancer Center. They were maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 ug streptomycin, and 400 ug/ml G418.

3.3 PEPTIDES AND REAGENTS

Synthetic Ova peptide corresponding to the CTL epitope of chicken ovalbumin (SIINFEKL) was purchased from Peptides International Inc. (Louisville, KY) and dissolved in 1XPBS at a concentration of 2.5mg/ml. Ova protein was purchased from Sigma (St Louis, MO) and re-suspended in 1x phosphate-buffered saline (PBS; Gibco, Carlsbad, CA) at 40mg/ml.

The synthetic glycolipid activator of NKT cells, α-GalCer or KRN7000, was purchased from Diagnocine LLC (Hackensack, NJ) and dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO) to a concentration of 1mg/ml.

Custom peptides corresponding to CD8 epitopes of the E6 and E7 oncoproteins of HPV-16 were obtained from GenWay Biotech (San Diego, CA) at a purity of >95%. $E6^{43–57}$

peptide, Q15L (QLLRREVYDFAFRDL), and $E7^{44–62}$ peptide, Q19D (QAEPDRAHVYNIVTFCCKCD) were modified to include upstream amino acids in order to facilitate purification, yielding (KQQLLRREVYDFAFRDL) and (GQAEPDRAHYNIVTFCCKCD), respectively. Peptides were resuspended at 3% and 5% DMSO to concentrations of 5mg/mL and 10mg/mL, respectively.

3.4 IMMUNIZATIONS

For dosing scheme experiments, mice were immunized by the intranasal or intravenous route 1-4 times at 5 to 7 day intervals, as described per experiment, following the procedures reported earlier (221): Mice received Ova protein alone (at 100 ug/mouse/dose) or admixed with the adjuvant synthetic glycolipid α-GalCer (at 2 ug/mouse/dose), diluted in PBS to 20ul or 200ul respectively) (10ul/nostril). For tumor challenge experiments, mice were immunized by the intranasal route, twice at 5-day intervals before TC-1 challenge or 2-3 times at varying intervals post-tumor challenge, with a mixture of the Q19D and Q15L peptides (100 ug of each/mouse) along with either CT (1 ug/mouse) or α-GalCer (at 2 ug/mouse) in a volume of 15 ul/nostril diluted in PBS. For all immunizations, mice were anaesthetized by the intraperitoneal (IP) injection of a ketamine-xylazine mixture (10mg/mL ketamine and 1mg/mL xylazine in bacteriostatic water), with volume ranging from 100-200 uL/mouse based on weight. At various time-points post immunization, mice were sacrificed and cell suspensions were prepared from the spleen by homogenization and from the lung, liver and lymph nodes by enzymatic dissociation for one and a half hours by incubation with 1 mg/mL collagenase type IV (Sigma, St. Louis, MO) solution re-suspended in complete RPMI medium. Lungs were perfused with 10 mL of 1x PBS prior to tissue collection. Lymphocytes from liver were further isolated by centrifugation through a percoll (Sigma, St. Louis, MO) gradient where 44% and 67% percoll solutions were made with serum free RPMI. Cells were suspended in the 44% percoll solution and the 67% percoll solution was layered below using a 14 gauge pipetting needle (Fisher Scientific, Waltham, MA). The solution was then centrifuged for 20 minutes at 2700 rpm, and the interphase was collected and washed in RPMI. Red blood cells were removed by incubation with ACK lysing buffer (1ml per pellet) for 5 minutes (Lonza Bioscience, Basel, Switzerland), which was then inactivated with 5ml complete RPMI.

3.5 IFNγ ELISPOT ASSAY

Cells isolated from spleen, liver, lung and mediastinal lymph nodes (MdLN) of immunized mice were subjected to Elispot assay to enumerate antigen specific IFNγ-producing cells as described earlier (221) using the reagent kit from BD Biosciences (San Jose, CA). The spots on the membrane, representing individual IFNγ-producing cells as spot forming colonies (SFC), were enumerated by the independent agency Zellnet Consulting Inc., New York, NY using the KS-ELISPOT automatic system (Carl Zeisis Inc., Thornwood, NY). Responses were considered positive when they were above 10 SFC/well and at least double the number obtained in cells cultured with medium alone.

3.6 FLUORESCENCE LABELED ANTIBODIES AND FLOW CYTOMETRY

Single-cell suspensions isolated from the various tissues of immunized mice were analyzed for the phenotype and function of NKT cells by flow cytometry. All the reagents, unless otherwise mentioned, were purchased from BD Biosciences, San Jose, CA. The NKT cells were stained first with Aqua Live/Dead reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were then washed and incubated with APC-conjugated mouse CD1d tetramer loaded with PBS57 (provided by NIAID tetramer facility at Emory University, Atlanta, GA, USA) at a concentration of 0.25ul in 50ul 1x PBS for 30 min in the dark at 37℃. Cells were then additionally incubated with a combination of surface markers, Pacific Blue-conjugated CD3 (clone 500A2, BD Biosciences), FITC-conjugated PD-1 (clone J43, eBioscience, San Diego, CA,USA), and PE-Cy7 CD69 (H1.2F3, eBiosciences) for an additional 30 min at 4℃ in the dark. Cells were then washed and fixed in 100ul of 4% Paraformaldehyde diluted in 1x PBS for 10 min at 0℃ in the dark. The percentages of DCs and their activation status were analyzed by staining for FITC-conjugated CD11b (clone M1/70, BD Biosciences), APC-conjugated CD11c (clone HL3, BD Biosciences), and PE conjugated CD86 (clone GL1, BD Biosciences) for 30 min at 0℃. After staining, all cells were analyzed on an LSRII flow cytometer (BD Biosciences) and the data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA). For NKT cell analysis, lymphocytes were first gated using the forward scatter and side scatter plots. Next, live cells were gated using side scatter and Aqua plots. Finally, the NKT cell population was determined by plotting PB-CD3 against the CD1d tetramer and these cells were analyzed further for surface marker expression and cytokine production. For DC analysis, cells were

first gated using the forward scatter and side scatter plots. Next CD11c+CD11b^{low} cells were gated and then CD86 expression was determined by histogram plots.

3.7 INTRACELLULAR CYTOKINE STAINING

For intracellular cytokine staining all cells were incubated with GolgiPlug (1ul/well in 200ul complete RPMI) (BD Biosciences, San Jose, CA) for 4.5 hours at 37℃ before any cellular staining. Cells were stained for surface markers and fixed as described above. Cells were then washed and incubated with cytokine antibodies in 1x BD Perm/Wash Buffer (BD Biosciences, San Jose, CA) for 60 minutes at 4℃. The cytokine specific antibody used in these experiments was PE-conjugated anti-IFNγ (clone XMG1.2, BD Biosciences, San Jose, CA). Cells were then washed two more times in the Perm/Wash buffer and resuspended in PBS containing 5% FBS, and samples were analyzed on the LSRII as described above.

3.8 STATISTICAL ANALYSIS

The data were analyzed for statistical significance using the student's t-test for paired samples and a p-value of <0.05 was considered significant. Statistical significance between immunizations routes was calculated using two-way ANOVA of repeated measures, and a p-value of <0.05 was considered significant. Data shown is representative of results obtained from individual experiments.

CHAPTER 4: RESULTS

4.1 EFFECT OF α-GALCER WHEN DELIVERED VIA THE IV-(IV/IN) SCHEME

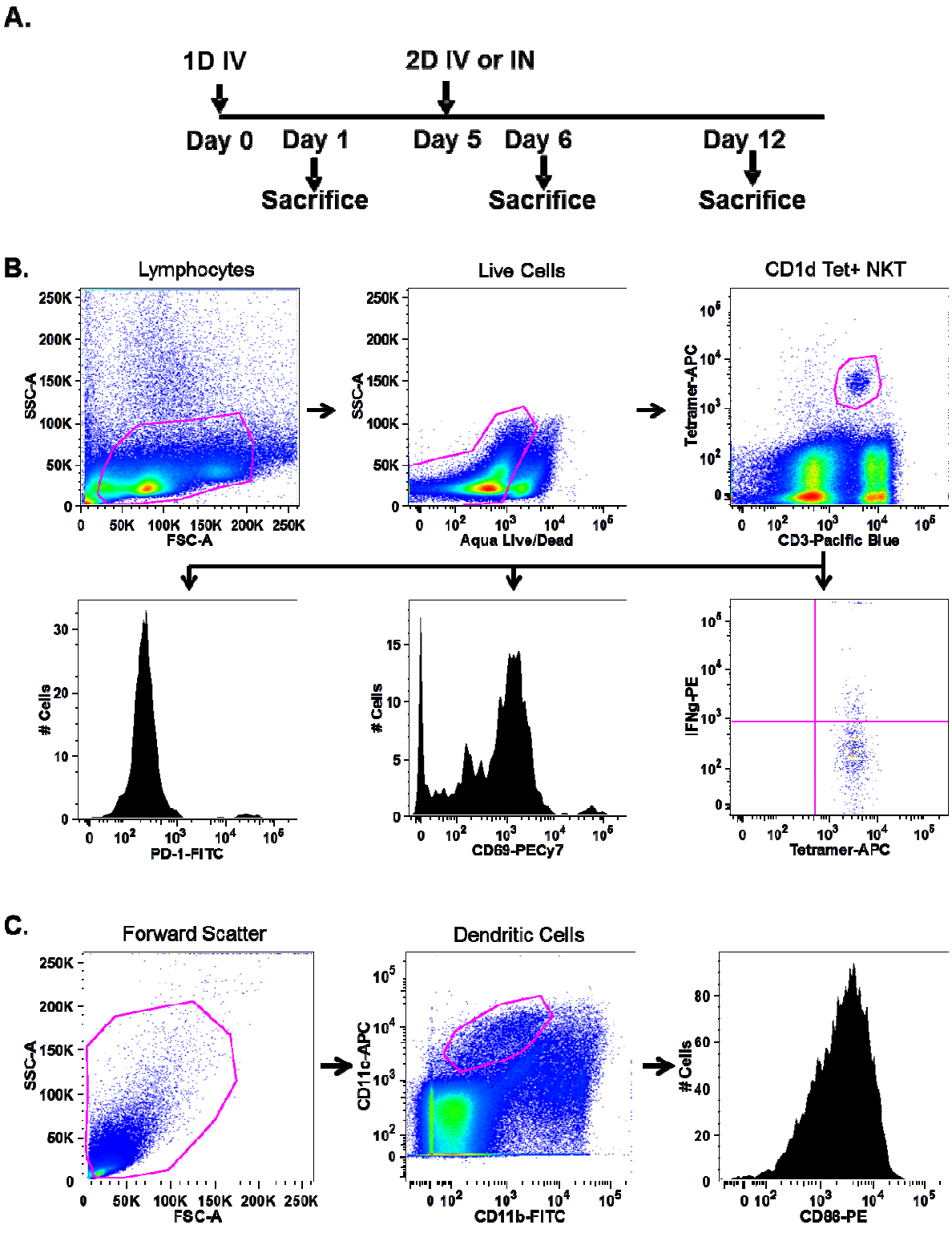
It is known that a single administration of intravenous α-GalCer strongly activates NKT cells to produce IFNγ and IL-4 (193). We and others have reported that these responses exert a potent adjuvant effect by strongly promoting adaptive humoral and cellular immune responses to co-administered antigen. However, it has also been shown that immunization with α-GalCer by the systemic intravenous route results in NKT cell anergy, a state in which a subsequent dose of α-GalCer does not activate NKT cells to produce IFNγ and does not lead to induction of an adaptive T cell response. In contrast, our previous studies have shown that multiple doses of α-GalCer can be administered via the intranasal route resulting in the reactivation of NKT cells to produce IFNγ and improved adaptive immune responses with each additional dose, up to a total of three tested. We therefore hypothesized that the route of α-GalCer delivery influences whether or not NKT cells develop anergy and that repeated activation of NKT cells and the production of IFNγ are important for the sustained adjuvant effect observed after mucosal (intranasal) versus systemic (intravenous) administration of α-GalCer. We investigated whether use of a combination of the intravenous and intranasal routes for the administration of α-GalCer could circumvent the induction of NKT cell anergy, thus allowing repeated activation and IFNγ production to facilitate sustained and/or improved adaptive immune responses to co-administered antigen.

We first tested whether administration of Ova (+/- α-GalCer) by the intravenous route followed by a second intranasal dose (IV-IN regimen), versus a second intravenous dose (IV-IV regimen), would reactivate NKT cells to produce IFNγ and elicit sustained and/or stronger Ova-specific adaptive T cell responses. As shown in Figure 4, separate groups of mice were administered the first dose of Ova (+/- α-GalCer) by the intravenous route. One day later, one set of mice was sacrificed and single cell suspensions from the spleen, liver, lung, and lung-draining mediastinal lymph node (MdLN) tissues were analyzed to determine NKT and dendritic cell responses. The second group of mice received a second dose of Ova (+/- α-GalCer) by the intranasal route five days after the first dose (IV-IN regimen). From among these, one set of mice was sacrificed on day six (one day after the second dose) to measure NKT cell responses as well as adaptive immune responses to the first round of

immunization by the IV route, specifically IFNγ production by CD8+ T cells in response to in vitro stimulation with an Ova-specific CD8+ T cell epitope peptide. Finally, a third group of mice was sacrificed on day twelve, which was seven days after the second round of immunization by the IN route, to measure adaptive immune responses subsequent to the second intranasal dose of Ova (+/- α-GalCer). In parallel, additional groups of mice were administered a second dose of Ova (+/- α-GalCer) by the intravenous route (IV-IV regimen) and subjected to similar analyses. Using fluorescently labeled antibodies and flow cytometry, NKT cells were identified and analyzed for activation in terms of production of IFNγ and expression of CD69 and PD-1. First, lymphocytes were gated using a forward scatter versus side scatter plot. Next, live cells were gated using a side scatter versus Aqua live/dead staining. The live lymphocytes were then gated using CD3-Pacific Blue versus CD1d Tetramer-APC staining to identify the double positive population as the NKT cells (Figure 4 B). The NKT cells were further analyzed for production of IFNγ as an indication of functional activation. We also analyzed the IFNγ positive population for the expression of CD69 activation marker using anti-CD69-PECy7 antibody and PD-1 anergy marker using anti-PD-1-FITC antibody. Additionally, dendritic cells were analyzed by flow cytometry after staining for the CD11c+ population to characterize expression of CD86 by mean fluorescence intensity (MFI) histogram. Finally, the adaptive cellular immune responses to the co-administered Ova antigen were analyzed by the IFNγ Elispot methodology employing the Ova-specific CD8+ T cell epitope peptide.

Figure 4. Immunization scheme and gating strategy for flow cytometry analyses of NKT and dendritic cells. (A) Mice were immunized with Ova +/- α-GalCer first by the intravenous route followed by second immunization either by the intravenous or intranasal route at day 5 after the primary immunization. Mice were sacrificed on days 1, 6, and 12 post-primary immunization. (B) Single cell suspensions prepared from the spleens, lungs, livers, and mediastinal lymph nodes of immunized mice were stained with fluorescently labeled reagents to detect NKT cells using: anti-CD3-PB, CD1d tetramer-APC, anti-IFNγ-PE, and Aqua live/dead stain. Aqua live/dead stain, anti-CD3-PB, CD1d Tetramer-APC, anti-IFNγ-PE, anti-PD-1-FITC, and anti-CD69-PECy7. The gating strategy for enumerating live CD1dTet+CD3+ cells expressing PD-1, CD69, and IFNγ is shown. (C) The dendritic cells isolated from the spleens, lungs, livers, and mediastinal lymph nodes were identified by staining with fluorescently labeled anti-CD11b-FITC, anti-CD11c-APC, and anti-CD86-PE to enumerate $CD11b^{low}$CD11c+ dendritic cell population and to measure activation by CD86 expression. Gating strategy is shown. Panels show a representative spleen from a mouse that received aGC+Ova by the intranasal route.

Figure 4. Immunization scheme and gating strategy for flow cytometry analyses of NKT and dendritic cells.



4.1.1 Analyses of cells from the spleen

A single IV dose of α-GalCer elicited strong activation of NKT cells in the spleen as evidenced by potent IFNγ production, PD-1 expression, and significant CD69 expression (Figure 5 A,B,C). Concurrently, we observed strong activation of DCs as measured by CD86 expression (Figure 5 D). We also observed significantly higher levels of adaptive immune response in terms of Ova peptide-specific IFNγ producing cells in mice that received α-GalCer+Ova relative to those that received Ova alone (Figure 5 E). Mice administered a second IV immunization of α-GalCer+Ova (IV-IV) showed greatly reduced activation of NKT cells which was however significantly higher than that observed in mice immunized twice with Ova alone by the IV route (Figure 5 A). Nevertheless, ANOVA of the data for fold change in IFNγ production by NKT cells from mice immunized twice by the IV route with Ova +/- α-GalCer revealed significantly lower responses compared to that seen in mice subjected to one IV immunization (Figure 5 A). Also, the NKT cells from mice immunized twice with Ova +/- α-GalCer by the IV route did not show any differences in the expression of CD69 while PD-1 levels remained elevated above control values, indicating anergy of NKT cells (Figure 5 B,C). Similarly there was a lack of DC activation subsequent to the second intravenous administration of α-GalCer (Figure 5 D). Furthermore, the numbers of Ova peptide-specific IFNγ-producing cells were higher but did not reach significance in mice immunized with α-GalCer+Ova as compared to those in the control group that received Ova alone (Figure 5 E). These results are consistent with reports from our group and those of others in the literature, suggesting that NKT cells were anergic in mice following the IV-IV regimen of immunization which may have adversely affected the development of adaptive immune responses. Similar results were obtained after a second dose of Ova +/- α-GalCer was delivered by the intranasal route to mice that received the first dose by the intravenous route (IV-IN regimen), after which NKT cells showed a lack of IFNγ production, a significant decrease in CD69, but increased PD-1 expression as compared to control mice that received Ova alone (Figure 5 A,B,C), all indicative of NKT cell anergy. However, the IV-IN regimen of immunization resulted in a statistically significant adaptive immune response as measured by Elispot assay (Figure 5 E) coinciding with significant activation of dendritic cells relative to the respective responses in mice that received only Ova protein by the IV-IN regimen (Figure 5 D). One possibility is that intranasal immunization targets presentation of α-GalCer and/or the Ova antigen to a different subset(s) of antigen presenting cells, sufficiently changing the interaction of APCs with NKT cells to avoid overstimulation and

anergy induction. Overall, these data show that a second immunization either by the IV or IN route subsequent to primary IV immunization equally and significantly reduced IFNγ production by the NKT cells relative to that after the first IV immunization (Figure 6 A). This data also coincided with that from the analysis of DCs where a single IV immunization resulted in significantly more activated DCs than those in mice immunized either twice by the IV route or first by IV followed by IN, and also there was no significant difference in DC activation when comparing the two routes for the second immunization (Figure 6 D). This trend also applied to data from the analysis of PD-1 expression on the NKT cells (Figure 6 B). However, while a single IV immunization of α-GalCer+Ova led to significantly higher CD69 expression by NKT cells as compared to that in mice immunized following the IV-IV regimen, IV-IV mice continued to express significantly more CD69 than mice following the IV-IN regimen, reflecting that delivery of α-GalCer by the IV route activated NKT cells more strongly than via the IN route (Figure 6 C).

Figure 5. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the spleen after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1 and 6) for determining activation of NKT cells by flow cytometry (panels A-D) and at six days after each dose (day 6 and 12) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of spleen cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from spleen in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 5. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the spleen after immunization employing α-GalCer as an adjuvant.

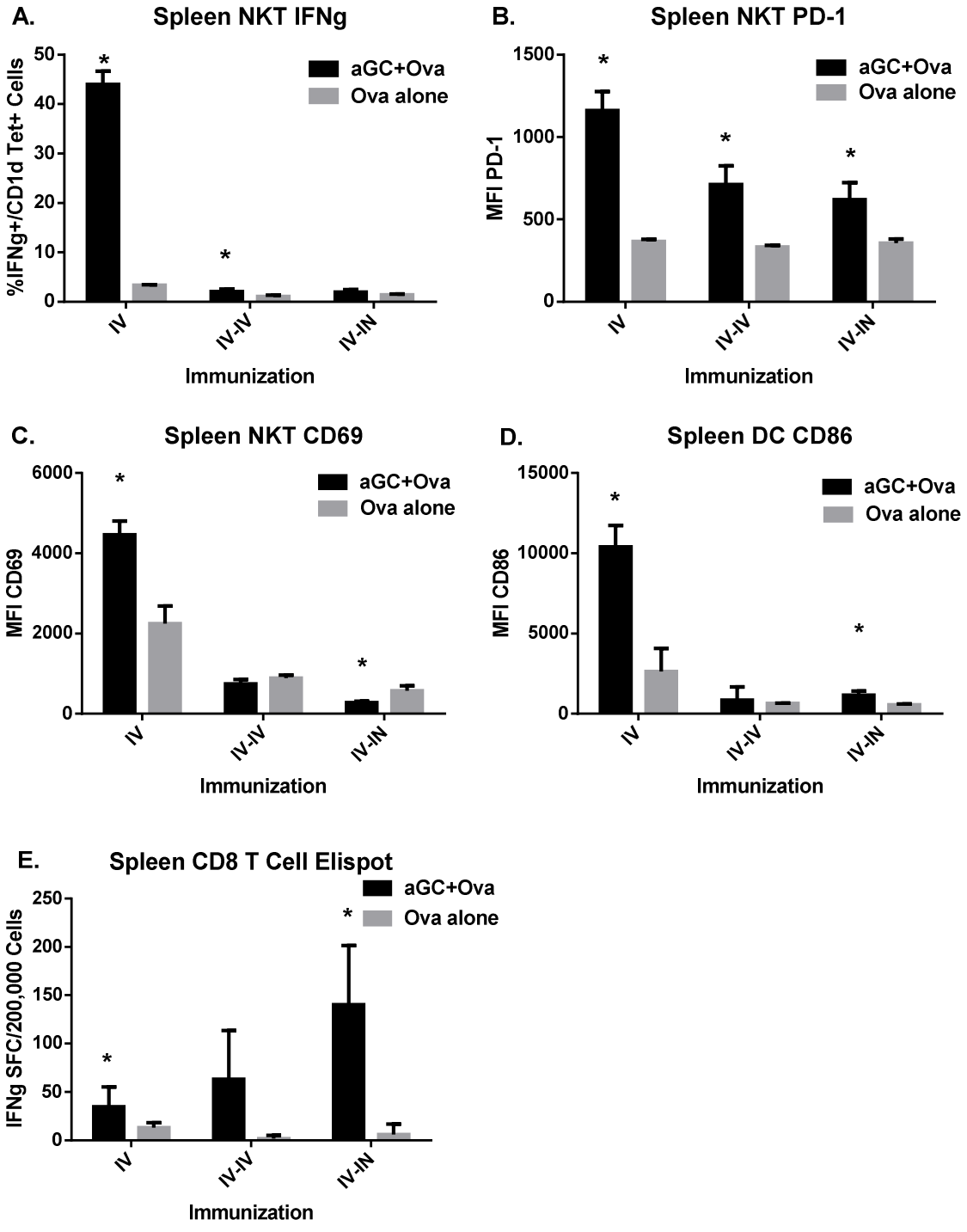
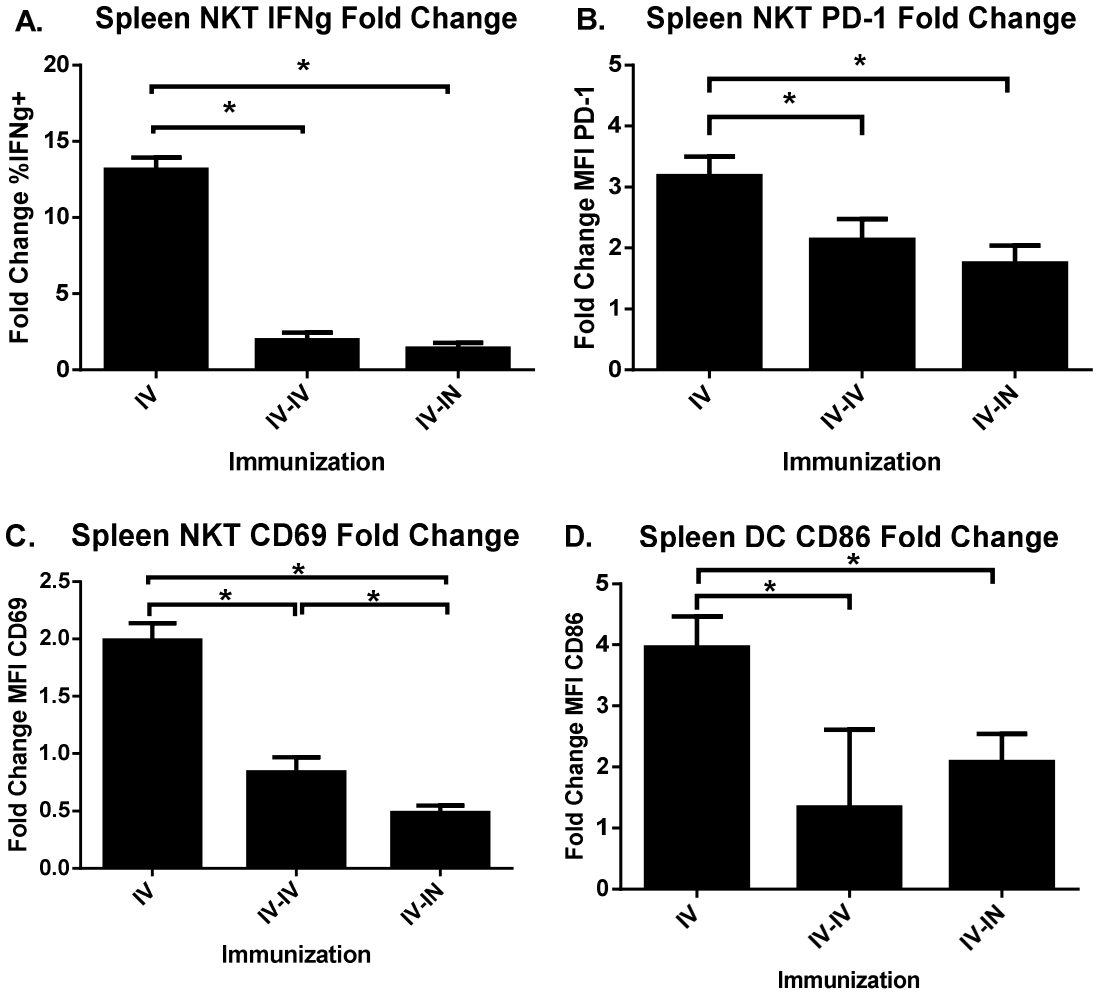


Figure 6. Combinatorial route effect on NKT cell and DC activation in the spleen after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 6. Combinatorial route effect on NKT cell and DC activation in the spleen after immunization employing α-GalCer as an adjuvant.



4.1.2 Analyses of cells from the liver

The first intravenous immunization of α-GalCer+Ova, relative to immunization with Ova alone, induced significantly higher production of IFNγ and expression of PD-1 and CD69 activation markers by the NKT cells in the liver (Figure 7 A,B,C). This relative increase in NKT cell activation coincided with activation of DCs as measured by CD86 expression (Figure 7 D). Interestingly, positive adaptive response, in terms of significantly higher number of IFNγ-producing Ova peptide-specific CD8+ T cells relative to the assay background value, was observed not only in mice immunized with α-GalCer+Ova but also, to similar extent, in those immunized with Ova alone (Figure 7 E). Subsequent to the second intravenous immunization, the NKT cells in the liver exhibited functional and phenotypic characteristics of anergy of NKT cells in terms of lack of significant IFNγ production along with significantly decreased CD69 expression and significantly higher levels of PD-1, all relative to the values for mice immunized with Ova alone (Figure 7 A-C). Additionally, comparable levels of CD86 expression between mice immunized with Ova +/- α-GalCer indicated a lack of DC activation (Figure 7 D). These results also coincided with an adaptive immune response that, however, was not significantly higher than that observed in mice immunized twice with Ova alone (Figure 7 E). Similar results were obtained after the second dose of α-GalCer+Ova was delivered by the intranasal route, where NKT cells exhibited an anergic phenotype (Figure 7 A-C), DCs were not activated (Figure 7 D), and the adaptive immune response, despite being strong, was not significant relative to that in mice receiving Ova alone (Figure 7 E). Overall, IFNγ production as well as expression of CD69 and PD-1 markers by the NKT cells in the liver was significantly reduced after a second dose regardless of the route of immunization, and also there was no difference in these parameters in mice immunized by the IV-IV or IV-IN regimens (Figure 8 A-C). Similar results were observed for the activation of DC in the liver (Figure 8 D).These data suggest that the intranasal administration of α-GalCer is not able to reverse the anergy of NKT cells induced by the prior intravenous dosing, which was reflected by a lack of significant enhancement of adaptive immune response by additional immunization, regardless of the route.

Figure 7. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the liver after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1 and 6) for determining activation of NKT cells by flow cytometry (panels A-D) and at six days after each dose (day 6 and 12) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of liver cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from liver in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 7. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the liver after immunization employing α-GalCer as an adjuvant.

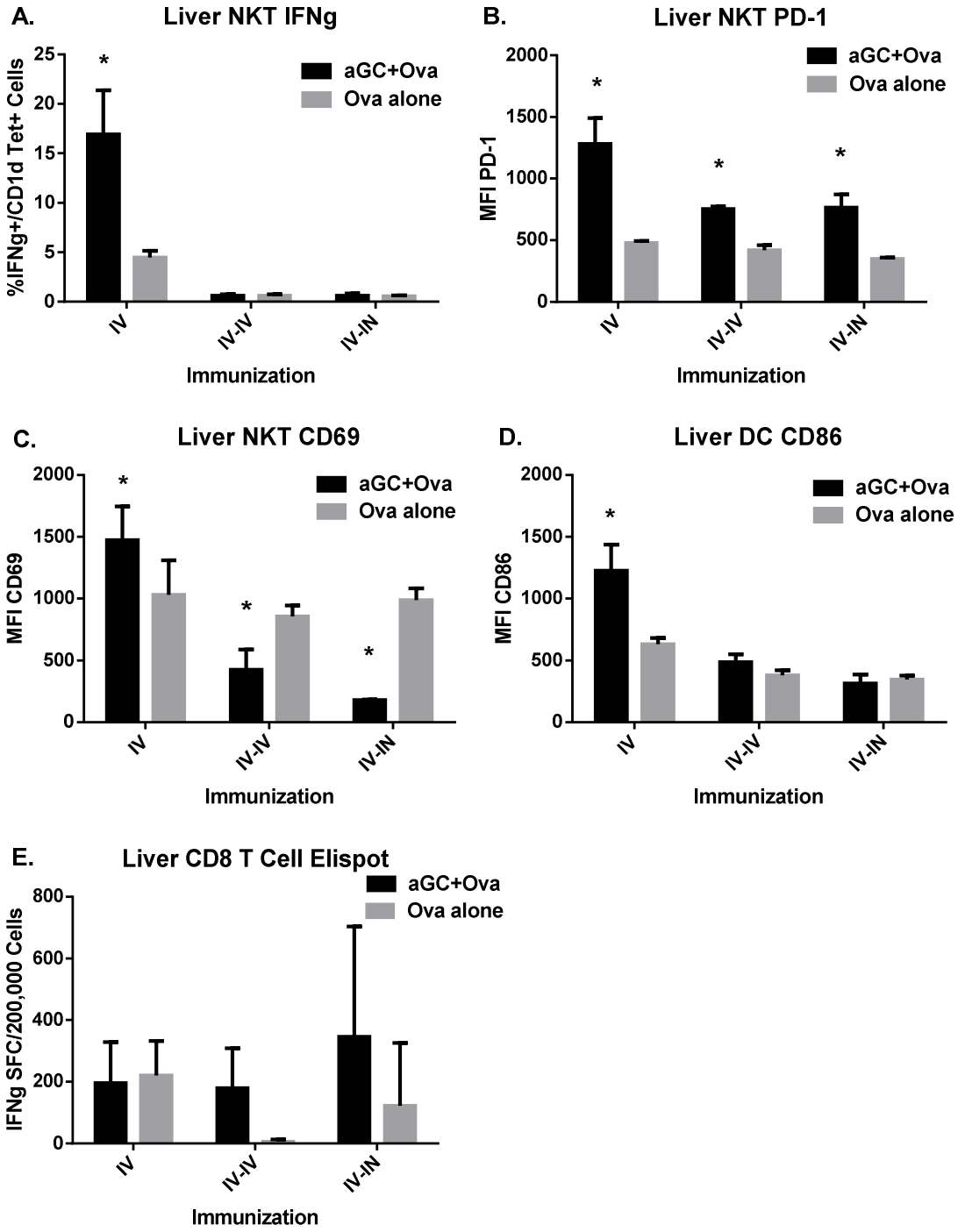
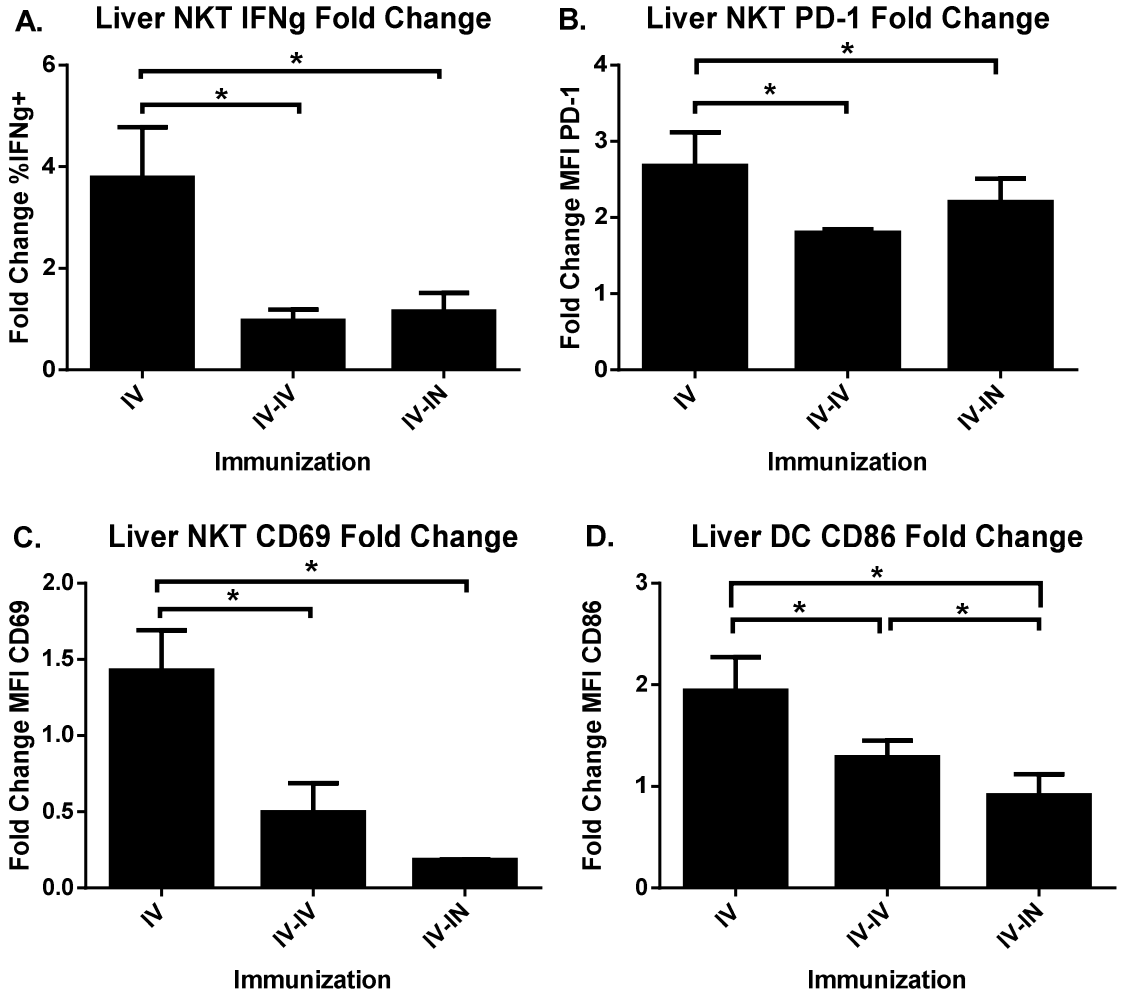


Figure 8. Combinatorial route effect on NKT cell and DC activation in the liver after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 8. Combinatorial route effect on NKT cell and DC activation in the liver after immunization employing α-GalCer as an adjuvant.



4.1.3 Analyses of cells from the lung

While the lung is not considered to be a responding tissue to systemic immunization routes, it is the main responding tissue to intranasal immunization.(4) After an initial intravenous immunization with α-GalCer+Ova, the NKT cells in the lung exhibited characteristics of functional activation in terms of producing significant levels of IFNγ as well as expressing PD-1 and CD69 activation markers (Figure 9 A-C). We also observed that the dendritic cells were activated as evidenced by the expression of significant levels of CD86 relative to cells from the lungs of mice that received Ova alone (Figure 9 D). Additionally, we observed a modest adaptive immune response, as measured by the Elispot analysis, which, however, did not reach significance when compared to that in mice immunized with Ova alone (Figure 9 E). Upon secondary immunization by the intravenous route, IFNγ production and CD69 expression by the NKT cells were reduced (Figure 9 A, C). However, the PD-1 expression on the NKT cells was persistent and also significant in mice immunized with α-GalCer+Ova relative to mice immunized with Ova alone by the IV-IV regimen (Figure 9 B). Despite the apparent lack of significant functional activation of NKT cells in the lung in terms of IFNγ production after two successive immunizations by the IV route, the DCs were significantly activated as measured by the CD86 expression in mice administered a second intravenous dose of α-GalCer+Ova relative to those immunized with Ova alone (Figure 9 D), though the differential increase was less dramatic and greatly reduced compared to that after the first IV immunization. The presence of significant DC activation corresponded to a significant and also improved adaptive response to the second IV immunization of α-GalCer+Ova in this tissue, seemingly unrelated to the lack of significant IFNγ production by the NKT cells (Figure 9 E). A secondary intranasal immunization with α-GalCer+Ova, relative to Ova alone, elicited significant IFNγ production by the NKT cells while also exhibiting significant PD-1 expression (Figure 9 A,B) but no differential levels of CD69 expression, unlike in the similar secondary IV immunization (Figure 9 C). The level of CD86 expression by dendritic cells after IV-IN immunization was higher but did not reach statistical significance in mice immunized with α-GalCer+Ova compared to that in mice immunized with Ova alone (Figure 9 D), but the fold change above Ova alone control was significantly higher than that observed after the first IV immunization (Figure 10 D). Interestingly, coinciding with significant levels of IFNγ production by NKT cells, there was a significantly positive adaptive immune response in the lung to the secondary intranasal immunization of α-GalCer+Ova relative to Ova alone (Figure 9 E), and this differential response was higher

than that elicited by a single intravenous immunization. Overall, the initial immunization by the IV route with α-GalCer+Ova relative to Ova alone showed a significantly higher fold change in the levels of IFNγ production by the NKT cells in the lung, and a secondary immunization by the IV route dampened this response while a secondary IN immunization sustained it (Figure 10 A). It is noteworthy that despite sustained or increased IFNγ production by NKT cells after the second immunization by the IV or IN routes, the CD69 expression was significantly reduced while the PD-1 levels were persistent and significantly higher than those observed after a single IV immunization (Figure 10 C, B). This, however, did not correspond to the activation of DCs, in which a single IV immunization activated DCs significantly more than after the second immunization by either route, and levels of activation for DCs in mice from IV-IV and IV-IN regimens were comparable (Figure 10 D). Thus, a potential link between NKT cell activation and induction of adaptive immune responses appears to be less consistent in this tissue, with dendritic cell activation not necessarily corresponding with induction of adaptive immunity.

Figure 9. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the lung after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1 and 6) for determining activation of NKT cells by flow cytometry (panels A-D) and at six days after each dose (day 6 and 12) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of lung cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from lung in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 9. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the lung after immunization employing α-GalCer as an adjuvant.

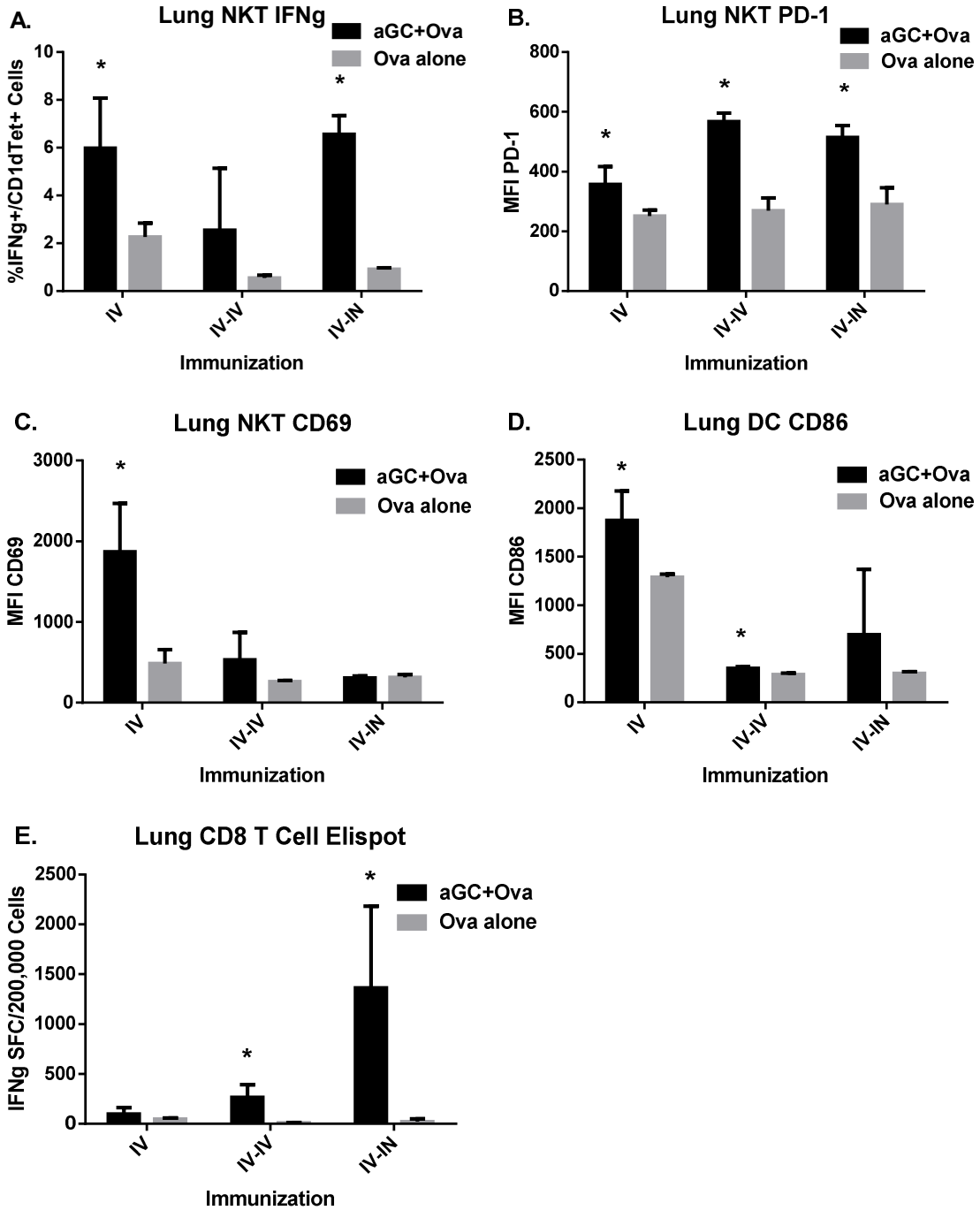
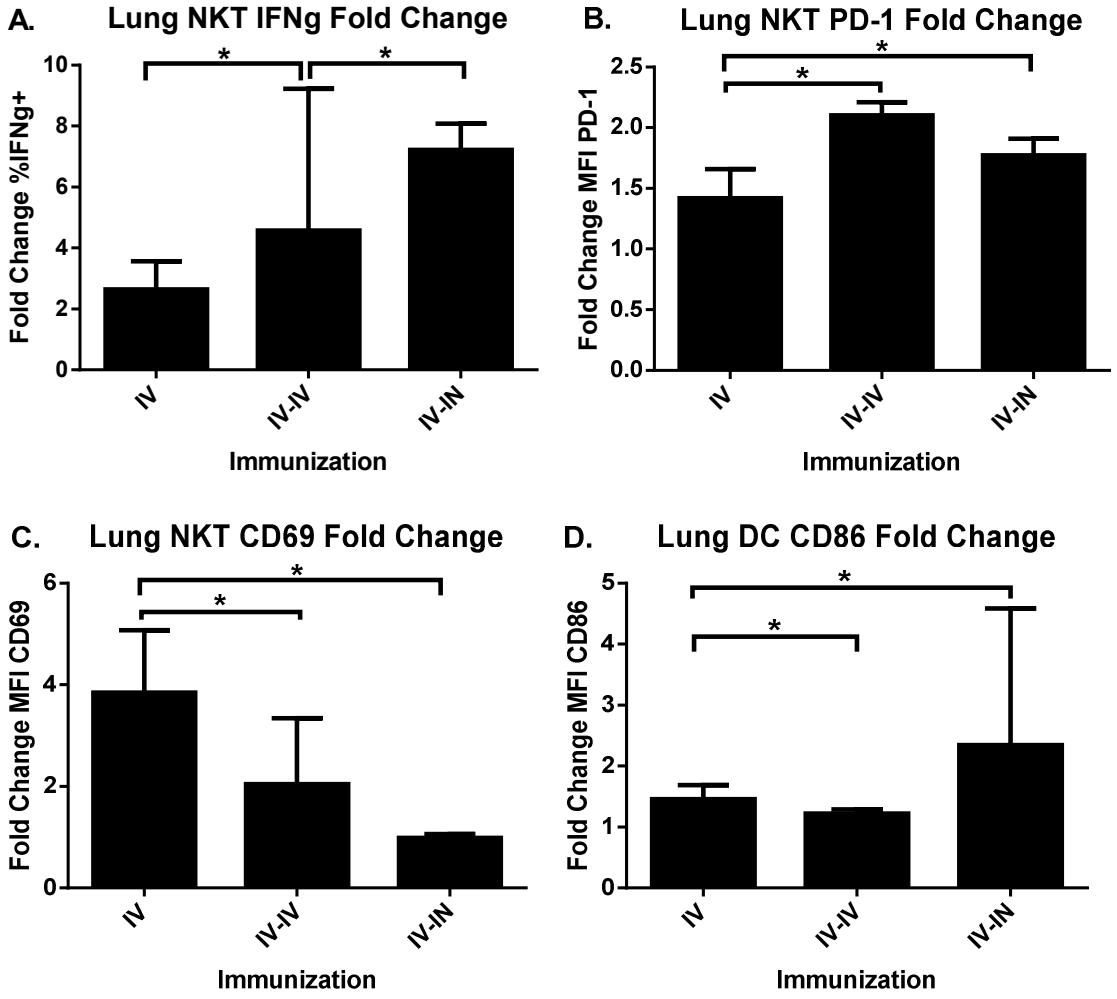


Figure 10. Combinatorial route effect on NKT cell and DC activation in the lung after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with OVA+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effectivene activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 10. Combinatorial route effect on NKT cell and DC activation in the lung after immunization employing α-GalCer as an adjuvant.



4.1.4 Analyses of cells from the mediastinal lymph nodes (MdLN)

Finally, we analyzed cells isolated from the lung-draining mediastinal lymph nodes of mice immunized with Ova+/-α-GalCer first by the IV route and followed in separate groups by a second immunization by either the IV or IN route. After the first IV immunization with α-GalCer+Ova, relative to Ova alone, the NKT cells produced a higher amount of IFNγ but showed no difference in the expression of either PD-1 or CD69, suggesting poor activation (Figure 11 A-C). Correspondingly, there was minimal activation of DC which was not significantly different from what was detected in mice immunized with Ova alone, and also no adaptive immune response was observed (Figure 11 D). These results suggest that the observed IFNγ+ NKT cells may not be those which are resident in the MdLN and/or possibly drained to this tissue from the lung, a tissue in which NKT cells produced significant amounts of IFNγ (Figure 9 A). A second immunization with α-GalCer+Ova by the IV route did not result in reactivation of NKT cells in terms of IFNγ production or CD69 expression, but PD-1 levels were significantly higher than those in mice immunized with Ova alone (Figure 11 A,C,B). Even though the second IV immunization with α-GalCer+Ova resulted in significant DC activation relative to that in mice immunized with Ova alone, the overall level of DC activation in the mice from the IV-IV immunization regimen was lower than what was observed after the first IV immunization with α-GalCer+Ova (Figure 11 D). However, this low but significant level of DC activation in mice immunized with α-GalCer+Ova, relative to mice immunized with Ova alone, did not translate to a positive adaptive immune response (Figure 11 E). As speculated above, it is possible that the activated dendritic cells observed in the MdLN were a result of migration from another tissue. In response to a secondary intranasal immunization with α-GalCer+Ova, once again there was no activation of NKT cells as measured by a lack of IFNγ production, significantly higher expression of PD-1, and significantly decreased CD69 expression as well as no DC activation when compared to mice immunized with Ova alone (Figure 11 A-D). Despite the lack of functional activation of NKT cells and DC in mice from the IV-IN regimen, there was a significantly positive adaptive immune response in this tissue relative to that in mice administered Ova alone (Figure 11 E), which might also be a result of migration of antigen-specific CD8+ T cells from the lung. Overall, ANOVA of data from mice immunized with Ova +/- α-GalCer once or twice by the IV route (IV and IV-IV regimens respectively) or first by the IV route followed by the IN route (IV-IN regimen) showed no significant difference in regards to IFNγ production as well as PD-1 expression by the NKT cells in the MdLN (Figure 12 A-B). However, with respect to

CD69 expression by the NKT cells, significantly higher levels were observed in mice that received a single IV immunization when compared to those in mice that followed the IV-IN regimen, while mice following the IV-IV regimen showed levels that were comparable to those in mice administered a single IV immunization (Figure 12 C). Also, CD69 expression was comparable between the IV-IV and IV-IN groups of mice (Figure 12 C). Finally, the ANOVA for these data revealed that activation of DCs was significantly higher in mice from the single IV regimen than those in both the IV-IV and IV-IN regimens, while the data for the mice in the IV-IV and IV-IN regimens were comparable (Figure 12 D).Thus, it appears that in this tissue, activation of DCs may be a result of migration from another tissue, as all other factors are negative for activation.

Overall, in the liver, a tissue generally considered to be mainly responsive to IV immunization, it appears that a single IV dose of α-GalCer strongly activates NKT cells leading to an anergic phenotype in those cells. Subsequent dosing, regardless of route, was not able to overcome this anergy. However, in the lung, a tissue that may not be considered a main responder to systemic immunization, it appears that activation of NKT cells as measured by IFNγ production in these tissues is able only to induce a minimal adaptive immune response. While subsequent IV dosing does not elicit response in these tissues, it appears that the resident NKT cells remain responsive to intranasal immunization.

Figure 11. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the MdLN after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1 and 6) for determining activation of NKT cells by flow cytometry (panels A-D) and at six days after each dose (day 6 and 12) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of MdLN cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from MdLN in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 11. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the MdLN after immunization employing α-GalCer as an adjuvant.

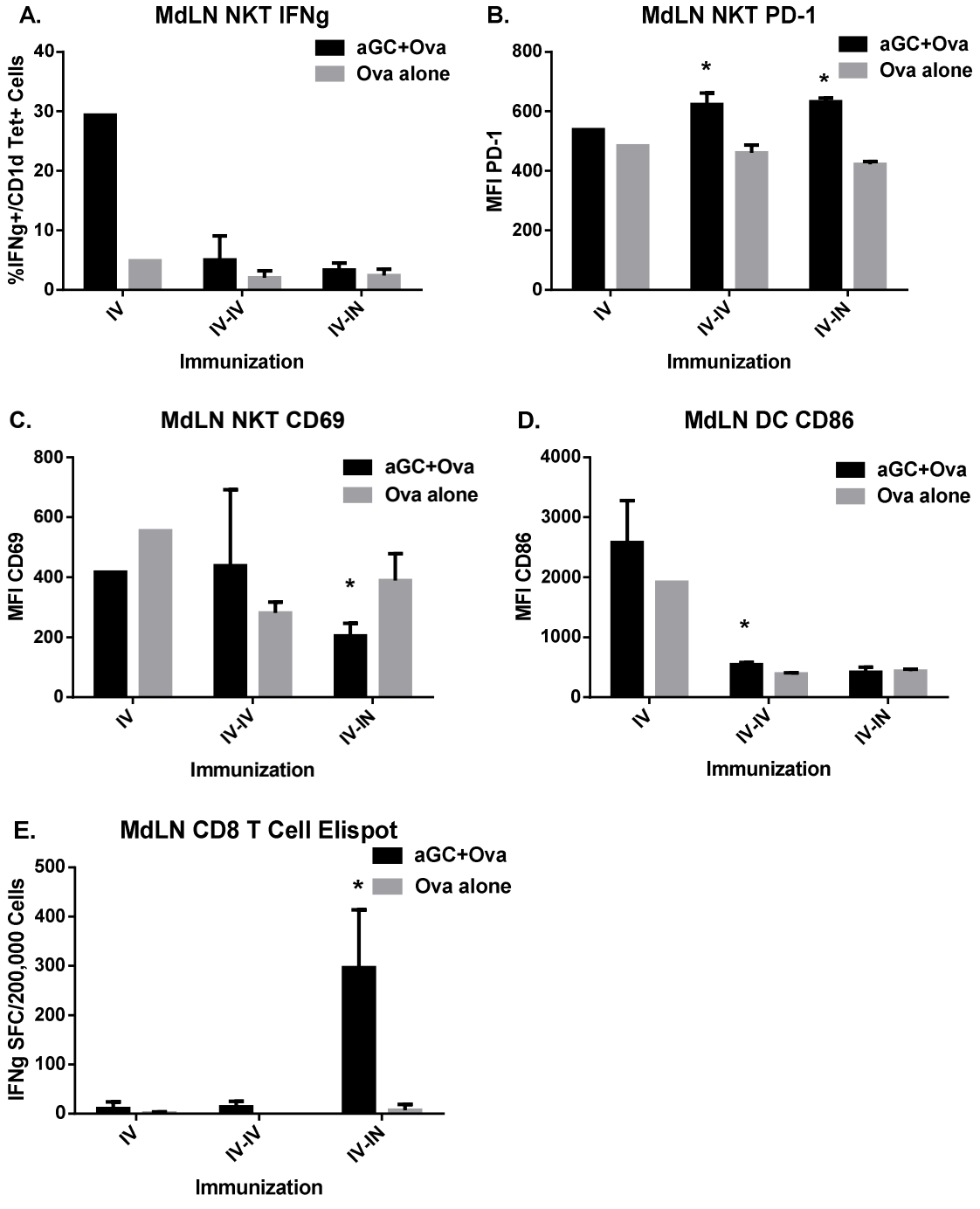
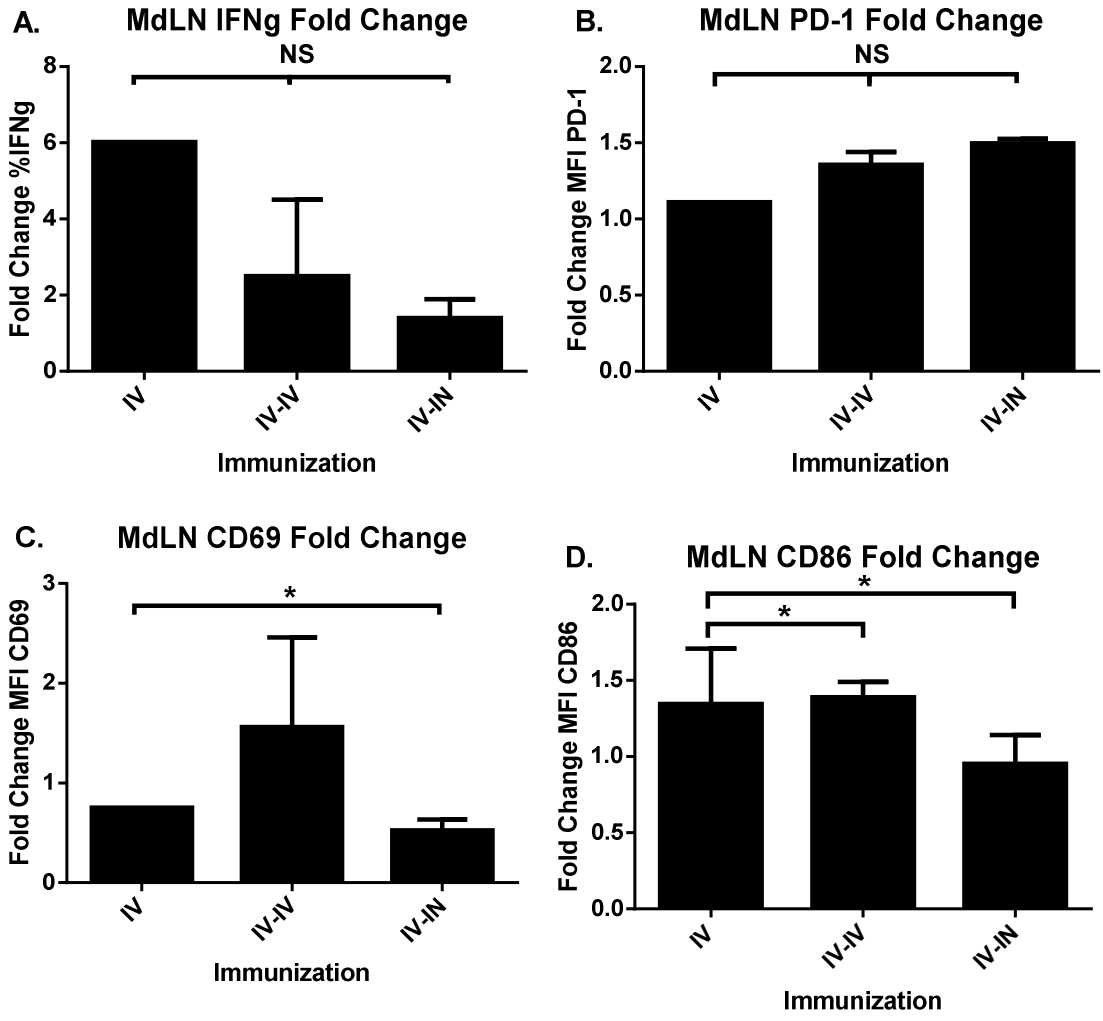


Figure 12. Combinatorial route effect on NKT cell and DC activation in the MdLN after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 12. Combinatorial route effect on NKT cell and DC activation in the MdLN after immunization employing α-GalCer as an adjuvant.



4.2 EFFECT OF A-GALCER WHEN DELIVERED VIA THE IN-(IV-IN)-IN SCHEME

We next investigated the effects of a priming intranasal immunization of Ova+/- α-GalCer followed by a boosting dose delivered by the intravenous versus intranasal route (Figure 13) on the activation of NKT cells and DCs and induction of adaptive immunity to the Ova antigen. All results for these experiments were presented as a comparison of responses in mice immunized with α-GalCer+Ova to those in mice immunized with Ova alone.

Figure 13. IN-IN/IV-IN immunization scheme.

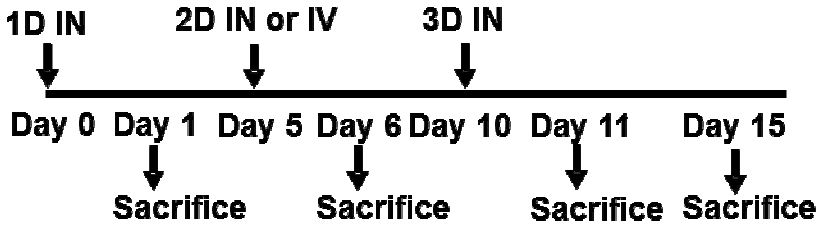


Figure 13. Mice were immunized with Ova+/- α-GalCer first by the intranasal route followed by second immunization either by the intravenous or intranasal route at day 5 after the primary immunization. Both IN-IN and IN-IV groups received a third intranasal immunization. Mice were sacrificed on days 1, 6, 11 and 15 post-primary immunization.

4.2.1 Analyses of cells from the spleen

After the first immunization with α-GalCer+Ova by the IN route, we observed strongly activated NKT cells in terms of production of IFNγ as well as expression of PD-1 and CD69, but DC activation was not significantly different from control mice that were administered Ova alone (Figure 14 A-D). However, we observed a significant amount of adaptive response as measured by the IFNγ Elispot analysis (Figure 14 E). A second intranasal immunization with α-GalCer+Ova reactivated NKT cells to produce significant amounts of IFNγ along with significant expression of PD-1 and CD69 (Figure 14 A-C). However, the activation of DCs in terms of CD86 expression, while higher in mice administered α-GalCer+Ova than those receiving Ova alone, did not reach significance, and

correspondingly adaptive immune response was higher but was not significantly different from control value in mice immunized with Ova alone (Figure 14 D-E). A second immunization with α-GalCer+Ova delivered by the IV route to mice that initially were immunized by the IN route resulted in significant activation of NKT cells in terms of IFNγ production as well as expression of PD-1 and CD69, with corresponding significant DC activation and a positive adaptive immune response, which was higher than control values from mice immunized with Ova alone but did not reach statistical significance (Figure 14 A-E). Sequential immunizations with α-GalCer+Ova, relative to Ova alone, first by the IN route followed by IV route (IN-IV) resulted in slightly higher levels of IFNγ-producing NKT cells when compared to that after the first IN immunization (Figure 15 A). However, two sequential immunization by the IN route (IN-IN) with α-GalCer+Ova, relative to Ova alone, resulted in NKT cells producing significantly lower levels of IFNγ than those in mice that followed the IN or IN-IV immunization regimen (Figure 15 A). Similar ANOVA of the PD-1 expression by the NKT cells showed comparable levels between IN and IN-IV groups, but significant reduction when values for mice in the IN-IN group were compared to those for mice in the IN and IN-IV groups (Figure 15 B). The CD69 expression is significantly elevated in mice immunized by the IN-IV and also the IN-IN groups relative to mice immunized once by the IN route. We also observed that CD69 expression in mice from the IN-IV group was significantly higher than that in mice from the IN-IN group (Figure 15 C). With respect to DC activation, immunization by the IN-IN as well as IN-IV regimens resulted in significantly elevated CD86 expression above one dose, while the DC activation after IN-IV immunization was significantly elevated above that after IN-IN immunization (Figure 15 D). Finally, a third consecutive dose of α-GalCer delivered by the IN route (IN-IN-IN scheme) showed reactivation of NKT cells to produce IFNγ, along with significant expression of PD-1and CD69 activation markers (Figure 14 A-C). The DC activation in mice immunized with α-GalCer+Ova by the IN-IN-IN regimen was minimal but significantly increased above that in mice administered Ova alone, and the adaptive response was also similarly higher but did not reach significance (Figure 14 D-E). A third immunization with α-GalCer+Ova by the IN route to mice that previously received two successive immunizations by the IN-IV regimen (IN-IV-IN scheme) also reactivated NKT cells to produce IFNγ, although the levels were reduced as compared to mice that did not receive a third IN immunization (IN-IV regimen) (Figure 14 A). The level of PD-1 expression on the NKT cells in mice from the IN-IV-IN immunization scheme was significant, but CD69 expression was not, and also no significant DC activation was observed (Figure 14 B-D). However, in the mice immunized with α-

GalCer+Ova by the IN-IV-IN regimen, the adaptive immune response as measured by the IFNγ Elispot assay indicated a significant increase above that in control mice immunized with Ova alone (Figure 14 E). Overall, the level of IFNγ production by NKT cells was not significantly changed by a third immunization following the IN-IV or IN-IN regimens, making each three dose scheme comparable (Figure 15 A). Immunization by the IN-IV regimen followed by a third IN immunization resulted in significant up-regulation of PD-1 expression on NKT cells in the spleen, and similar results were obtained from mice that received a third IN immunization (IN-IN-IN) (Figure 15 B). However, PD-1 levels in mice from the IN-IV-IN immunization scheme were significantly higher than those in mice from the IN-IN-IN scheme (Figure 15 B). Similarly, a third IN immunization following the IN-IV scheme (IN-IV-IN) resulted in a significant drop in CD69 expression which was not the case with the IN-IN-IN scheme that remained comparable to the values observed in mice from the IN-IN scheme, making CD69 expression on NKT cells in mice from the IN-IV-IN group significantly less than that from mice in the IN-IN-IN group (Figure 15 C). The level of DC activation in terms of CD86 expression in mice following the IN-IV-IN regimen was significantly reduced compared to that in mice following the IN-IV regimen (Figure 15 D). However, CD86 expression on DC induced by IN-IN-IN immunization was comparable to that observed for mice in the IN-IN as well as IN-IV-IN groups (Figure 15 D). These results suggest that repeated activation of NKT cells occurs after multiple rounds of intranasal immunization in the spleen, but this does not correspond strictly to the induction of positive adaptive immune responses, which appear to be more dependent on the activation status of the antigen presenting DCs.

Figure 14. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the spleen after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1, 6, and 11) for determining activation of NKT cells by flow cytometry (panels A-D) and at 5-6 days after each dose (day 6, 11, 15) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of spleen cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from spleen in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 14. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the spleen after immunization employing α-GalCer as an adjuvant.

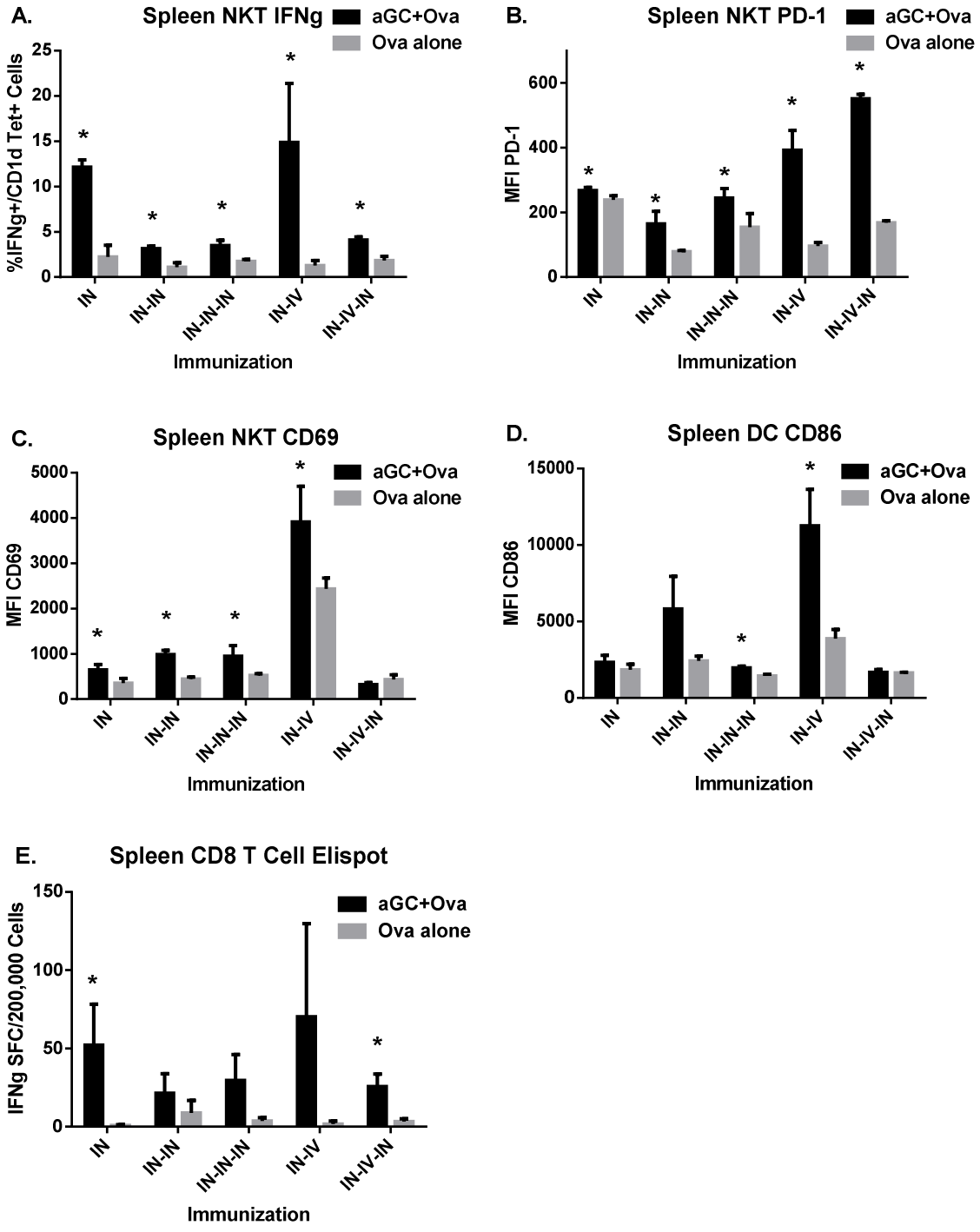
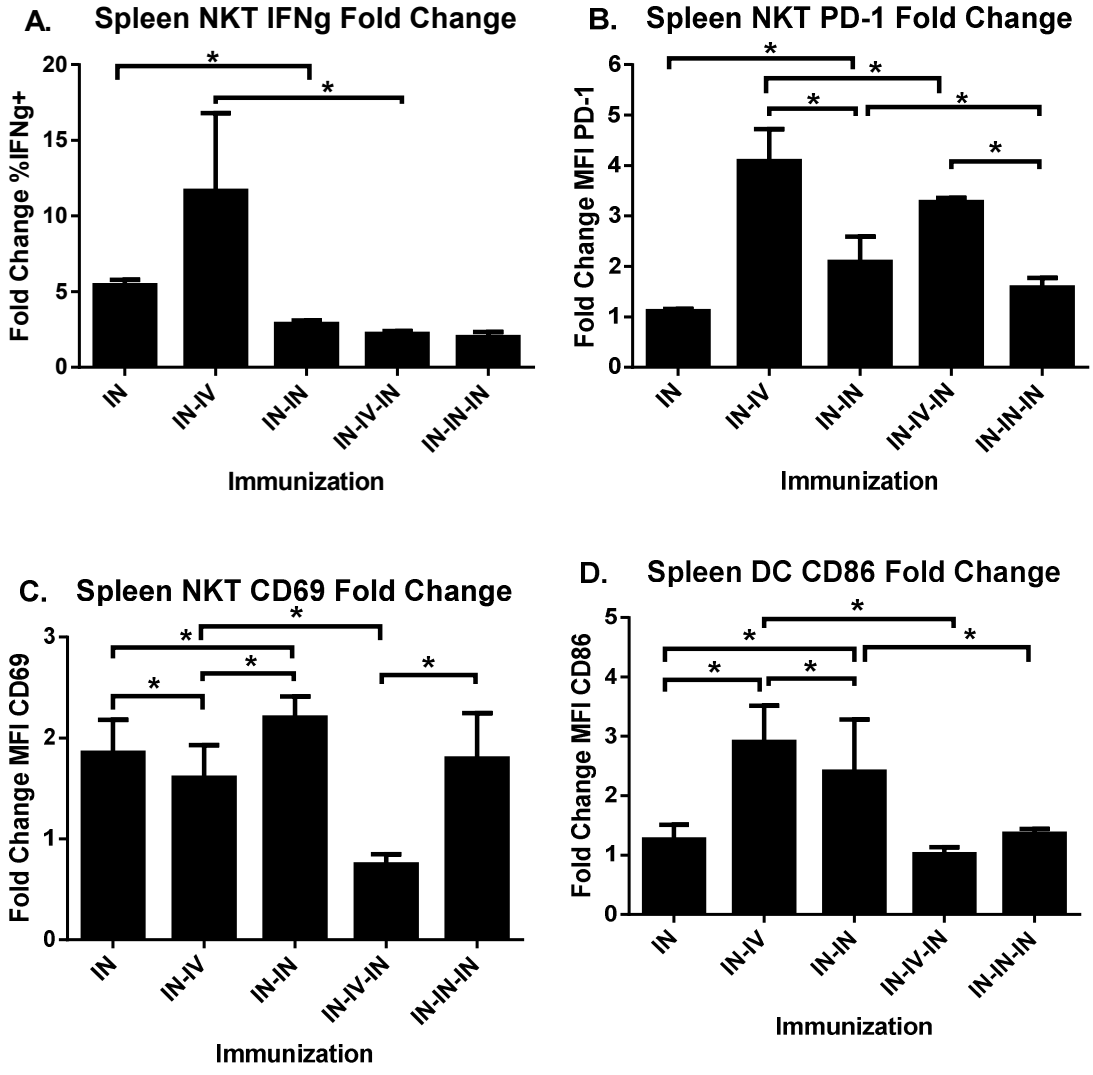


Figure 15. Combinatorial route effect on NKT cell and DC activation in the MdLN after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 15. Combinatorial route effect on NKT cell and DC activation in the spleen after immunization employing α-GalCer as an adjuvant.



4.2.2 Analyses of cells from the liver

An initial intranasal dose of α-GalCer+Ova, relative to Ova alone, induced stronger activation of NKT cells in the liver in terms of significant levels of IFNγ production and CD69 expression but no difference in the level of PD-1 expression (Figure 16 A,C,B). In parallel, we observed significant activation of DCs in terms of CD86 expression and a strong adaptive immune response (Figure 16 D-E). A second dose of α-GalCer+Ova delivered by the intranasal route (IN-IN regimen) resulted in negligible IFNγ production by NKT cells and no difference in the expression of PD-1 or CD69 in comparison to mice the received Ova alone (Figure 16 A-C). Although not significantly activated, DCs in the liver showed levels of CD86 expression comparable to those after the first intranasal dose, and adaptive immune responses in the liver remained at a level significantly higher than that in mice immunized with Ova alone (Figure 16 D-E). Analyses of cells from mice administered a second intravenous immunization subsequent to the first immunization by the intranasal route (IN-IV regimen) also showed reactivation of NKT cells to produce IFNγ, in addition to expression of significant amounts of PD-1 (Figure 16 A-B). While high levels of CD69 expression were observed in the mice receiving repeat doses of α-GalCer+Ova following the IN-IV regimen, they were comparable to those in mice in the control group immunized with Ova alone (Figure 16 C). Furthermore, DCs were significantly activated in mice administered two doses of α-GalCer+Ova, relative to Ova alone, following the IN-IV regimen, which coincided with positive adaptive immune response (Figure 16 D-E). ANOVA of data from the liver cells of mice that received a single IN immunization showed significantly more IFNγ production by NKT cells than that in mice administered a second dose by either the IN or IV route (IN-IN and IN-IV regimens, respectively) and that a second IV dose (IN-IV regimen) resulted in significantly higher IFNγ production by NKT cells than that observed after a second IN dose (IN-IN regimen) (Figure 17 A). The PD-1 levels on NKT cells in mice administered a single IN dose were comparable to those in mice from the IN-IV regimen, but PD-1 levels on NKT cells in mice following the IN-IN regimen were significantly less than in mice that were immunized either once by the IN route or after IN-IV immunization (Figure 17 B). With respect to the CD69 expression on NKT cells, the levels in mice from both IN-IV and IN-IN regimens were significantly higher than in mice that received a single IN immunization (Figure 17 C). Also, mice following the IN-IV regimen showed significantly more CD69 expression than those following the IN-IN regimen. Finally, DCs in the livers of mice from the IN-IN group expressed CD86 levels comparable to those in mice administered a single IN

immunization, but a second IV immunization (IN-IV regimen) following the initial IN immunization led to significantly more CD86 expression on DC (Figure 17 D). Nevertheless, the DC activation levels, in terms of CD86 expression, were comparable in mice from both the IN-IV and IN-IN regimen groups. Since a single dose of α-GalCer delivered by the intravenous route induces anergy of NKT cells (as shown in the current study as well as in literature reports), we investigated whether or not the second intravenous dose of α-GalCer delivered to mice that originally received an initial intranasal dose of α-GalCer would also result in anergy of the NKT cells. We therefore administered a third intranasal immunization of α-GalCer +/- Ova to mice from the IN-IN and IN-IV regimen groups. Cells from the livers of mice administered α-GalCer+Ova, relative to Ova alone, by the IN-IN-IN regimen did not show induction of significant IFNγ production by NKT cells (Figure 16 A). Correspondingly, expression levels of both CD69 and PD-1 were similar to that in the control group of mice receiving Ova alone by the IN-IN-IN regimen (Figure 16 B-C). However, levels of DC activation remained significant, and this coincided with a positive adaptive immune response in mice immunized with α-GalCer+Ova relative to Ova alone control by the IN-IN-IN regimen (Figure 16 D-E). In the case of the mice following the IN-IV-IN immunization scheme, subsequent to the third dose, the NKT cells did not show IFNγ production, but PD-1 expression was significant, suggesting anergy (Figure 16 A-B). Additionally, CD69 expression was significantly down-regulated compared to Ova alone control, and DCs were also not significantly activated (Figure 16 C-D). Adaptive immune response after the IN-IV-IN immunization regimen remained comparable to that observed in mice receiving two immunizations by the IN-IV regimen, but was not significantly different than those in mice that received Ova alone by the IN-IV-IN scheme (Figure 16 E). In summary, a third intranasal immunization to mice that were earlier immunized by the IN-IV scheme resulted in significantly less IFNγ production by NKT cells than after IN-IV dosing, whereas a third intranasal immunization subsequent to IN-IN immunization resulted in comparable IFNγ production by NKT cells to that after two successive IN immunizations (IN-IN regimen) (Figure 17 A). Also, mice immunized using the IN-IV-IN scheme showed NKT cells producing significantly less IFNγ than those from mice following the IN-IN-IN regimen. The PD-1 expression levels in mice following the IN-IV and IN-IV-IN schemes were comparable, but the IN-IN-IN immunization scheme resulted in significantly more PD-1 expression on NKT cells than in mice following the IN-IN scheme (Figure 17 B). Overall, three immunizations, regardless of the route used for the second dose, resulted in comparable expression of PD-1 by NKT cells. Following the IN-IV scheme of immunization with a third

dose resulted in significantly less CD69 expression, but the levels for mice in the IN-IN and IN-IN-IN groups were comparable (Figure 17 C). Also, NKT cells in the livers of mice following the IN-IV-IN and IN-IN-IN schemes showed CD69 expression that were significantly different from one another. Lastly, CD86 expression on DCs after a third immunization was significantly reduced as compared to that in mice receiving IN-IV immunization, but was similar to CD86 levels on DCs in mice following the IN-IN regimen (Figure 17 D). Thus, the IN-IN-IN immunization regimen resulted in significantly higher CD86 expression on DC than the IN-IV-IN regimen. In the liver tissue, an initial dose of α-GalCer delivered by the IN route followed by a second IV dose seemed to have induced anergy in NKT cells as indicated by high expression of PD-1, but did not blunt the adaptive immune response which was the case after two successive doses of α-GalCer delivered by the IV route. Multiple doses of α-GalCer delivered by the IN route did not induce reactivation of NKT cells in the liver, but significant DC activation and positive adaptive immune responses were observed after each dose, suggesting that perhaps activated DCs are homing to the liver from elsewhere and influencing the induction of adaptive immune response in this tissue.

Figure 16. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the liver after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1, 6, and 11) for determining activation of NKT cells by flow cytometry (panels A-D) and at 5-6 days after each dose (day 6, 11, 15) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of liver cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from liver in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 16. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the liver after immunization employing α-GalCer as an adjuvant.

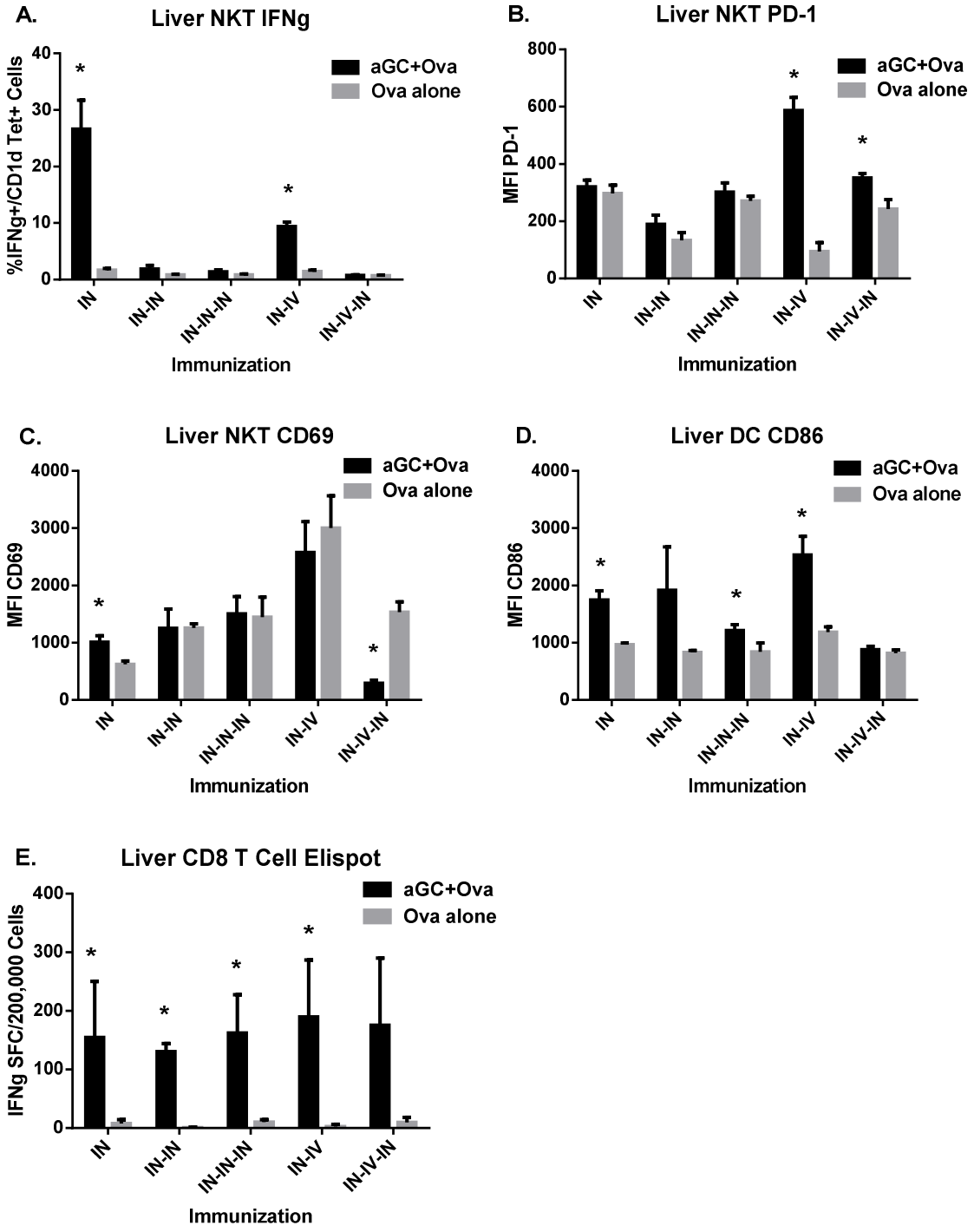
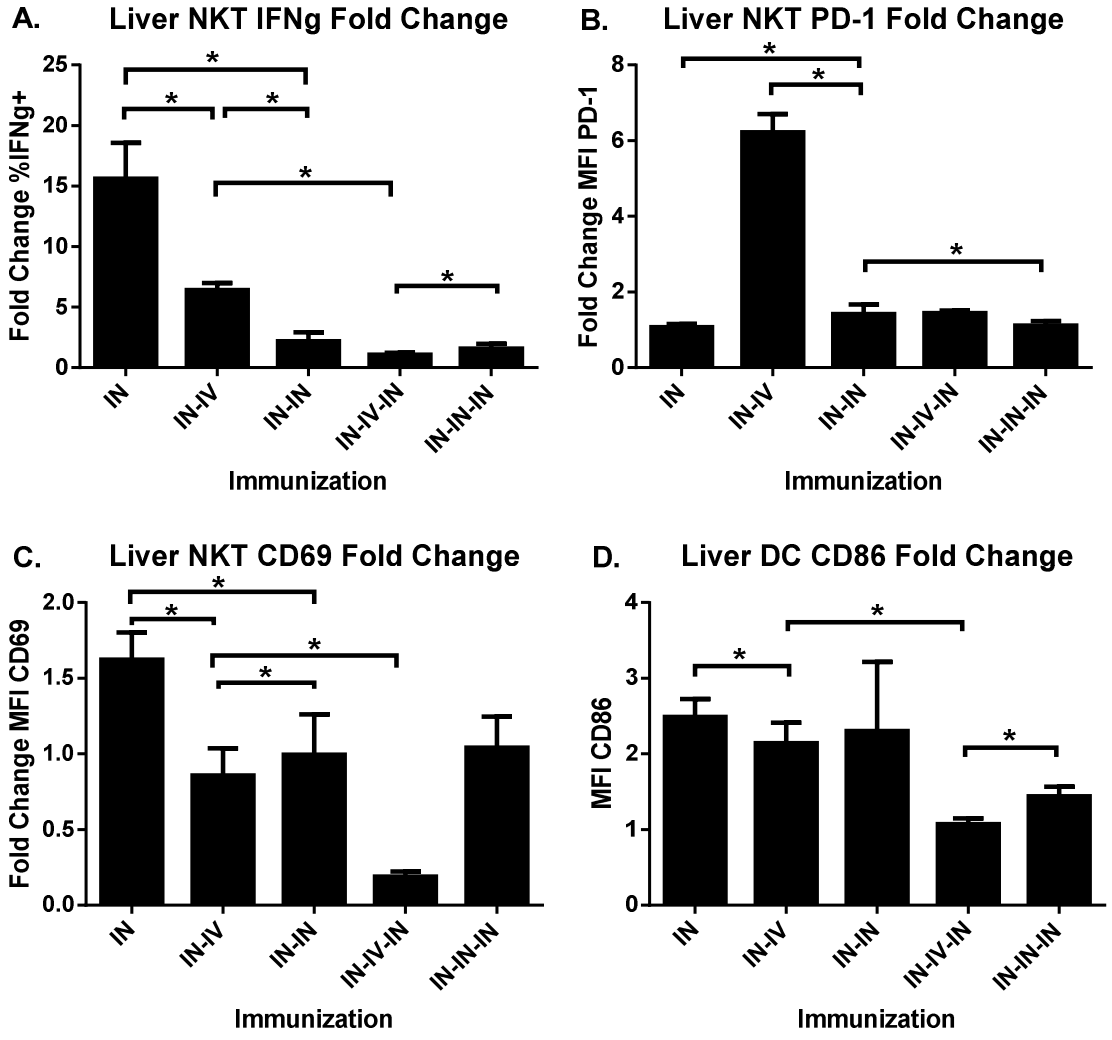


Figure 17. Combinatorial route effect on NKT cell and DC activation in the liver after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 17. Combinatorial route effect on NKT cell and DC activation in the liver after immunization employing α-GalCer as an adjuvant.



4.2.3 Analyses of cells from the lung

One immunization by the IN route with α-GalCer+Ova induced strong activation of NKT cells in the lung leading to significant IFNγ production as well as expression of PD-1 and CD69 relative to that observed in the control group of mice immunized with Ova alone (Figure 18 A-C). We also observed DCs in this tissue were significantly activated after the IN immunization, and correspondingly a positive adaptive immune response, in terms of Ova-specific IFNγ-producing CD8+ T cells, was observed (Figure 18 D-E). Administration of the second IN immunization of α-GalCer+Ova led to significant IFNγ production by NKT cells, albeit at reduced levels as compared to those after the first immunization (Figure 18 A). The second intranasal immunization also induced significant expression of both CD69 and PD-1on the NKT cells (Figure 18 B-C). We also observed significant activation of DCs and positive adaptive immune response after the second successive IN immunization with α-GalCer+Ova (Figure 18 D-E). In contrast to this, a second IV dose of α-GalCer following an initial IN dose did not reactivate NKT cells to produce significant amounts of IFNγ, but PD-1 levels remained significant, and CD69 was strongly and significantly up-regulated relative to mice immunized with Ova alone by the IN-IV regimen (Figure 18 A-C). Likewise, dendritic cells were activated and induction of a positive adaptive immune response was observed, suggesting that perhaps IFNγ production by the NKT cells might not be the sole factor influencing DC activation in this tissue to promote induction of adaptive immunity (Figure 18 D-E). The levels of IFNγ production by NKT cells in response to immunization with α-GalCer+Ova, relative to Ova alone, in mice from the IN-IN and IN-IV regimens were significantly less than that observed after the initial IN immunization, but the responses were comparable among mice immunized by the two regimens (IN-IV and IN-IN) (Figure 19 A). While similar comparisons for the expression of PD-1 on NKT cells in mice immunized by the IN-IN or IN-IV regimen were each comparable to that after the first IN immunization, mice following the IN-IN immunization regimen showed PD-1 expression that was significantly less than that observed in the IN-IV regimen mice (Figure 19 B). Furthermore, CD69 expression on the NKT cells was significantly higher in mice from the IN-IV immunization regimen as compared to that observed after both a single or two sequential immunizations by the IN route, with responses in the latter two groups being comparable to one another (Figure 19 C). Parallel analyses of the DCs in terms of CD86 expression in the lungs of mice immunized with α-GalCer+Ova versus Ova alone revealed significantly more

activation after the second immunization, whether by the IN or IV routes, and the levels of CD86 expression on the DCs in mice from the IN-IN and IN-IV groups were similar to one another (Figure 19 D). Finally, a third consecutive immunization with α-GalCer+Ova, relative to Ova alone, by the IN-IN-IN scheme did not induce significant IFNγ production by NKT cells that, however, showed significant levels of expression of PD-1 and CD69 activation markers (Figure 18 A-C). Likewise, DCs were activated and a positive adaptive CD8+ T cell immune response was observed in the mice following the IN-IN-IN immunization regimen, albeit not significant above background (Figure 18 D-E). These results suggest that IFNγ production by NKT cells subsequent to the third successive dose of α-GalCer+Ova delivered by the IN route may be dispensable for the activation of DCs and/or induction of adaptive immunity to co-administered antigen, particularly in the lung tissue. Analyses of the cells from the lungs of mice that received α-GalCer+Ova using the IN-IV-IN dosing scheme showed no activation of NKT cells in terms of IFNγ production and CD69 expression, but PD-1 levels were significantly higher than in the control mice that received Ova alone in this scheme (Figure 18 A,C,B). While the DCs were not activated in these mice, adaptive immune response remained significant but decreased as compared to that in mice that did not receive the third IN immunization (i.e. mice in the IN-IV regimen) (Figure 18 D-E). In the lung, the third immunization by the IN route to mice that were immunized previously by either the IN-IV or IN-IN regimens induced significantly more IFNγ production by NKT cells, supporting the effectiveness of the IN route of α-GalCer delivery to repeatedly activate NKT cells, regardless of the route of immunization used in the earlier second immunization (Figure 19 A). The difference in the PD-1 expression by NKT cells between mice following the IN-IV and IN-IN regimens was significant, with IN-IV inducing higher PD-1 expression that was not significantly different from IN-IV-IN. IN-IN-IN immunization resulted in a significant drop in PD-1 expression compared to two doses IN-IN (Figure 19 B). Also, mice in the IN-IV-IN and IN-IN-IN groups showed levels of PD-1 expression in the lung NKT cells that were comparable to one another. The level of CD69 expression on the NKT cells was significantly reduced in mice from the IN-IV-IN group as compared to that in mice from the IN-IV group, but was significantly increased in the mice from the IN-IN-IN group versus those in both the IN-IN and IN-IV-IN groups (Figure 19 C). Lastly, DCs in the lung were significantly more activated after immunization by the IN-IV scheme than what was observed in mice following the IN-IV-IN scheme, whereas two and three immunizations delivered by the IN route led to similar levels of DC activation (Figure 19 D). The DCs in the mice from the IN-IN-IN group showed CD86 expression that was significantly higher than what was

observed in mice from the IN-IV-IN immunization group. Overall, these results from the analyses of cells from the lung tissue of mice immunized with α-GalCer+Ova versus Ova alone by the IN-IV +/- IN regimens suggest that IFNγ production by the NKT cells in response to α-GalCer may not be the sole underlying mechanism for the activation of DCs and/or eventual induction of adaptive immune response to the co-administered antigen, but might be related to CD69 expression by NKT cells and, further downstream, DC activation status.

Figure 18. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the lung after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1, 6, and 11) for determining activation of NKT cells by flow cytometry (panels A-D) and at 5-6 days after each dose (day 6, 11, 15) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of lung cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from lung in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 18. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the lung after immunization employing α-GalCer as an adjuvant.

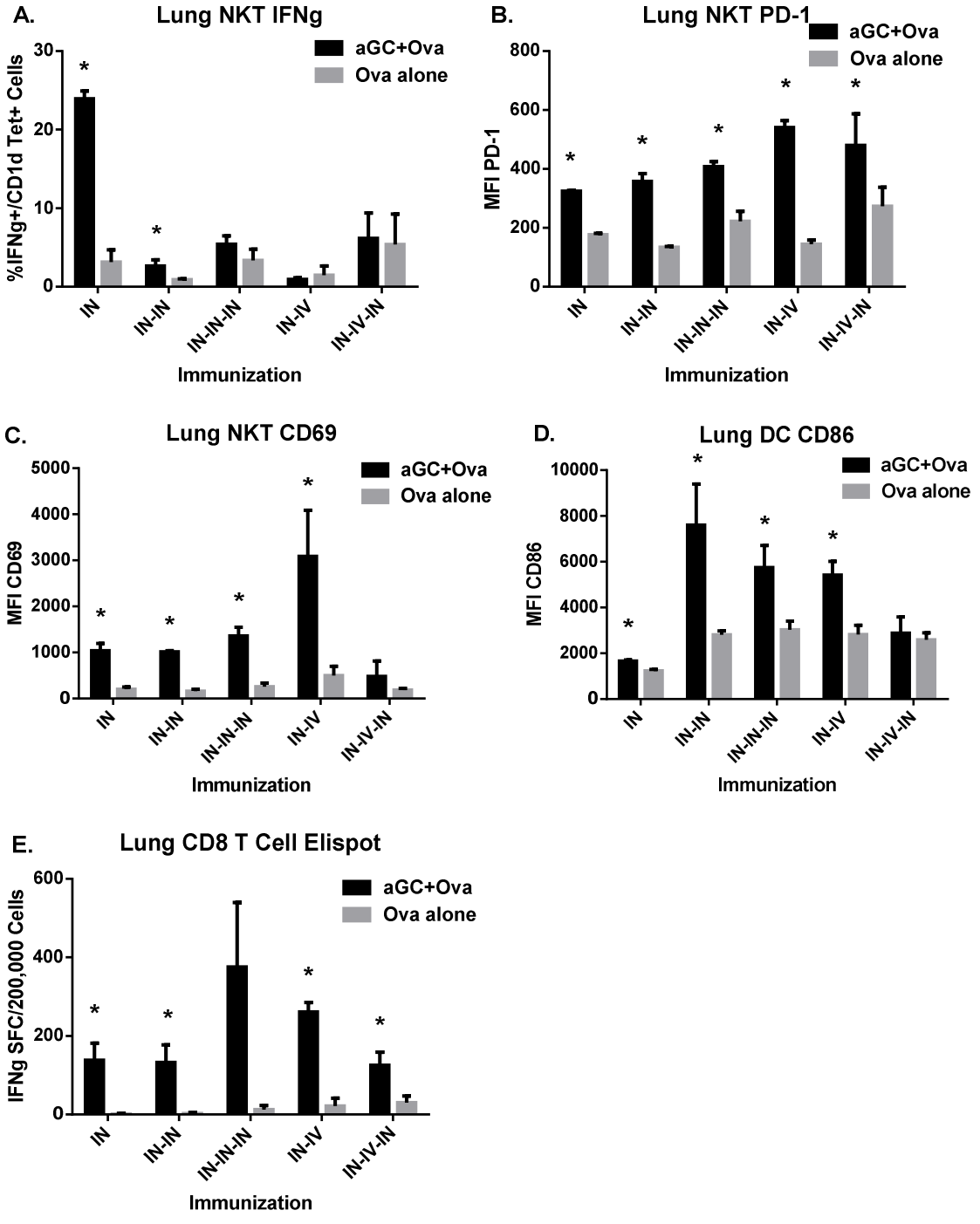
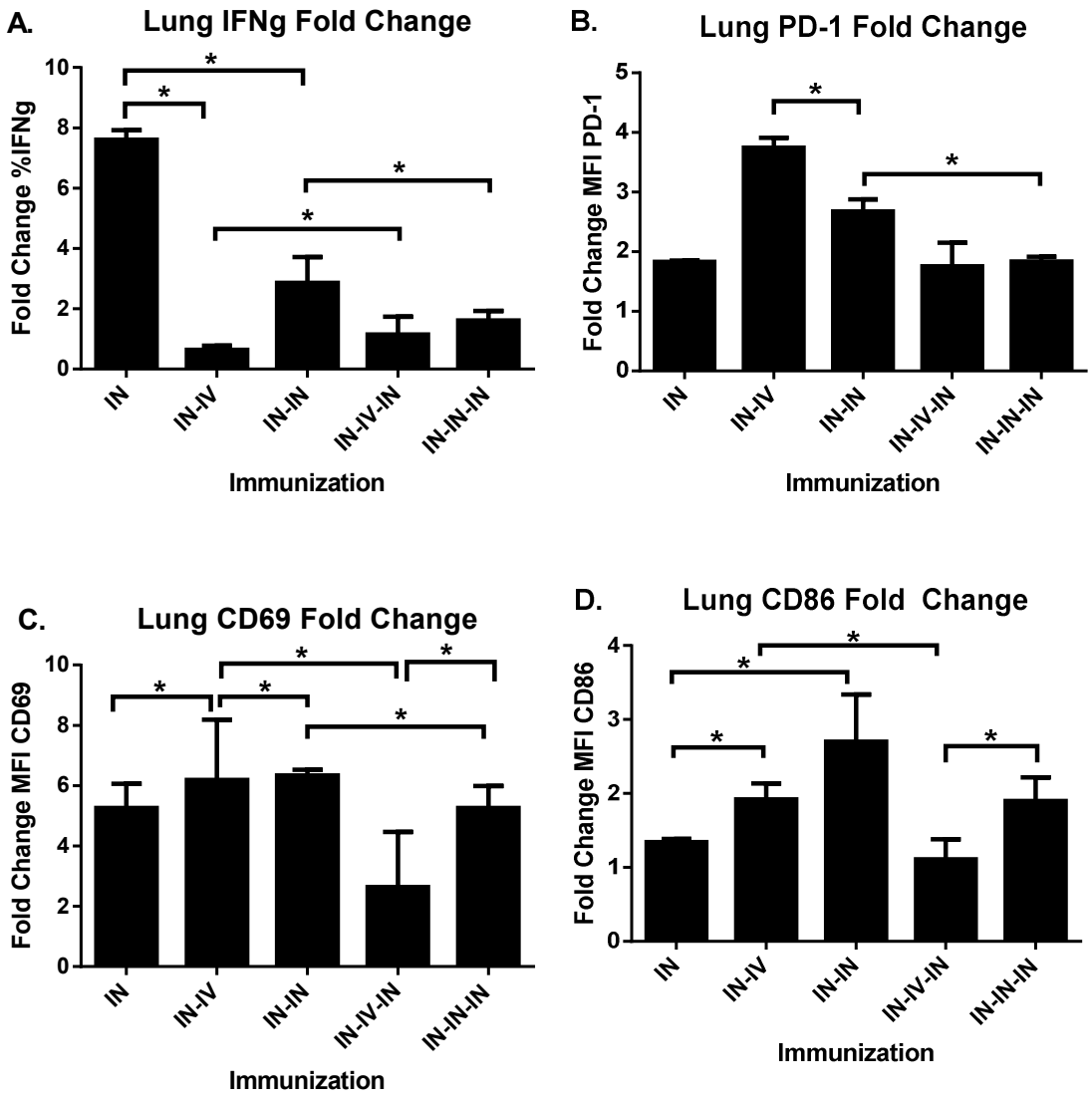


Figure 19. Combinatorial route effect on NKT cell and DC activation in the lung after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 19. Combinatorial route effect on NKT cell and DC activation in the lung after immunization employing α-GalCer as an adjuvant.



4.2.4 Analyses of cells from the mediastinal lymph node (MdLN)

An initial intranasal immunization with α-GalCer+Ova induced strong IFNγ production by NKT cells in the lung-draining mediastinal lymph node (MdLN), and the expression of PD-1 and CD69 activation markers was higher than background levels (Figure 20 A-C). While the DCs did not exhibit strong activation, a significantly positive adaptive immune response was observed (Figure 20 D-E). It is possible that the Ova-specific CD8+ T cells responsible for the adaptive immune response drained to the MdLN from the lung or another tissue. All subsequent doses of α-GalCer delivered by either the IN or IV route did not induce IFNγ production by the NKT cells in the MdLN, and there was also a lack of significant CD69 expression by the NKT cells (Figure 20 A,C). We also did not observe DC activation, although mice administered α-GalCer by the IN-IN and IN-IV schemes showed a trend for increased DC activation (Figure 20 D). The PD-1 expression on the NKT cells was also not significant following α-GalCer delivery by any route (Figure 20 B), although it was interesting to note that the biggest fold change in PD-1 expression between mice receiving Ova alone and α-GalCer+Ova was observed in the IN-IV scheme (Figure 21 B), consistent with an anergy phenotype of NKT cells. While mice immunized with α-GalCer+Ova, relative to Ova alone, by any of the regimens except IN-IN-IN showed higher adaptive immune response in terms of Ova-specific IFNγ-producing CD8+ T cells, the levels were significant only in mice that received on IN immunization and those that were immunized following the IN-IV regimen (Figure 20 E). Overall, it appears that the lack of adaptive response in the MdLN following the IN-IN-IN immunization scheme was due simply to lack of sufficient activation of NKT cells rather than anergy, as also indicated by a lack of significant DC activation. In comparing data from all immunization regimens by ANOVA of mice administered α-GalCer+Ova versus Ova alone, the only point at which there is a significant difference in IFNγ production is after a single IN dose, which is significantly higher than either of the subsequent second immunizations (Figure 21 A). Similar analyses revealed that PD-1 expression was significantly higher after a single IN immunization than after a second immunization by either the IN or IV route, and the IN-IV-IN immunization regimen induced a significant increase in PD-1 expression above that observed in mice that followed the IN-IV immunization scheme (Figure 21 B). However this level of PD-1 expression induced by IN-IV-IN immunization was comparable to that seen in mice from the IN-IN and IN-IN-IN immunization regimens. A single IN immunization with α-GalCer+Ova, relative to Ova alone, resulted in significantly higher CD69 expression on NKT cells than that observed after either

of the second immunizations, and mice immunized by the IN-IV regimen showed significantly higher levels compared to that in mice immunized by the IN-IN regimen (Figure 21 C). The third IN immunization following the IN-IV regimen (IN-IV-IN) resulted in a significant decrease in CD69 expression, but expression after a third IN immunization following the IN-IN regimen (IN-IN-IN) was comparable to that observed after IN-IN immunization. Both three dose schemes resulted in similar levels of CD69 expression on NKT cells. Lastly, IN immunization followed by either a second IN or IV immunization resulted in significantly higher CD86 expression on DC, with IN-IN and IN-IV regimens being comparable (Figure 21 D). A third IN dose after each of these schemes resulted in significant decrease in CD86 expression as compared to the relative two dose scheme, with both three dose schemes being comparable to one another.

Figure 20. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the MdLN after immunization employing α-GalCer as an adjuvant. Mice were immunized once by the IV or twice by a combination of IV and IN routes at five day intervals with either Ova alone or admixed with α-GalCer (aGC+Ova). Separate groups of mice were sacrificed one day after each dose (days 1, 6, and 11) for determining activation of NKT cells by flow cytometry (panels A-D) and at 5-6 days after each dose (day 6, 11, 15) for determining antigen-specific T cell responses by IFNγ-Elispot assay (panel E). NKT cells and DCs were stained as in Figure 4. Flow cytometry analyses of IFNγ production (A), expression of PD-1 (B) and CD69 (C) by NKT cells and CD86 expression by DC (D) of MdLN cells from mice immunized with Ova with or without α-GalCer (Ova alone or aGC+Ova, respectively) are shown. Numbers of IFNγ producing cells from MdLN in response to in vitro stimulation with the CD8 T cell epitope Ova peptide are shown as spot forming colonies (SFC) per $2x10^5$ plated cells. Data are presented as mean and standard deviation of values collected for three mice and significance was calculated using the student t-test with significance at p-values ≤ 0.05(*).

Figure 20. Activation of NKT cells and DCs, and induction of antigen-specific immune responses in the MdLN after immunization employing α-GalCer as an adjuvant.

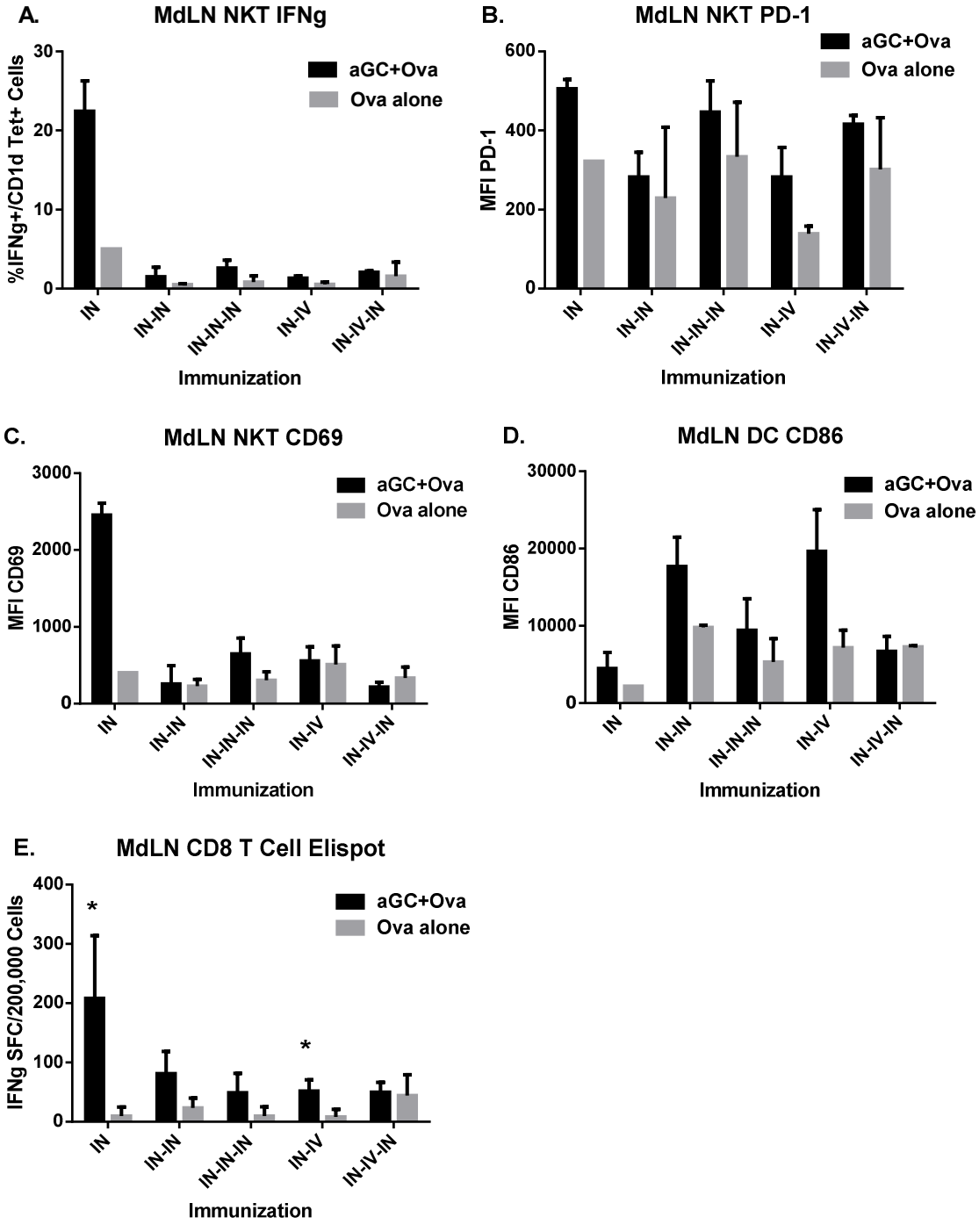
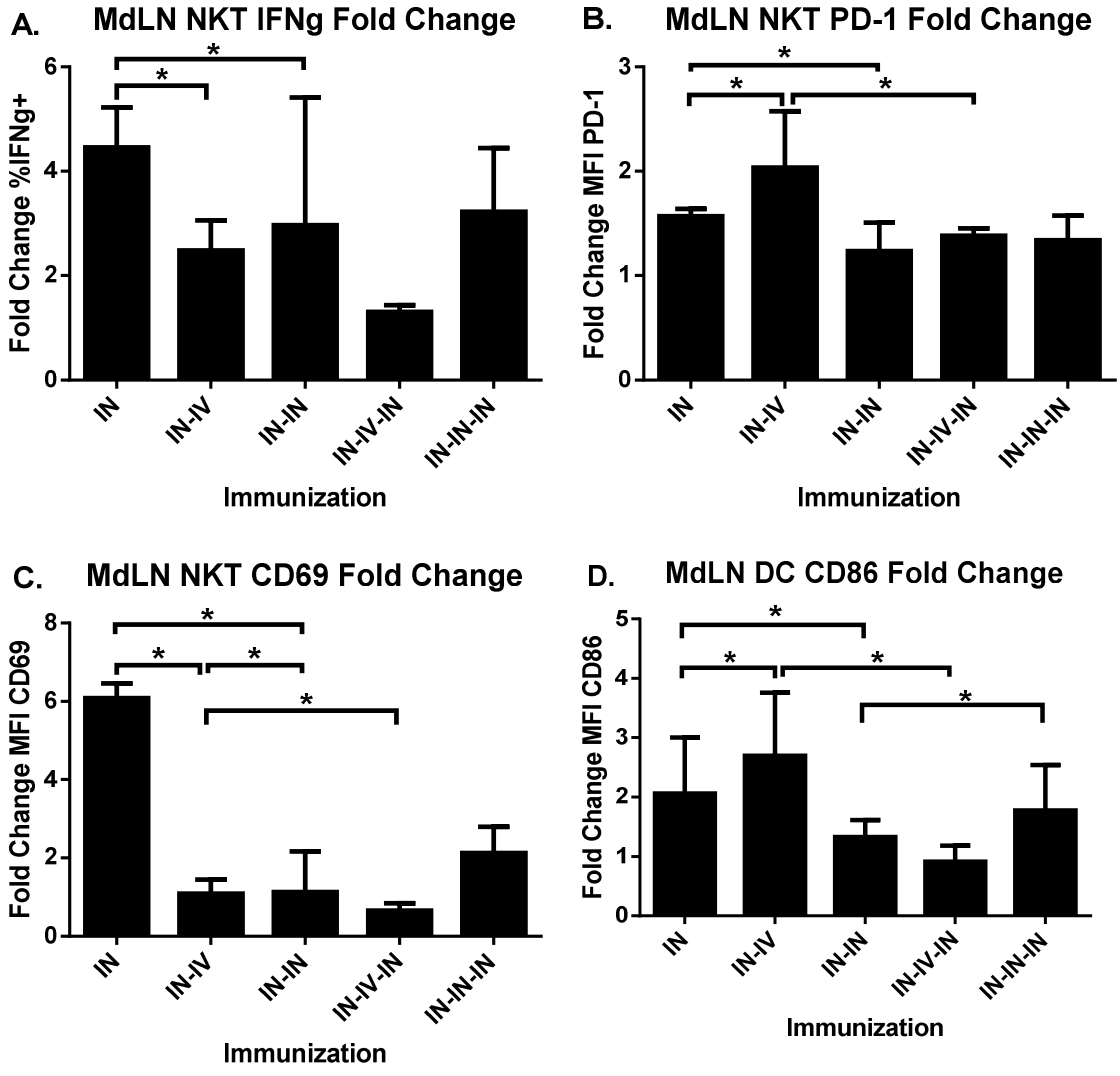


Figure 21. Combinatorial route effect on NKT cell and DC activation in the MdLN after immunization employing α-GalCer as an adjuvant. Fold change in values (Mean + SD) for activation of NKT cells (in terms of IFNγ production as well as expression of PD-1 and CD69 in panels A-C), and DCs (in terms of CD86 expression in panel D) between mice immunized with Ova alone or with Ova+α-GalCer. Data were subjected to two-way repeated measure analysis of variance (ANOVA) to determine significance of effective activation between immunization regimens, indicated by p-values ≤ 0.05(*).

Figure 21. Combinatorial route effect on NKT cell and DC activation in the MdLN after immunization employing α-GalCer as an adjuvant.



4.3 CONSECUTIVE INTRANASAL IMMUNIZATIONS VIA THE IN-IN-IN-IN SCHEME

As described above, experiments testing combinations of immunization by the IN and IV routes showed that multiple immunizations, up to three doses, by the intranasal route afforded reactivation of NKT cells along with DC activation and induction of adaptive immune response in the lung tissue. We extended this investigation by testing the effectiveness of four sequential immunizations by the IN route to induce adaptive immune responses. Briefly, mice were administered four intranasal doses of α-GalCer+Ova at five day intervals, and separate groups of mice were sacrificed five days after each dose to analyze various tissues for IFNγ production by the CD8+ T cells using the Elispot assay (Figure 22). As described above, a single immunization with α-GalCer+Ova, relative to Ova alone, induced a positive adaptive immune response in the spleen, lung and MdLN tissues (Figure 23). A second intranasal immunization also induced a positive adaptive immune response in these tissues, with a trend of improvement in the lung and MdLN tissues, but decrease in the spleen. A third immunization again led to a positive adaptive response in these three tissues, but the levels were reduced relative to those seen after the first and second IN immunizations. After the fourth and final immunization, while the response in all tissues was still positive, that in the lung was reduced to a level that was only slightly above the background cutoff value, and while it was significantly positive in the spleen, adaptive response in this tissue decreased relative to one, two, and three immunizations by the IN route.

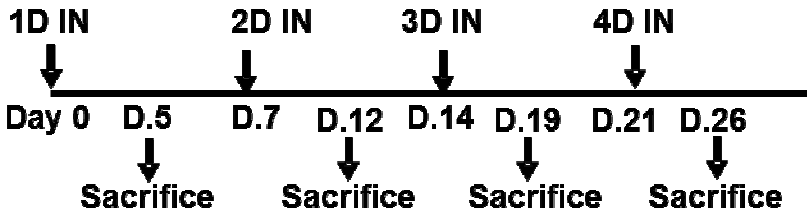


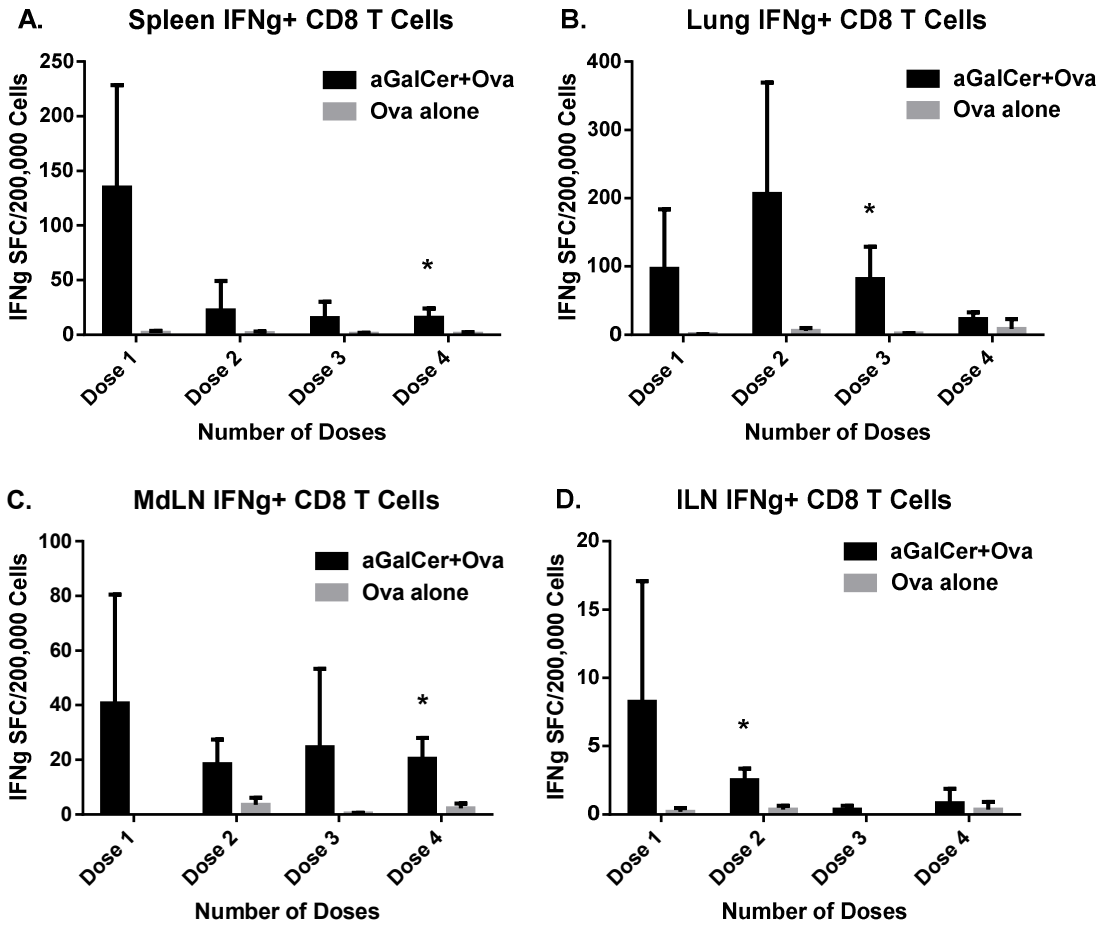
Figure 22. Scheme for sequential immunization four times by the intranasal route.

Mice were immunized from one to four times (1D to 4D) at seven day intervals by the intranasal (IN) route with either Ova alone or admixed with α-GalCer and different groups of mice were sacrificed five days after each immunization (days 5, 12, 19, and 26 post-primary immunization).

Figure 23. Induction of Ova peptide-specific IFNγ-producing cells by four consecutive intranasal immunizations with the Ova +/- α-GalCer as adjuvant.

Cells isolated from spleen, lung, MdLN and ILN of C57BL/6 mice immunized from one to four times at 5 day intervals by the intranasal route were analyzed for IFNγ producing cells in response to in vitro stimulation with Ova-specific CD8 T cell epitope peptide by the Elispot assay as described in the methods section. Average values and errors bars were calculated from one experiment using three mice in each group and shown as IFNγ spot forming colonies (SFC) for $2x10^5$ plated cells after adjusting for background values from cells cultured in medium alone. Responses were considered positive when they were above 10 SFC/well and double the number obtained in cells cultured with medium alone. The *p*-values were calculated comparing with the medium alone control and values ≤0.05 (*) were considered significant.

Figure 23. Induction of Ova peptide-specific IFNγ-producing cells by four consecutive intranasal immunizations with the Ova +/- α-GalCer as adjuvant.



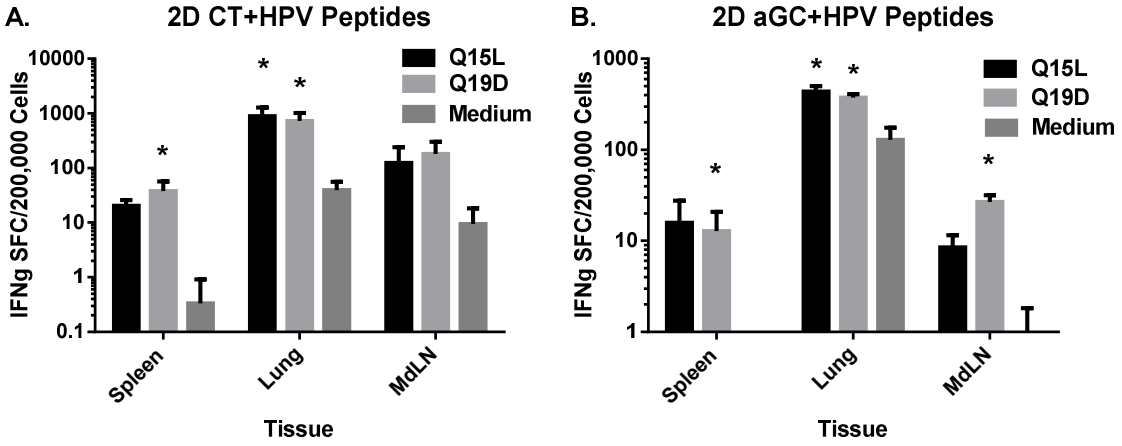
CHAPTER 5: TUMOR CHALLENGE MODEL

5.1 Induction of HPV Peptide-Specific Adaptive Immunity

We tested whether multiple rounds of intranasal immunization delivering α-GalCer to induce repeated activation of NKT cells and sustained adaptive immune responses to co-administered antigens will also translate into protective efficacy in a tumor challenge model in mice. As antigens for this study, we chose two T cell epitope peptides from the E6 and E7 oncoproteins of the high-risk human papillomavirus type 16, the virus associated with a variety of orogenital neoplasia including cervical cancer and oropharyngeal lesions. These HPV-16 E6 and E7 peptide sequences were identified in earlier published studies from our laboratory to be associated with HPV-specific memory immunity in patients with recurrence-free survival post-ablative treatment for HPV-induced cervical intraepithelial neoplasia, and thus were recognized to serve as potential therapeutic vaccine candidates. Previously, these peptides (Q15L from the E6 region and Q19D from the E7 region) were shown to induce adaptive CD4+ and CD8+ T cells immune responses when delivered to mice in two doses by the intranasal route using admixed cholera toxin (CT) adjuvant.(4) We adopted this model for the current experiments by including an additional group of mice that received α-GalCer adjuvant along with the HPV-16 E6/E7 peptides. Mice were immunized twice at five day intervals by the intranasal route with either the HPV peptides alone or admixed with α-GalCer or CT. On day ten after the first immunization (five days after the second immunization), mice were sacrificed to measure adaptive immune responses using IFNγ Elispot assay to ascertain the effectiveness of α-GalCer adjuvant, relative to CT. Mice receiving CT+HPV peptides showed potent adaptive immune response to each peptide in the lung, as well as positive responses in the MdLN and spleen with significant levels above the medium alone control in the spleen and lung (Figure 24 A). Immunization with α-GalCer+HPV peptides also resulted in strong immune responses to both of the peptides in the lung, spleen, and MdLN at a similar level as observed in mice immunized with CT+HPV (Figure 24 B, A).

Figure 24. Induction of antigen-specific immune responses by intranasal immunization with HPV peptides admixed with either cholera toxin (CT) or α-GalCer (aGC) as adjuvant. Groups of C57BL/6 mice were immunized twice at five day intervals by the intranasal route with a mixture of Q19D and Q15L peptides corresponding to the E6 and E7 oncoproteins of HPV-16, respectively, admixed with either CT adjuvant (A) or aGC adjuvant (B). Cells isolated from spleen, lung, and MdLN were analyzed for peptide-specific IFNγ-producing cells by the Elispot assay as described in the methods section and shown as IFNγ spot forming colonies (SFC) for $2x10^5$ input cells. Average values and standard errors were calculated for three mice in each group. Responses were considered positive when they were above 10 SFC/well and double the number obtained in cells cultured with medium alone. The *p*-values were calculated by comparing with the medium alone control and values ≤0.05 (*) are considered significant.

Figure 24. Induction of antigen-specific immune responses by intranasal immunization with HPV peptides admixed with either cholera toxin (CT) or α-GalCer (aGC) as adjuvant.

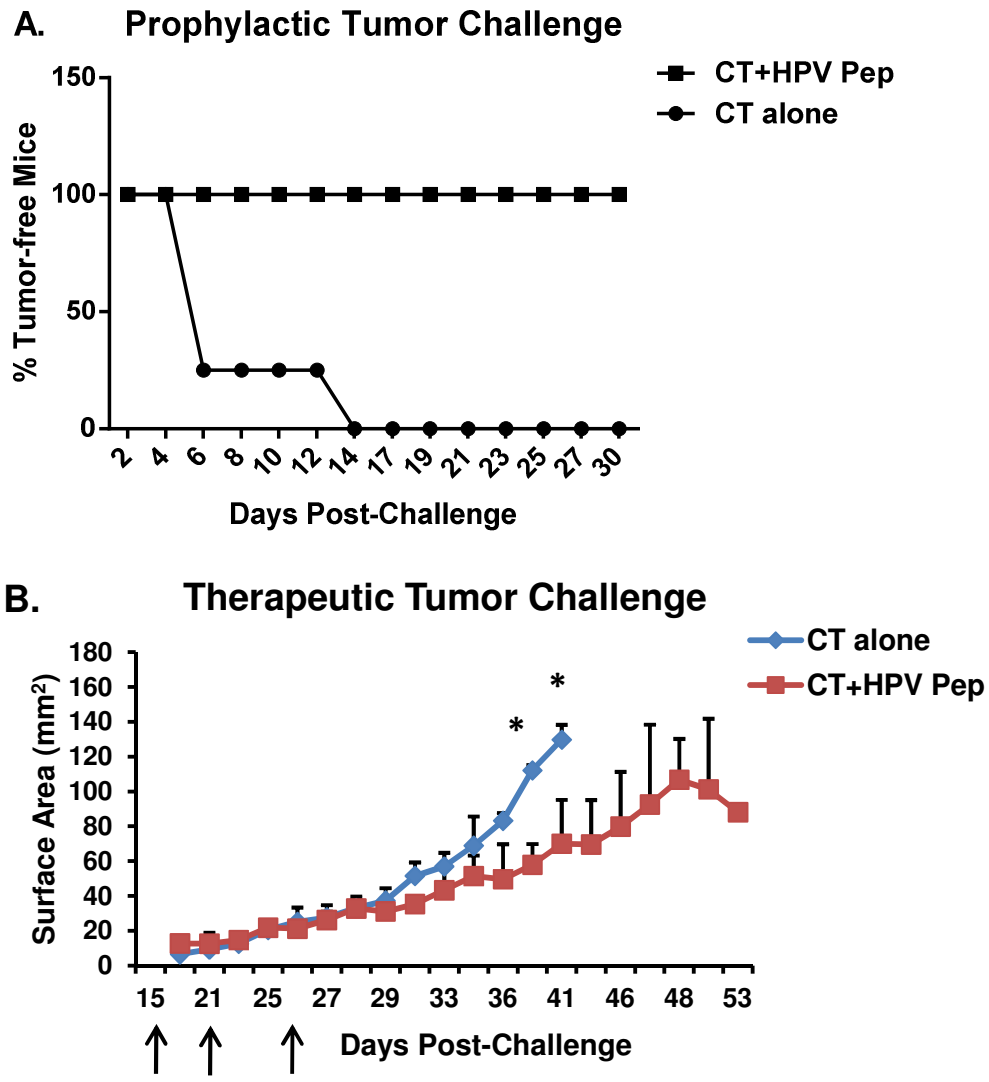


5.2 Vaccination against HPV Protein-Expressing Tumor using CT Adjuvant

Having confirmed the effectiveness of α-GalCer adjuvant for inducing immune responses to the co-administered HPV peptide antigens, we next tested the efficacy of this immunization regimen. We first performed tumor challenge experiments in mice using the CT adjuvant shown earlier to be effective. Mice were administered the prophylactic immunization twice at five days intervals by the intranasal route with a mixture of CT and HPV peptides Q15L and Q19D. At ten days after the first dose, mice were challenged subcutaneously with TC-1 tumor cells, an EL4 mouse tumor cell line engineered to express the E6 and E7 proteins (219). Tumor burden was determined by measuring the surface area every two days using vernier calipers. Control mice immunized with the CT adjuvant alone were all sacrificed by 14 days post-tumor challenge due to large tumor burden, whereas mice receiving CT+HPV peptides showed complete protection against tumor growth (Figure 25 A). We next tested this model in a therapeutic setting in which mice received the first immunization of CT+HPV peptides by the intranasal route at the first sign of tumor growth and received subsequent doses at 5-6 day intervals up to three doses. Mice immunized with CT+HPV peptides showed a slowing trend in tumor growth starting around 29 days after tumor challenge, with significant differences in tumor growth relative to the CT alone control group at day 40 post-challenge (Figure 25 B). Regression could not be followed further due to large tumor burden in control mice. These results suggest that earlier and perhaps more frequent therapeutic immunization may further slow tumor growth in these mice.

Figure 25. Intranasal immunization with HPV peptides using cholera toxin (CT) adjuvant protects mice against challenge with TC-1 tumor cells. (A) Prophylactic tumor challenge study: Two experimental groups of C57BL/6 mice were immunized by the intranasal route with the mixture of Q19D and Q15L peptides (corresponding to the E6 and E7 oncoproteins of HPV-16, respectively) along with CT adjuvant (CT+HPV Pep) or CT alone twice at 5 day intervals, and 1 week after the last immunization injected subcutaneously on the right flank with $2x10^5$ TC-1 tumor cells (expressing the HPV-16 E6 and E7 oncogenes) per mouse. The percentages of tumor-free mice were plotted over a period of 30 days. (B) Therapeutic tumor challenge study: Naïve mice were challenged on the right flank with $2x10^5$ TC-1 tumor cells per mouse as described above, and one group of mice was then immunized thrice (correspondingly with the arrows) with either the mixture of Q19D and Q15L peptides along with CT adjuvant (CT+HPC Pep) or with adjuvant alone (CT alone). The size of the tumors (length x width) was measured at several times and expressed as surface area (mm2). Mice immunized with CT+HPV peptides exhibited a delay in tumor growth which was significant 40 days post-tumor challenge, as determined for the individual days by the student t-test with significance at $p \leq .05$(*).

Figure 25. Intranasal immunization with HPV peptides using cholera toxin (CT) adjuvant protects mice against challenge with TC-1 tumor cells.



5.3 Vaccination against HPV Protein-Expressing Tumor using α-GalCer Adjuvant

Based on these encouraging results, we tested the prophylactic and therapeutic immunizations using α-GalCer+HPV peptides delivered by the intranasal route. Prophylactic immunizations were given twice, five days apart, and mice were tumor challenged ten days after the first dose. As with CT, α-GalCer+HPV peptides afforded complete protection against syngeneic TC-1 tumor cells expressing E6 and E7 (Figure 26). Mice that were treated therapeutically were immunized twice at a six day intervals starting at the first sign of tumor growth. Relative to the mice immunized with α-GalCer alone, mice immunized with α-GalCer+HPV peptides were observed to show significant differences in tumor size by day 11 post-tumor challenge (Figure 27 A). In this experiment, an additional group of mice was immunized with α-GalCer+HPV peptides that were earlier injected with the EL4 tumor cells expressing an irrelevant antigen, OVA, known as EG7.Ova. These tumors grew at a drastically accelerated rate compared to TC-1 tumors and, therefore, the mice were euthanized by day 28, by which time a significant difference in tumor size was observed relative to that in immunized mice challenged with the TC-1 tumors (Figure 27 A).

A repeat experiment was performed comparing protective efficacy of intranasal immunization with α-GalCer+HPV peptides to α-GalCer alone, but eliminating challenge with the unrelated EG7.Ova tumor and including control groups of mice mock immunized with PBS or immunized with the HPV peptides alone without inclusion of α-GalCer adjuvant. Immunizations were delivered 14 days apart. Mice immunized with α-GalCer+HPV peptides showed a decreased tumor growth rate compared to PBS alone control, but the differences did not reach significance (Figure 27 B). Of note, the mice immunized twice with α-GalCer alone experienced accelerated tumor growth as compared to PBS control (Figure 27 B).

Figure 26. Prophylactic intranasal immunization with HPV peptides using α-GalCer adjuvant protects against HPV tumor growth. Prophylactic tumor challenge study: Four experimental groups of C57BL/6 mice were immunized by the intranasal route with the mixture of Q19D and Q15L peptides (corresponding to the E6 and E7 oncoproteins of HPV-16, respectively) along with α-GalCer adjuvant (aGC+HPV) or α-GalCer alone twice at 5 day intervals, and 1 week after the last immunization injected subcutaneously on the right flank with $2x10^5$ TC-1 tumor cells (expressing the HPV-16 E6 and E7 oncogenes) or $3x10^6$ EG7.Ova cells (control) per mouse. The percentages of tumor-free mice were plotted over a period of 40 days.

Figure 26. Prophylactic intranasal immunization with HPV peptides using α-GalCer adjuvant protects against HPV tumor growth.

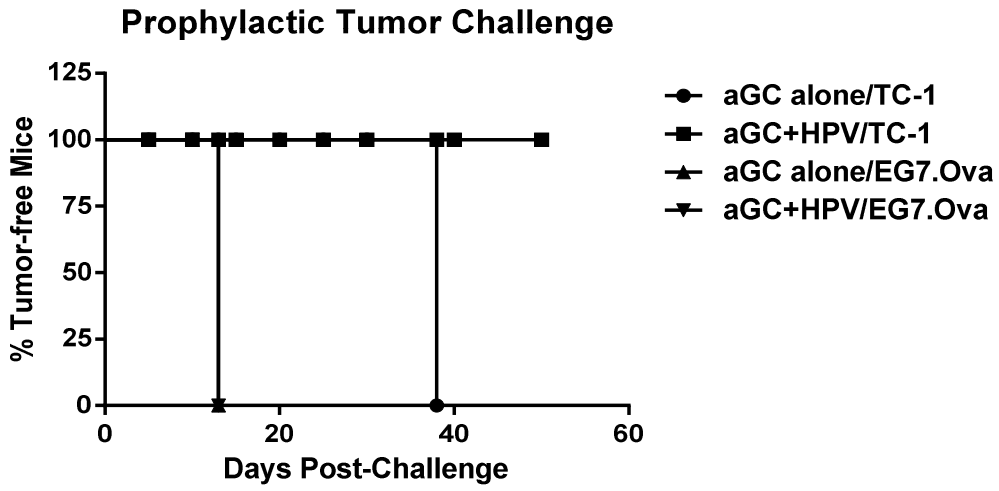
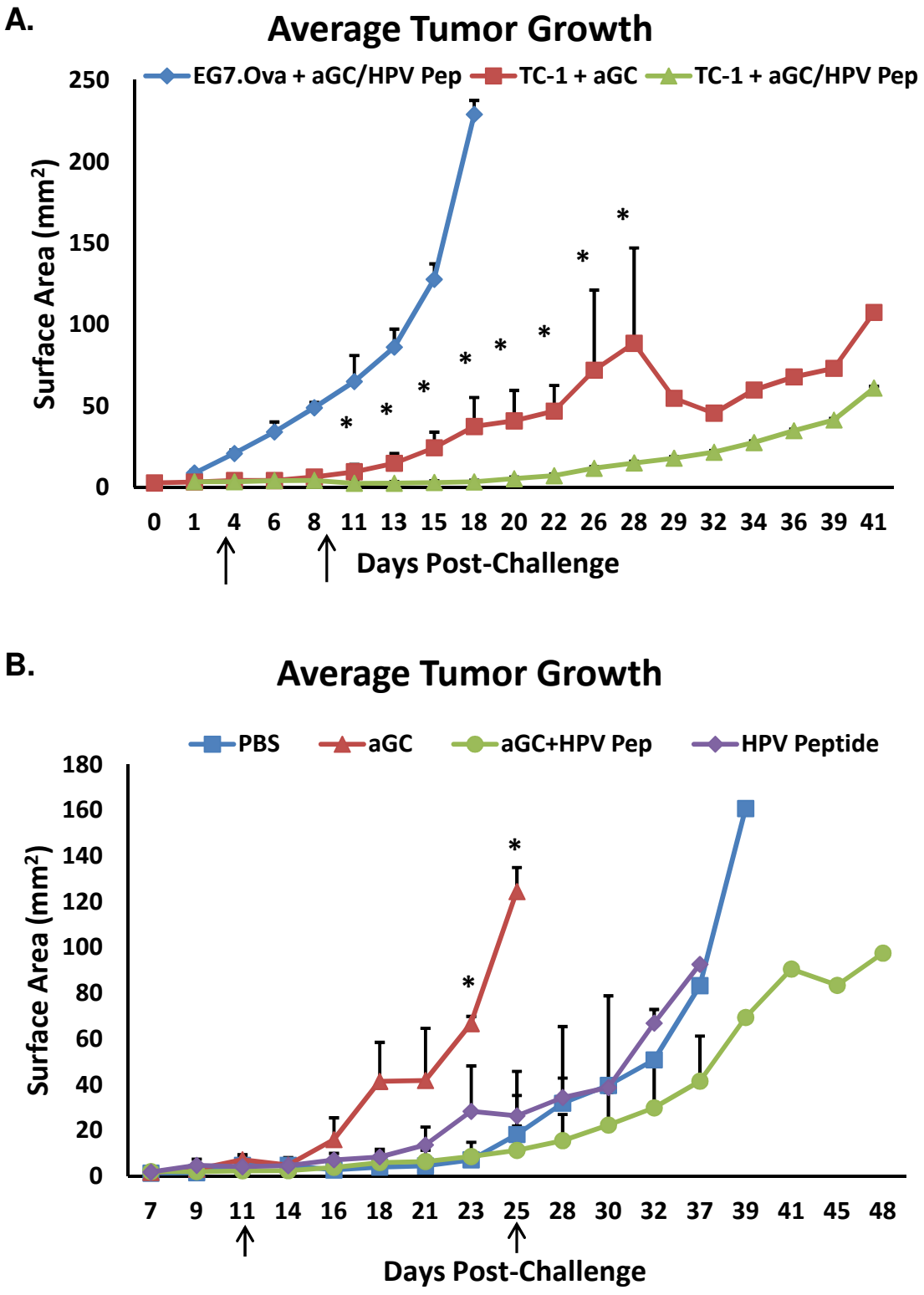


Figure 27. Therapeutic intranasal immunization with HPV peptides using α-GalCer adjuvant delays HPV tumor growth. (A) Naïve mice were challenged on the right flank with $2x10^5$ TC-1 cells (expressing E6 and E7 oncogenes of HPV-16) or EG7.Ova (HPV-negative) tumor cells per mouse. Mice were then immunized by the intranasal route twice correspondingly with the depicted arrows with either α-GalCer adjuvant alone or along with the mixture of Q19D and Q15L peptides (corresponding to the E6 and E7 oncoproteins of HPV-16, respectively). The size of the tumors (length x width) was measured o as surface area (mm^2). Mice immunized with aGC+HPV peptides exhibited a delay in TC-1 tumor growth as compared to mice immunized with α-GalCer alone which was significant between 11 and 28 days post-tumor challenge. Mice injected with EG7.Ova tumor cells and immunized with aGC+HPV peptides showed no control of the tumor growth. (B) Naïve mice were challenged on the right flank with $2x10^5$ TC-1 tumor cells and immunized twice by the intranasal route at 11 and 25 days post-challenge with PBS, α-GalCer alone, HPV peptides alone or mixture of α-GalCer and HPV peptides. Intranasal immunization with aGC+HPV peptides slowed TC-1 tumor growth compared to mock immunization with PBS or HPV peptides alone, but immunization with α-GalCer adjuvant alone resulted in acceleration of tumor growth. The difference in tumor size was which significant between mice immunized with α-GalCer alone and those immunized with aGC+HPV peptides at 23 and 25 days post-tumor challenge, as measured by the student t-test with significance at $p \leq .05$(*).

Figure 27. Therapeutic intranasal immunization with HPV peptides using α-GalCer adjuvant delays HPV tumor growth.



CHAPTER 6: DISCUSSION

The purpose of this study was to investigate the hypothesis that the route utilized to administer α-GalCer adjuvant leads to differential responses by NKT cells, i.e. activation versus anergy, as measured by IFNγ production. We also hypothesized that the IFNγ-producing capacity of NKT cells in response to α-GalCer directly influences induction of adaptive immune responses to co-administered antigens. We tested this by combining intravenous and intranasal routes for sequential immunizations with ovalbumin +/- α-GalCer, and subsequently measuring activation of NKT cells and DC, and induction of antigen-specific T cell activity. Based on past and present studies from our group and those reported in the literature, we surmised the liver and lung to be the primary responding tissues after intravenous and intranasal immunization, respectively, for scoring immune responses specific to α-GalCer and antigen administrations.

A single intravenous immunization activated NKT cells to produce IFNγ in all tissues tested. However, this activated NKT phenotype did not translate to positive adaptive responses in any tissue but the spleen. The first dose was followed by either an IV or IN secondary dose. As expected, the second IV dose resulted in NKT cell anergy in the liver, which is the responding tissue to IV immunization, accompanied by a lack of DC activation indicating that the anergy induced by the first IV immunization could not be overcome by immunization via the mucosal intranasal route in this tissue. This was corroborated by a lack of adaptive immune response in the liver. In the lung, however, the trend was different. In mice that received the IV-IV regimen of immunization, the initial dose strongly activated NKT cells, but subsequent to the second dose there was a lack of IFNγ production combined with expression of other “anergy” markers (e.g. PD-1). However, NKT cells from mice that followed the IV-IN regimen were able to produce large amounts of IFNγ in response to the second dose of α-GalCer, indicating reactivation of NKT cells. Thus, in the lung, it appears that mucosal immunization is able to overcome the anergy induced by the initial systemic immunization (as evident by results from the IV-IV mice), and this is not the case in the systemic-responding liver tissue. The spleen and MdLN followed a similar trend as the liver, where NKT cells were not reactivated after the second IN dose. However, strong adaptive immune responses occurred in both tissues after IV-IN immunization, suggesting that IFNγ production by the activated NKT cells may not be the only mechanism of adjuvant effect induced by α-GalCer administration. This experiment, however, shows that mucosal

intranasal immunization is able to overcome NKT cell anergy induced by initial systemic immunization, at least in the lung tissue, the main responding tissue to intranasal immunization.

When the immunization scheme began with an intranasal dose, NKT cells from all tissues tested were strongly activated to produce IFNγ. A second intranasal dose reactivated NKT cells in the responding lung tissue, but not in the liver. In addition, lung DCs were strongly activated as was adaptive immunity. When the second dose was administered intravenously, NKT cells were reactivated in the liver (responding tissue to this route of immunization) suggesting a lack of anergy development subsequent to the initial intranasal α-GalCer delivery. However, after the IN-IV regimen, NKT cells were not reactivated in the lung. The results of the IN-IV and IN-IN immunizations strongly indicate that NKT cell activation is a tissue specific response that may also depend on the route of immunization. The third intranasal dose was administered in order to see the expected anergy phenotype after IN-IV immunization as well as to determine if IN-IN immunization induces anergy. In the lung, a third IN dose did not activate NKT cells to produce IFNγ, either after IN-IV or IN-IN immunization. This contrasts the trend seen in the lung after IV-IN immunization, where mucosal delivery overcame the anergy induced by systemic immunization. Perhaps by including the additional primary IN dose, which very strongly activated the NKT cells, the capacity to be reactivated to the same degree was diminished. It stands to reason that despite the strength of IV immunization, immunizing via a route that targets a particular tissue will activate the cells more strongly than immunizing via a route that targets tissues other than that of interest. This strong activation may present roadblocks to further activation, not dissimilar to anergy induction after IV immunization in the liver. In fact, the study of four consecutive intranasal immunizations indicates that anergy may in fact be induced by multiple intranasal immunizations, but that this happens over the course of several immunizations rather than one. Interestingly, the IN-IN-IN scheme maintained the ability to activate DCs despite lack of IFNγ production, but the IN-IV-IN scheme did not. A third IN dose was not able to reactivate either IN-IV or IN-IN mouse NKT cells in the liver, indicating again that IN immunization is not able to overcome anergy in systemic-responding tissues as well as the fact that route of immunization plays a role in the ability to activate target NKT cells in particular tissues. Once again, other tissues tested revealed mixed results. Adaptive immunity induction did not correspond in that in many instances where NKT cells were not activated, adaptive immune responses remained positive or even

improved on the first IN dose (which induced strong NKT cell activation). This observation points to the idea that adaptive immune induction does not depend solely on IFNγ production by the NKT cells, and that the adjuvanticity provided by α-GalCer may follow a more complex mechanism of action for downstream events.

This evidence raises several questions about the nature of NKT cell IFNγ production and its role in enhancing adaptive immune responses. Our previous studies point to the necessity of α-GalCer for inducing these adaptive immune responses (221). In fact, IFNγ production in general has been shown to be indispensable for induction of adaptive immunity (222). Furthermore, it is known that NKT cell activation influences events beyond trans-activation of DCs such as activation of NK cells, which have been implicated as the active cells responsible for the anti-tumor activity of α-GalCer (222). And despite acquiring an anergic phenotype, NKT cells maintain their ability to produce IL-4 in response to α-GalCer (175), pointing to the fact that anergy in these cells affects certain cell processes more than others. Therefore, it is unclear whether IFNγ production by NKT cells in response to activation by α-GalCer leads directly to adaptive immune responses or perhaps other cytokines produced by these cells, even in their anergic state, could be responsible for trans-activating DCs and/or influencing the induction of adaptive immune responses to co-administered antigens. It is also possible that the network of communication between different immune cell subsets that is responsible for induction of adaptive immunity is more complicated and involves additional factors beyond NKT cells and DCs.

From observations of these data, I believe there are two factors that may determine how the route of α-GalCer administration and subsequent IFNγ production by NKT cells influence the induction of NKT cell anergy. First, NKT cells must reach a certain threshold of activation to become anergic. This may explain why systemic immunization, which is more potent than mucosal immunization at activating NKT cells to produce high levels of IFNγ, induces anergy after a single dose while it takes multiple mucosal immunizations to do so. The second factor may be that the route of immunization targets the adjuvant effect to certain tissues, thus allowing more potent activation of NKT cells in the targeted tissues than would occur at the same site using a different route. Thus, the level of NKT cell activation, as measured by IFNγ production, is dependent on both the route of α-GalCer administration (to target the tissue of interest) and a threshold of activation. These factors together could dictate the proficiency with which subsequent doses of α-GalCer reactivate NKT cells. For example, a single IV immunization will not activate NKT cells in the lung as proficiently as an

IN dose. This may explain why immunization following an IV-IN scheme results in strong reactivation of NKT cells after the second dose; reactivation is likely afforded both by the initial activation not reaching its peak, and by the second immunization targeting the response to the desired tissue. When the second dose (following IV) was delivered by the IV route, NKT cells produced IFNγ to a lesser degree than after a second IN dose because lung NKT cells were not targeted, but to a measurable degree because the first IV dose did not activate NKT cells to the same threshold as would an initial IN dose. Differential tissue-dependent responses may be due to several factors including harnessing different APCs to present α-GalCer or changes in microenvironment of the tissue.

Interestingly, these studies suggested that a stronger IFNγ response by NKT cells activated by α-GalCer does not necessarily translate to better adaptive immunity. In multiple instances, undetectable IFNγ levels corresponded to strong adaptive immune response. As we have previously shown that α-GalCer is indispensable for inducing these adaptive immune responses after each dose (221), these observations indicate that perhaps the strength of NKT IFNγ production resulting from the initial dose of α-GalCer is more important than in subsequent doses, where perhaps other cell types have been primed to play a more significant role in enhancing adaptive responses than the NKT cells themselves. These other cell types may require NKT cell activation but not necessarily repeated IFNγ production by NKT cells in order to be activated themselves, allowing downstream events to enhance adaptive immunity.

I have also obtained data supporting the idea that multiple intranasal doses (up to three) of α-GalCer may induce NKT cell anergy. In NKT cells which have become anergic due to systemic immunization, the cells are polarized to Th2 responses, which are believed to play a suppressive role in cytotoxic immunity (223, 224). For example, after initial exposure to α-GalCer, subsequent exposure leads to decreased NKT cell IFNg and decreased activation of NK cells to produce IFNg (157). NK cells have been implicated as the primary cell type responsible for the anti-tumor effects of α-GalCer (222). If this is also true after the delivery of multiple doses of α-GalCer by the mucosal route, it could offer a possible explanation as to why, in the therapeutic tumor study, multiple doses of α-GalCer alone delivered by the intranasal route resulted in progression of subcutaneous tumor growth; the NKT cells may have become anergic and polarized to Th2 phenotype which would promote tumor growth rather than suppress it. Dosing with α-GalCer+antigen controlled tumor growth, indicating that perhaps antigen is necessary early on to prime and

activate downstream immunity which can compensate for the effect of NKT cell anergy if it is being induced after multiple doses of intranasal α-GalCer. Future studies will be important to determine if this differential effect will be seen in repeat experiments as well as to determine the mechanism behind it and the downstream immune players mediating the immune response.

We have yet to discover if there is a point at which IFNγ is no longer produced after intranasal administration and how this complete lack of production may affect adaptive immunity. In our study employing four intranasal doses of α-GalCer, the data points to the possibility that the level of activation of adaptive immunity is tissue dependent, and that it is possible that multiple doses of intranasal α-GalCer will eventually lead to a state in which adaptive immunity is no longer activated. This, however, is in contrast to the trend seen in the lung after three IN doses. What we are able to induce is that if NKT cells are playing a less significant role in induction of adaptive immunity after each dose (as suggested by the combined route studies), perhaps there is a point at which their activation becomes unnecessary and other immune cells are able to take over. Thus, more studies are necessary to determine the role of repeated vaccination with α-GalCer+antigen in both induction of NKT cell anergy and in inducing adaptive responses. Activation of NKT cells must be better characterized by measuring other cytokines and when they are produced. The role of other immune players, including APCs and NK cells, must also be studied. Additionally, efforts should be made to better characterize the mechanism by which activated NKT cells may directly induce adaptive immunity in various tissues. A difference in NKT cell biology, APC receptor expression, and even tissue microenvironment may be playing a role in inducing differential responses in a tissue dependent manner. Perhaps these differences can account for the varying degrees of NKT cell anergy in multiple tissues in response to the same immunization scheme. If this is the case, this information can be used to target NKT cells to more strongly activate them as well as to avoid anergy, both important factors in moving α-GalCer adjuvant studies out of mouse models and into primate models and human clinical trials.

CHAPTER 7: SUMMARY AND CONCLUSIONS

This study identified that varying the route of α-GalCer administration results in differential NKT cell activation and induction of anergy which is tissue-dependent. In this study, we showed that a single IV dose of α-GalCer+Ova activated NKT cells to produce IFNγ in the systemic-responding liver tissue and induced adaptive immune responses. In agreement with published studies (193), we found that a second systemic immunization with α-GalCer resulted in NKT cell anergy in the liver in terms of IFNγ production. Delivery of the second dose by the mucosal intranasal route was unable to overcome anergy in the liver. In contrast, while a second IV dose revealed the lung NKT cells to be anergic as well, a second IN dose overcame the anergy in this mucosal-responding tissue to induce IFNγ production by NKT cells and positive adaptive immune responses which were greatly elevated above a single IV dose. The liver lacked significant adaptive responses to secondary immunization. The induction of anergy, or lack thereof, following these two immunization regimens (IV-IV vs. IV-IN) reveals this tissue-dependence of NKT cells which varies by the route utilized.

Furthermore, in the comparison of IN-IN-IN immunization to IN-IV-IN immunization, we found a similar trend indicating a tissue-dependence of the NKT cell response based on immunization route. Two intranasal doses activated NKT cells in the mucosal-responding lung tissue, but not in the systemic-responding liver. When the second dose was delivered systemically, the NKT cells in the liver were reactivated but not in the lung. In order to determine the presence or absence of anergy, we delivered a third intranasal dose and found that NKT cells were not reactivated in the lung or the liver following IN-IV-IN or IN-IN-IN immunization. This examination provided further evidence of the tissue-dependent activation of NKT cells based on route of immunization. Furthermore, this study indicated both that multiple intranasal doses can induce NKT cell anergy, which we confirmed in our regimen utilizing four IN doses, and also that the degree to which NKT cells are activated plays a role in whether or not anergy is induced. Those cells that are activated to a lesser degree after a dose of α-GalCer are less susceptible to anergy than cells that are more highly activated, allowing them to be reactivated by a second dose of α-GalCer.

While the mechanism of NKT cell anergy is not yet well understood, it is clear that NKT cells play a vital role in the effectiveness of α-GalCer as an adjuvant, as lack of inclusion in our mucosal vaccination strategy resulted in a lack of adaptive response (221).

In these studies, we found time and again evidence that the activation status of NKT cells in terms of IFNγ production did not directly correlate to induction of adaptive immune responses. The mechanism for activation of adaptive responses in this system is not yet well defined. If the mechanisms of α-GalCer-mediated induction of adaptive immunity can be elucidated, α-GalCer will show further potential as an effective adjuvant for vaccines against infectious diseases or cancer. We reported earlier that repeated immunization by the intranasal or oral route using α-GalCer as an adjuvant induced systemic and mucosal immune responses to co-administered antigens that improved with each subsequent administration (221). These data are consistent with reports from other groups showing protection against viral infections and tumor challenge with intranasal vaccination employing α-GalCer adjuvant (225, 226). The studies presented here suggest that while intranasal immunization does not completely circumvent the induction of NKT cell anergy, the differential NKT cell activation based on responding tissue suggests that immunization route can be an important tool to circumvent anergy in order to allow for multiple doses of α-GalCer and thus improved immune responses that may extend beyond adaptive immunity. This is highly significant because clinical utility of α-GalCer could be fully realized by identifying and harnessing these differential responses to α-GalCer immunization in order to optimize α-GalCer adjuvant activity and to avoid its suppressive effects. Future experiments should be aimed at providing the mechanistic understanding of the benefits of mucosal immunization to capitalize on the ability of α-GalCer to induce cytotoxic immunity and to allow vaccinations to better target the NKT cells to the desired responses.

Finally, our tumor challenge model utilizing HPV peptides with α-GalCer adjuvant delivered by the intranasal route revealed an interesting phenomenon. While immunization with α-GalCer+Ova was able to control tumor growth to a degree, multiple doses of α-GalCer alone by the intranasal route resulted in acceleration of tumor growth. This could be due to multiple factors. If NKT cells are in fact being anergized by multiple intranasal doses of α-GalCer, perhaps the lack of Th1 responses is lending itself to enhancing suppressive effects, including responses by NK cells and IL-13 production by type II NKT cells (155).

Overall, this study has revealed that NKT cell activation shows tissue dependence when mice are immunized by systemic versus mucosal means. The reason for anergy induction after systemic immunization has been attributed to the preferential presentation of α-GalCer by B cells rather than DCs, which we suspect to be the main APCs active after mucosal immunization. This tissue dependence may be harnessed in order to overcome

anergy induced in some tissue by targeting presentation of α-GalCer to different APCs. In addition, these studies strongly suggest that IFNγ production by NKT cells is not the only factor involved in inducing adaptive immune responses with mucosal α-GalCer immunization. Even without IFNγ expression, cells were able to produce significant adaptive immune responses. Thus, other cytokines made by the NKT cells may play a role in α-GalCer-mediated adaptive immunity. This investigation suggests that, while α-GalCer has proven to be a potent adjuvant in vaccine and anti-tumor formulations, the mechanism of its action must be further studied to fully harness its potential.

CHAPTER 8: FUTURE DIRECTIONS

These studies sought to determine the consequence of NKT cell activation and reactivation on the quality and quantity of adaptive immune responses to co-administered antigen, as well as the practical significance of activation of NKT cells in inducing protection in a tumor challenge model resulting from mucosal delivery of α-GalCer adjuvant with antigen. We initially hypothesized that IFNγ production by NKT cells was responsible for induction of strong and improving adaptive immune responses. However, our studies have revealed that in the absence of IFNγ production by NKT cells, we are still able to induce, and even see improvement in, adaptive immune responses in several tissues tested. When anergy of NKT cells is induced by IV immunization, certain tissues, specifically the lung, are still able to respond weakly to additional IV immunization and strongly to IN immunization. This may be due to differential homing tendencies of α-GalCer-loaded APCs after immunization by these routes, or perhaps production of cytokines other than IFNγ by anergic NKT cells may be capable of activating antigen presenting cells which then directly activate other cell types such as T cells and NK cells, renewing the adaptive responses initiated by activated NKT cells.

8.1 THE ROLE OF NKT CELL IFNγ IN INDUCING ADAPTIVE IMMUNITY

Our findings have brought to light several unknowns in regards to the mechanism by which α-GalCer acts as an adjuvant to induce adaptive immune responses. The first and most obvious question highlighted by these studies is that the role of NKT cell IFNγ production in the adjuvant effect of α-GalCer is unclear, and further efforts should be made to determine the importance of IFNγ production by NKT cells in inducing adaptive immune responses. While the importance of IFNγ has been highlighted in studies using IFNγ knockout mice (222), this has the effect of wiping out activity by other cell types functioning through this cytokine that could be playing a role in the development of immune response. To test the effect of lack of IFNγ production by the NKT cells alone, these cells can be isolated from IFNγ knockout mice and adoptively transferred into Ja18 knockout mice, which lack NKT cells. After adoptive transfer, mice will be immunized by the intranasal or intravenous routes, NKT cells will be identified and analyzed by flow cytometry, and Elispot and CTL assays can be used to measure activation of adaptive immunity.

8.2 IDENTIFICATION, LOCATION, AND ROLE OF APCS IN α-GALCER PRESENTATION

It is also important to characterize the cell type primarily responsible for α-GalCer presentation in each tissue following the various immunization routes. To do this, we can isolate the three major cell types with the potential to present α-GalCer, the DCs, macrophages, and B cells, by magnetic microbead and FACS isolation methods after delivering α-GalCer or mock immunization to the mice by the intravenous or intranasal routes. Then, by co-culturing in vitro with the NKT cell hybridoma DN32.D3 and estimating IL-2 production as reported earlier (124), we can determine which cell type is picking up and presenting the α-GalCer in each tissue (218). Previous work in our lab has led us to believe that DCs primarily present α-GalCer after intranasal immunization (218). The Batf3$^{-/-}$ strain of mice, which are deficient in migratory subsets of CD8α^{+} DC (227), hold some interest in that they can be used to elucidate whether migration of DCs is necessary to induce the responses in any of the tissues studied, particularly in the MdLN. Add something about different DC subtypes.

Along these lines, we must investigate the potential contributions of the resident versus migratory subsets of the three major cells types capable of α-GalCer presentation (i.e. B cells, DC, and macrophages) to better understand the mechanism of α-GalCer presentation and the influence of these presenting populations on the breadth and strength of adaptive immunity. We can do this by first measuring the activation time course of α-GalCer presenting cells by isolating immune cells from various tissues after α-GalCer administration, and then analyzing by flow cytometry (228) the specific cell surface markers such as CD11b on B cells, the maturation markers MHC class II, CD80, and CD86 on both CD8α^{+} and CD11c^{+} DC, and CD169 on macrophages along with chemokine receptors used for homing such as CXCR5, CCR6, and CCR7. These cells can then be tested to measure their capacity to activate the NKT cell hybridoma DN32.D3 in vitro as reported earlier (124). Studies have revealed that expression of cell surface markers and even location within the GALT changes the ability of DCs to present antigen and activate lymphocytes or promote a tolerogenic environment (8, 229-231).

It is in our interest to determine not only the primary APCs active in presenting α-GalCer after immunization via various routes, but also the specific sites of α-GalCer presentation and how they are affected by route of immunization, whether they are at the

local tissues after intranasal and intravenous administration and/or at distant sites after transportation by presenting cells. There are multiple strategies to be employed in answering this question. One option is to deliver fluorescently labeled α-GalCer by intranasal or intravenous routes, and sacrifice mice at various time points between immunization and 24 hours. By staining the various antigen presenting cell types and NKT cells using immunohistochemistry techniques, we can determine the location of α-GalCer presentation. A second option is to identify DCs, macrophages, and B cells, after delivering fluorescently-labeled α-GalCer, by sacrificing immunized mice at multiple time points and staining single-cell suspensions with fluorescently-labeled antibodies to analyze the location of α-GalCer and the cells on which it is presented using flow cytometry.

In addition to studying the migration patterns of the potential antigen-presenting cells, it is possible that NKT cells themselves may be migrating to distant tissues to induce adaptive responses distally, an idea that is supported by the data presented in this study, e.g. MdLN. An important aspect of the adjuvant potential of NKT cells relates to their capacity to activate DC for efficient induction of antigen-specific T and B cell responses. Therefore, we should also perform functional analysis of the NKT cells, similar to analysis of APCs, to determine the role of chemokines and chemokine receptors (e.g. CXCL16/CXCR6, CCL22/CCR4, CXCL9/CXCR3) influencing migration (232-234) by flow cytometry, immunohistochemistry, and qPCR of RNA isolated from different tissues. Additionally, we can compare the preferential migration of NKT cells using an adoptive transfer model by isolating the NKT cells from CD45.1 B6 mice after intranasal immunization with α-GalCer and adoptively transferring to CD45.2 B6. After 24 hours, we can stain for and view congenic NKT cells using flow cytometry to enumerate cells in each tissue and determine if activated NKT cells preferentially migrate to the spleen or the lung compared to un-stimulated NKT cells. In addition, we must simultaneously measure activation markers of the DCs as well as any APCs we determine are playing a presenting role from the previous future study to correlate with the effect of stimulated NKT cells.

8.3 IDENTIFICATION OF NKT CELL MARKERS OF ANERGY AND ACTIVATION

Finally, it has long been in our interest to determine the differences in gene expression between anergic NKT cells and activated NKT cells. For example, PD-1 expression has been shown to play a role in induction of anergy, as blockage of the PD-

1/PD-L1 interaction avoids induction of anergy after systemic immunization (199, 235). It is possible, then, that other phenotypic and functional markers play a role both in induction of anergy and selective re-activation of NKT cells after mucosal immunization. For instance, inhibitory receptors such as Qa-1b ligand NKG2A and CTLA-4 have been previously studies (236, 237). Global microarray analysis identifies differentially expressed genes, in our case in NKT cells activated by mucosal versus systemic α-GalCer delivery. It is possible that certain pathways/mechanisms involved in classical T cell anergy could also be involved in NKT anergy, including the blockade of the MAP-kinase pathway, upregulation of Egr-2 via the NF-AT pathway (238), and impaired NF-kB pathway signaling (239). We will collect cells from liver tissues of mice at different time points (2, 6, 24 and 72h) after intravenous or intranasal α-GalCer delivery using the Pan T cell isolation kit II (from Miltenyi Biotech) followed by flow sorting to purify NKT cells on the basis of the $Aqua^{-}$,$CD3^{+}$,$CD8^{-}$,$NK1.1^{+}$ phenotype. Since the T cell receptor on activated NKT cells is down regulated soon after α-GalCer presentation, tetramer staining may not identify the NKT cells and therefore, we can additionally use the natural killer cell receptor CD160 to guide isolation of pure populations of NKT cells. The RNA must be isolated and quality confirmed. Then total RNA must be amplified and biotin-labeled through an Eberwine procedure and hybridized to Illumina WG6 mouse whole-genome arrays. It has been suggested that gene set enrichment analysis (GSEA) (240) is beneficial to match our results with previously identified gene sets from mSigDB, such as those for exhausted CD8 T cells (241). Subsequently, selected genes differentially expressed in the two NKT cell populations of interest can be validated by real-time qPCR analyses. This microarray analysis would provide both information about NKT cell gene expression as well as possible receptors or ligands that may be interacting with different APCs and how they modulate induction of NKT cell anergy.

8.4 IDENTIFYING DIFFERENTIAL ROLES OF α-GALCER IN CANCER TREATMENT

Our tumor experiments in this study have uncovered an interesting phenomenon in which intranasal immunization with α-GalCer alone has the effect of accelerating tumor growth. Previous studies have shown that a single intravenous immunization with α-GalCer in fact controls tumor growth, but multiple IV immunizations result in acceleration (189). Our observations could be due to the fact that multiple intranasal immunizations with α-GalCer alone are in fact anergizing NKT cells, as is the case with multiple IV immunizations, and thus NKT cells become skewed toward Th2 responses, namely production of IL-13 which represses cytotoxic activities and promotes tumor growth (155). However, when α-GalCer is co-administered with antigen, tumor growth rate was reduced as compared to growth after mock immunization with PBS. In this case, we believe our previous studies support the idea that the addition of antigen to the α-GalCer vaccine promotes Th1 responses and a cytotoxic environment that controls the tumor growth. Further studies need to be conducted to identify the cytokine and cell players active in our model. First, studies should be done on the effect of multiple intranasal immunizations with α-GalCer alone as have been done with α-GalCer+antigen to determine if there is a differential effect of NKT cells in the presence or absence of antigen. Flow cytometry methods and ELISA can be used to identify differences in cytokine production and NK activation between α-GalCer and α-GalCer+antigen treated mice; for example IFNγ and IL-4 profiles should be compared to levels of other Th2 cytokines such as IL-13 and IL-21, which has been shown to both stimulate CD8+ T cells and NK cells while inhibiting activation of DCs (242, 243). In addition, it is important to investigate the role of regularly recurring immunizations versus a set number of doses in controlling tumor growth. Perhaps consistent immunizations, meaning administered for longer duration or at a shorter intervals, will better control and perhaps even reduce tumor burden in these mice. Finally, it is important that we be able to test this vaccine in a more biologically relevant manner. A vaginal challenge with HPV E6 and E7 expressing virus appropriate for mouse use is necessary to validate the findings that this vaccine is effective at controlling the effects of HPV infection. This is important as this can show that immunization with this vaccine is not only applicable in treating cervical neoplasia but can also serve as a therapeutic treatment for patients that do not have cancer but are infected with the virus.

CHAPTER 9: REFERENCES

1. Pavot, V., N. Rochereau, C. Genin, B. Verrier, and S. Paul. 2012. New insights in mucosal vaccine development. Vaccine 30:142-154.
2. Woodrow, K. A., K. M. Bennett, and D. D. Lo. 2012. Mucosal vaccine design and delivery. Annu Rev Biomed Eng 14:17-46.
3. Neutra, M. R., E. Pringault, and J. P. Kraehenbuhl. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14:275-300.
4. Lycke, N. 2012. Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 12:592-605.
5. Neutra, M. R., and P. A. Kozlowski. 2006. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6:148-158.
6. Neutra, M. R., N. J. Mantis, and J. P. Kraehenbuhl. 2001. Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol 2:1004-1009.
7. Miller, C. J., M. McChesney, and P. F. Moore. 1992. Langerhans cells, macrophages and lymphocyte subsets in the cervix and vagina of rhesus macaques. Lab Invest 67:628-634.
8. Niess, J. H., S. Brand, X. Gu, L. Landsman, S. Jung, B. A. McCormick, J. M. Vyas, M. Boes, H. L. Ploegh, J. G. Fox, D. R. Littman, and H. C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 307:254-258.
9. Holt, P. G., M. A. Schon-Hegrad, and P. G. McMenamin. 1990. Dendritic cells in the respiratory tract. Int Rev Immunol 6:139-149.
10. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2:361-367.
11. Jang, M. H., M. N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P. D. Rennert, T. Hiroi, H. Tamagawa, H. Iijima, J. Kunisawa, Y. Yuki, and H. Kiyono. 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. Proc Natl Acad Sci U S A 101:6110-6115.
12. Iwasaki, A. 2007. Mucosal dendritic cells. Annu Rev Immunol 25:381-418.

13. Belyakov, I. M., and J. D. Ahlers. 2009. What Role Does the Route of Immunization Play in the Generation of Protective Immunity against Mucosal Pathogens? Journal of Immunology 183:6883-6892.
14. Ichinohe, T., H. K. Lee, Y. Ogura, R. Flavell, and A. Iwasaki. 2009. Inflammasome recognition of influenza virus is essential for adaptive immune responses. J Exp Med 206:79-87.
15. Hargreaves, D. C., and R. Medzhitov. 2005. Innate sensors of microbial infection. J Clin Immunol 25:503-510.
16. Medzhitov, R. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol 1:135-145.
17. Hysi, P., M. Kabesch, M. F. Moffatt, M. Schedel, D. Carr, Y. Zhang, B. Boardman, E. von Mutius, S. K. Weiland, W. Leupold, C. Fritzsch, N. Klopp, A. W. Musk, A. James, G. Nunez, N. Inohara, and W. O. Cookson. 2005. NOD1 variation, immunoglobulin E and asthma. Hum Mol Genet 14:935-941.
18. Kantele, A., M. Hakkinen, Z. Moldoveanu, A. Lu, E. Savilahti, R. D. Alvarez, S. Michalek, and J. Mestecky. 1998. Differences in immune responses induced by oral and rectal immunizations with Salmonella typhi Ty21a: evidence for compartmentalization within the common mucosal immune system in humans. Infect Immun 66:5630-5635.
19. Kunkel, E. J., and E. C. Butcher. 2003. Plasma-cell homing. Nat Rev Immunol 3:822-829.
20. Ogra, P. L. 1984. Mucosal immune response to poliovirus vaccines in childhood. Rev Infect Dis 6 Suppl 2:S361-368.
21. Belshe, R. B., P. M. Mendelman, J. Treanor, J. King, W. C. Gruber, P. Piedra, D. I. Bernstein, F. G. Hayden, K. Kotloff, K. Zangwill, D. Iacuzio, and M. Wolff. 1998. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. N Engl J Med 338:1405-1412.
22. Viret, J. F., D. Favre, B. Wegmuller, C. Herzog, J. U. Que, S. J. Cryz, Jr., and A. B. Lang. 1999. Mucosal and systemic immune responses in humans after primary and booster immunizations with orally administered invasive and noninvasive live attenuated bacteria. Infect Immun 67:3680-3685.
23. Perry, R. T., C. V. Plowe, B. Koumare, F. Bougoudogo, K. L. Kotloff, G. A. Losonsky, S. S. Wasserman, and M. M. Levine. 1998. A single dose of live oral cholera vaccine

CVD 103-HgR is safe and immunogenic in HIV-infected and HIV-noninfected adults in Mali. Bull World Health Organ 76:63-71.

24. Lai, S. K., D. E. O'Hanlon, S. Harrold, S. T. Man, Y. Y. Wang, R. Cone, and J. Hanes. 2007. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. Proc Natl Acad Sci U S A 104:1482-1487.
25. Lai, S. K., Y. Y. Wang, and J. Hanes. 2009. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev 61:158-171.
26. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. J Cell Biol 55:186-204.
27. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137:1142-1162.
28. Nussenzweig, M. C., R. M. Steinman, B. Gutchinov, and Z. A. Cohn. 1980. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. J Exp Med 152:1070-1084.
29. Bonifaz, L. C., D. P. Bonnyay, A. Charalambous, D. I. Darguste, S. Fujii, H. Soares, M. K. Brimnes, B. Moltedo, T. M. Moran, and R. M. Steinman. 2004. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 199:815-824.
30. van Duin, D., R. Medzhitov, and A. C. Shaw. 2006. Triggering TLR signaling in vaccination. Trends Immunol 27:49-55.
31. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med 198:267-279.
32. Kasturi, S. P., I. Skountzou, R. A. Albrecht, D. Koutsonanos, T. Hua, H. I. Nakaya, R. Ravindran, S. Stewart, M. Alam, M. Kwissa, F. Villinger, N. Murthy, J. Steel, J. Jacob, R. J. Hogan, A. Garcia-Sastre, R. Compans, and B. Pulendran. 2011. Programming the magnitude and persistence of antibody responses with innate immunity. Nature 470:543-547.
33. Blander, J. M., and R. Medzhitov. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature 440:808-812.
34. Cranage, M. P., C. A. Fraser, Z. Stevens, J. Huting, M. Chang, S. A. Jeffs, M. S. Seaman, A. Cope, T. Cole, and R. J. Shattock. 2010. Repeated vaginal

administration of trimeric HIV-1 clade C gp140 induces serum and mucosal antibody responses. Mucosal Immunol 3:57-68.

35. Wheeler, C. M., S. K. Kjaer, K. Sigurdsson, O. E. Iversen, M. Hernandez-Avila, G. Perez, D. R. Brown, L. A. Koutsky, E. H. Tay, P. Garcia, K. A. Ault, S. M. Garland, S. Leodolter, S. E. Olsson, G. W. Tang, D. G. Ferris, J. Paavonen, M. Steben, F. X. Bosch, J. Dillner, E. A. Joura, R. J. Kurman, S. Majewski, N. Munoz, E. R. Myers, L. L. Villa, F. J. Taddeo, C. Roberts, A. Tadesse, J. Bryan, L. C. Lupinacci, K. E. Giacoletti, M. James, S. Vuocolo, T. M. Hesley, and E. Barr. 2009. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in sexually active women aged 16-26 years. J Infect Dis 199:936-944.
36. Csaba, N., M. Garcia-Fuentes, and M. J. Alonso. 2009. Nanoparticles for nasal vaccination. Adv Drug Deliv Rev 61:140-157.
37. Peltola, H., H. Makela, H. Kayhty, H. Jousimies, E. Herva, K. Hallstrom, A. Sivonen, O. V. Renkonen, O. Pettay, V. Karanko, P. Ahvonen, and S. Sarna. 1977. Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. N Engl J Med 297:686-691.
38. Thapa, P., G. Zhang, C. Xia, A. Gelbard, W. W. Overwijk, C. Liu, P. Hwu, D. Z. Chang, A. Courtney, J. K. Sastry, P. G. Wang, C. Li, and D. Zhou. 2009. Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy. Vaccine 27:3484-3488.
39. Lemoine, D., M. Deschuyteneer, F. Hogge, and V. Preat. 1999. Intranasal immunization against influenza virus using polymeric particles. J Biomater Sci Polym Ed 10:805-825.
40. van der Lubben, I. M., J. C. Verhoef, G. Borchard, and H. E. Junginger. 2001. Chitosan for mucosal vaccination. Adv Drug Deliv Rev 52:139-144.
41. Amidi, M., E. Mastrobattista, W. Jiskoot, and W. E. Hennink. 2010. Chitosan-based delivery systems for protein therapeutics and antigens. Adv Drug Deliv Rev 62:59-82.
42. Moschos, S. A., V. W. Bramwell, S. Somavarapu, and H. O. Alpar. 2004. Adjuvant synergy: the effects of nasal coadministration of adjuvants. Immunol Cell Biol 82:628-637.

43. Jain, A. K., K. B. Chalasani, R. K. Khar, F. J. Ahmed, and P. V. Diwan. 2007. Muco-adhesive multivesicular liposomes as an effective carrier for transmucosal insulin delivery. J Drug Target 15:417-427.
44. Gluck, U., J. O. Gebbers, and R. Gluck. 1999. Phase 1 evaluation of intranasal virosomal influenza vaccine with and without Escherichia coli heat-labile toxin in adult volunteers. J Virol 73:7780-7786.
45. Treanor, J., C. Nolan, D. O'Brien, D. Burt, G. Lowell, J. Linden, and L. Fries. 2006. Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects. Vaccine 24:254-262.
46. Lindholm, C., A. Naylor, E. L. Johansson, and M. Quiding-Jarbrink. 2004. Mucosal vaccination increases endothelial expression of mucosal addressin cell adhesion molecule 1 in the human gastrointestinal tract. Infect Immun 72:1004-1009.
47. Stagg, A. J., M. A. Kamm, and S. C. Knight. 2002. Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. Eur J Immunol 32:1445-1454.
48. Alpan, O., G. Rudomen, and P. Matzinger. 2001. The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. J Immunol 166:4843-4852.
49. Azizi, A., H. Ghunaim, F. Diaz-Mitoma, and J. Mestecky. 2010. Mucosal HIV vaccines: a holy grail or a dud? Vaccine 28:4015-4026.
50. Zhou, B., Y. Zhang, X. Wang, J. Dong, B. Wang, C. Han, J. Yu, and D. Li. 2010. Oral administration of plant-based rotavirus VP6 induces antigen-specific IgAs, IgGs and passive protection in mice. Vaccine 28:6021-6027.
51. Daniell, H., S. J. Streatfield, and K. Wycoff. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219-226.
52. Song, J. H., H. H. Nguyen, N. Cuburu, T. Horimoto, S. Y. Ko, S. H. Park, C. Czerkinsky, and M. N. Kweon. 2008. Sublingual vaccination with influenza virus protects mice against lethal viral infection. Proc Natl Acad Sci U S A 105:1644-1649.
53. Song, J. H., J. I. Kim, H. J. Kwon, D. H. Shim, N. Parajuli, N. Cuburu, C. Czerkinsky, and M. N. Kweon. 2009. CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination. J Immunol 182:6851-6860.
54. Cuburu, N., M. N. Kweon, C. Hervouet, H. R. Cha, Y. Y. Pang, J. Holmgren, K. Stadler, J. T. Schiller, F. Anjuere, and C. Czerkinsky. 2009. Sublingual immunization with nonreplicating antigens induces antibody-forming cells and cytotoxic T cells in

the female genital tract mucosa and protects against genital papillomavirus infection. J Immunol 183:7851-7859.

55. Kozlowski, P. A., S. Cu-Uvin, M. R. Neutra, and T. P. Flanigan. 1997. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. Infect Immun 65:1387-1394.
56. Kozlowski, P. A., S. B. Williams, R. M. Lynch, T. P. Flanigan, R. R. Patterson, S. Cu-Uvin, and M. R. Neutra. 2002. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. J Immunol 169:566-574.
57. Lamm, M. E. 1997. Interaction of antigens and antibodies at mucosal surfaces. Annual Review of Microbiology 51:311-340.
58. Hutchings, A. B., A. Helander, K. J. Silvey, K. Chandran, W. T. Lucas, M. L. Nibert, and M. R. Neutra. 2004. Secretory immunoglobulin A antibodies against the sigma 1 outer capsid protein of reovirus type 1 Lang prevent infection of mouse Peyer's patches. Journal of Virology 78:947-957.
59. Seavey, M. M., and T. R. Mosmann. 2009. Estradiol-induced vaginal mucus inhibits antigen penetration and CD8(+) T cell priming in response to intravaginal immunization. Vaccine 27:2342-2349.
60. Seavey, M. M., and T. R. Mosmann. 2006. Paternal antigen-bearing cells transferred during insemination do not stimulate anti-paternal CD8+ T cells: role of estradiol in locally inhibiting CD8+ T cell responses. J Immunol 177:7567-7578.
61. Decrausaz, L., S. Domingos-Pereira, M. Duc, M. Bobst, P. Romero, J. T. Schiller, P. Jichlinski, and D. Nardelli-Haefliger. 2011. Parenteral is more efficient than mucosal immunization to induce regression of human papillomavirus-associated genital tumors. Int J Cancer 129:762-772.
62. Imaoka, K., C. J. Miller, M. Kubota, M. B. McChesney, B. Lohman, M. Yamamoto, K. Fujihashi, K. Someya, M. Honda, J. R. McGhee, and H. Kiyono. 1998. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. J Immunol 161:5952-5958.
63. Rudin, A., G. C. Riise, and J. Holmgren. 1999. Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infect Immun 67:2884-2890.

64. Gallichan, W. S., and K. L. Rosenthal. 1998. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. J Infect Dis 177:1155-1161.
65. Alpar, H. O., J. E. Eyles, E. D. Williamson, and S. Somavarapu. 2001. Intranasal vaccination against plague, tetanus and diphtheria. Adv Drug Deliv Rev 51:173-201.
66. Fiore, A. E., C. B. Bridges, and N. J. Cox. 2009. Seasonal influenza vaccines. Curr Top Microbiol Immunol 333:43-82.
67. Brandtzaeg, P. 2007. Induction of secretory immunity and memory at mucosal surfaces. Vaccine 25:5467-5484.
68. Brandtzaeg, P. 2003. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. Int J Pediatr Otorhinolaryngol 67 Suppl 1:S69-76.
69. Mestecky, J., R. C. Alexander, Q. Wei, and Z. Moldoveanu. 2011. Methods for evaluation of humoral immune responses in human genital tract secretions. Am J Reprod Immunol 65:361-367.
70. Mestecky, J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 7:265-276.
71. Kool, M., K. Fierens, and B. N. Lambrecht. 2012. Alum adjuvant: some of the tricks of the oldest adjuvant. J Med Microbiol 61:927-934.
72. Heijmans, S., M. De Meulemeester, P. Reynders, D. Giet, E. Demanet, P. Y. Devresse, G. Icardi, M. Drame, F. Roman, and P. Gillard. 2011. Immunogenicity profile of a 3.75-mug hemagglutinin pandemic rH5N1 split virion AS03A-adjuvanted vaccine in elderly persons: a randomized trial. J Infect Dis 203:1054-1062.
73. Leroux-Roels, I., A. Borkowski, T. Vanwolleghem, M. Drame, F. Clement, E. Hons, J. M. Devaster, and G. Leroux-Roels. 2007. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 370:580-589.
74. Tritto, E., F. Mosca, and E. De Gregorio. 2009. Mechanism of action of licensed vaccine adjuvants. Vaccine 27:3331-3334.
75. Calabro, S., M. Tortoli, B. C. Baudner, A. Pacitto, M. Cortese, D. T. O'Hagan, E. De Gregorio, A. Seubert, and A. Wack. 2011. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine 29:1812-1823.
76. Kool, M., T. Soullie, M. van Nimwegen, M. A. Willart, F. Muskens, S. Jung, H. C. Hoogsteden, H. Hammad, and B. N. Lambrecht. 2008. Alum adjuvant boosts

adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 205:869-882.

77. McKee, A. S., M. W. Munks, M. K. MacLeod, C. J. Fleenor, N. Van Rooijen, J. W. Kappler, and P. Marrack. 2009. Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity. J Immunol 183:4403-4414.
78. Mosca, F., E. Tritto, A. Muzzi, E. Monaci, F. Bagnoli, C. Iavarone, D. O'Hagan, R. Rappuoli, and E. De Gregorio. 2008. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci U S A 105:10501-10506.
79. Marichal, T., K. Ohata, D. Bedoret, C. Mesnil, C. Sabatel, K. Kobiyama, P. Lekeux, C. Coban, S. Akira, K. J. Ishii, F. Bureau, and C. J. Desmet. 2011. DNA released from dying host cells mediates aluminum adjuvant activity. Nat Med 17:996-1002.
80. Czerkinsky, C., F. Anjuere, J. R. McGhee, A. George-Chandy, J. Holmgren, M. P. Kieny, K. Fujiyashi, J. F. Mestecky, V. Pierrefite-Carle, C. Rask, and J. B. Sun. 1999. Mucosal immunity and tolerance: relevance to vaccine development. Immunol Rev 170:197-222.
81. Matzinger, P. 1994. Tolerance, danger, and the extended family. Annu Rev Immunol 12:991-1045.
82. Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. Nat Med 5:1249-1255.
83. Matzinger, P. 2001. Essay 1: the Danger model in its historical context. Scand J Immunol 54:4-9.
84. Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. Curr Opin Immunol 13:114-119.
85. Pradeu, T., and E. L. Cooper. 2012. The danger theory: 20 years later. Front Immunol 3:287.
86. Shields, A. M., G. S. Panayi, and V. M. Corrigall. 2011. Resolution-associated molecular patterns (RAMP): RAMParts defending immunological homeostasis? Clin Exp Immunol 165:292-300.
87. Broere, F., R. van der Zee, and W. van Eden. 2011. Heat shock proteins are no DAMPs, rather 'DAMPERs'. Nat Rev Immunol 11:565; author reply 565.
88. van Eden, W., R. Spiering, F. Broere, and R. van der Zee. 2012. A case of mistaken identity: HSPs are no DAMPs but DAMPERs. Cell Stress Chaperones 17:281-292.

89. Kono, H., and K. L. Rock. 2008. How dying cells alert the immune system to danger. Nat Rev Immunol 8:279-289.
90. Basu, S., R. J. Binder, R. Suto, K. M. Anderson, and P. K. Srivastava. 2000. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol 12:1539-1546.
91. Wolf, J. L., D. H. Rubin, R. Finberg, R. S. Kauffman, A. H. Sharpe, J. S. Trier, and B. N. Fields. 1981. Intestinal M cells: a pathway for entry of reovirus into the host. Science 212:471-472.
92. Weinstein, D. L., B. L. O'Neill, D. M. Hone, and E. S. Metcalf. 1998. Differential early interactions between Salmonella enterica serovar Typhi and two other pathogenic Salmonella serovars with intestinal epithelial cells. Infect Immun 66:2310-2318.
93. Yuki, Y., and H. Kiyono. 2003. New generation of mucosal adjuvants for the induction of protective immunity. Rev Med Virol 13:293-310.
94. McCluskie, M. J., R. D. Weeratna, P. J. Payette, and H. L. Davis. 2001. The use of CpG DNA as a mucosal vaccine adjuvant. Curr Opin Investig Drugs 2:35-39.
95. Kaisho, T., and S. Akira. 2002. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta 1589:1-13.
96. Yamamoto, S., H. Kiyono, M. Yamamoto, K. Imaoka, K. Fujihashi, F. W. Van Ginkel, M. Noda, Y. Takeda, and J. R. McGhee. 1997. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci U S A 94:5267-5272.
97. Yamamoto, S., Y. Takeda, M. Yamamoto, H. Kurazono, K. Imaoka, K. Fujihashi, M. Noda, H. Kiyono, and J. R. McGhee. 1997. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J Exp Med 185:1203-1210.
98. Hase, C. C., L. S. Thai, M. Boesman-Finkelstein, V. L. Mar, W. N. Burnette, H. R. Kaslow, L. A. Stevens, J. Moss, and R. A. Finkelstein. 1994. Construction and characterization of recombinant Vibrio cholerae strains producing inactive cholera toxin analogs. Infect Immun 62:3051-3057.
99. Lomada, D., R. Gambhira, P. N. Nehete, F. A. Guhad, A. K. Chopra, J. W. Peterson, and K. J. Sastry. 2004. A two-codon mutant of cholera toxin lacking ADP-ribosylating activity functions as an effective adjuvant for eliciting mucosal and systemic cellular immune responses to peptide antigens. Vaccine 23:555-565.

100. Manuri, P. R., B. Nehete, P. N. Nehete, R. Reisenauer, S. Wardell, A. N. Courtney, R. Gambhira, D. Lomada, A. K. Chopra, and K. J. Sastry. 2007. Intranasal immunization with synthetic peptides corresponding to the E6 and E7 oncoproteins of human papillomavirus type 16 induces systemic and mucosal cellular immune responses and tumor protection. Vaccine 25:3302-3310.
101. Natori, T., Y. Koezuka, and T. Higa. 1993. ChemInform Abstract: Agelasphins (I), Novel α-Galactosylceramides from the Marine Sponge Agelas mauritianus. ChemInform 24:no-no.
102. Natori, T., K. Akimoto, K. Motoki, Y. Koezuka, and T. Higa. 1997. [Development of KRN7000, derived from agelasphin produced by Okinawan sponge]. Nihon Yakurigaku Zasshi 110 Suppl 1:63P-68P.
103. Naidenko, O. V., Y. Koezuka, and M. Kronenberg. 2000. CD1-mediated antigen presentation of glycosphingolipids. Microbes Infect 2:621-631.
104. Sonnino, S., L. Cantu, M. Corti, D. Acquotti, and B. Venerando. 1994. Aggregative properties of gangliosides in solution. Chem Phys Lipids 71:21-45.
105. Zhou, D., J. Mattner, C. Cantu, 3rd, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y. P. Wu, T. Yamashita, S. Teneberg, D. Wang, R. L. Proia, S. B. Levery, P. B. Savage, L. Teyton, and A. Bendelac. 2004. Lysosomal glycosphingolipid recognition by NKT cells. Science 306:1786-1789.
106. Zhou, X. T., C. Forestier, R. D. Goff, C. Li, L. Teyton, A. Bendelac, and P. B. Savage. 2002. Synthesis and NKT cell stimulating properties of fluorophore- and biotin-appended 6"-amino-6"-deoxy-galactosylceramides. Org Lett 4:1267-1270.
107. Kos, F. J. 1998. Regulation of adaptive immunity by natural killer cells. Immunol Res 17:303-312.
108. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature 392:245-252.
109. Nishimura, T., H. Kitamura, K. Iwakabe, T. Yahata, A. Ohta, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, M. Taniguchi, M. Nakui, M. Sekimoto, and T. Koda. 2000. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. Int Immunol 12:987-994.
110. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. Annu Rev Immunol 21:483-513.

111. Imai, K., M. Kanno, H. Kimoto, K. Shigemoto, S. Yamamoto, and M. Taniguchi. 1986. Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. Proc Natl Acad Sci U S A 83:8708-8712.
112. Koseki, H., K. Imai, T. Ichikawa, I. Hayata, and M. Taniguchi. 1989. Predominant use of a particular alpha-chain in suppressor T cell hybridomas specific for keyhole limpet hemocyanin. Int Immunol 1:557-564.
113. Ohteki, T., and H. R. MacDonald. 1994. Major histocompatibility complex class I related molecules control the development of CD4+8- and CD4-8- subsets of natural killer 1.1+ T cell receptor-alpha/beta+ cells in the liver of mice. J Exp Med 180:699-704.
114. Adachi, Y., H. Koseki, M. Zijlstra, and M. Taniguchi. 1995. Positive selection of invariant V alpha 14+ T cells by non-major histocompatibility complex-encoded class I-like molecules expressed on bone marrow-derived cells. Proc Natl Acad Sci U S A 92:1200-1204.
115. Makino, Y., N. Yamagata, T. Sasho, Y. Adachi, R. Kanno, H. Koseki, M. Kanno, and M. Taniguchi. 1993. Extrathymic development of V alpha 14-positive T cells. J Exp Med 177:1399-1408.
116. Fowlkes, B. J., A. M. Kruisbeek, H. Ton-That, M. A. Weston, J. E. Coligan, R. H. Schwartz, and D. M. Pardoll. 1987. A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. Nature 329:251-254.
117. Budd, R. C., G. C. Miescher, R. C. Howe, R. K. Lees, C. Bron, and H. R. MacDonald. 1987. Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes. J Exp Med 166:577-582.
118. Sykes, M. 1990. Unusual T cell populations in adult murine bone marrow. Prevalence of CD3+CD4-CD8- and alpha beta TCR+NK1.1+ cells. J Immunol 145:3209-3215.
119. Ballas, Z. K., and W. Rasmussen. 1990. NK1.1+ thymocytes. Adult murine CD4-, CD8- thymocytes contain an NK1.1+, CD3+, CD5hi, CD44hi, TCR-V beta 8+ subset. J Immunol 145:1039-1045.
120. Arase, H., N. Arase, K. Nakagawa, R. A. Good, and K. Onoe. 1993. NK1.1+ CD4+ CD8- thymocytes with specific lymphokine secretion. Eur J Immunol 23:307-310.

121. Yoshimoto, T., and W. E. Paul. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. J Exp Med 179:1285-1295.
122. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. J Exp Med 180:1097-1106.
123. Bendelac, A. 1995. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. J Exp Med 182:2091-2096.
124. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse NK1+ T lymphocytes. Science 268:863-865.
125. Arase, H., N. Arase, K. Ogasawara, R. A. Good, and K. Onoe. 1992. An NK1.1+ CD4+8- single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor V beta family. Proc Natl Acad Sci U S A 89:6506-6510.
126. Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol 15:535-562.
127. Smyth, M. J., N. Y. Crowe, Y. Hayakawa, K. Takeda, H. Yagita, and D. I. Godfrey. 2002. NKT cells - conductors of tumor immunity? Curr Opin Immunol 14:165-171.
128. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? Nat Rev Immunol 4:231-237.
129. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol 23:877-900.
130. Assarsson, E., T. Kambayashi, J. K. Sandberg, S. Hong, M. Taniguchi, L. Van Kaer, H. G. Ljunggren, and B. J. Chambers. 2000. CD8+ T cells rapidly acquire NK1.1 and NK cell-associated molecules upon stimulation in vitro and in vivo. J Immunol 165:3673-3679.
131. McNab, F. W., D. G. Pellicci, K. Field, G. Besra, M. J. Smyth, D. I. Godfrey, and S. P. Berzins. 2007. Peripheral NK1.1 NKT cells are mature and functionally distinct from their thymic counterparts. J Immunol 179:6630-6637.
132. Lynch, L., D. O'Shea, D. C. Winter, J. Geoghegan, D. G. Doherty, and C. O'Farrelly. 2009. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. Eur J Immunol 39:1893-1901.

133. Eberl, G., R. Lees, S. T. Smiley, M. Taniguchi, M. J. Grusby, and H. R. MacDonald. 1999. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. J Immunol 162:6410-6419.
134. Kim, S., S. Lalani, V. V. Parekh, T. L. Vincent, L. Wu, and L. Van Kaer. 2008. Impact of bacteria on the phenotype, functions, and therapeutic activities of invariant NKT cells in mice. J Clin Invest 118:2301-2315.
135. Leite-De-Moraes, M. C., A. Hameg, A. Arnould, F. Machavoine, Y. Koezuka, E. Schneider, A. Herbelin, and M. Dy. 1999. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. J Immunol 163:5871-5876.
136. Gumperz, J. E., S. Miyake, T. Yamamura, and M. B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J Exp Med 195:625-636.
137. Lee, P. T., K. Benlagha, L. Teyton, and A. Bendelac. 2002. Distinct functional lineages of human V(alpha)24 natural killer T cells. J Exp Med 195:637-641.
138. Coquet, J. M., S. Chakravarti, K. Kyparissoudis, F. W. McNab, L. A. Pitt, B. S. McKenzie, S. P. Berzins, M. J. Smyth, and D. I. Godfrey. 2008. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. Proc Natl Acad Sci U S A 105:11287-11292.
139. Godfrey, D. I., S. Stankovic, and A. G. Baxter. 2010. Raising the NKT cell family. Nat Immunol 11:197-206.
140. Geissmann, F., T. O. Cameron, S. Sidobre, N. Manlongat, M. Kronenberg, M. J. Briskin, M. L. Dustin, and D. R. Littman. 2005. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. PLoS Biol 3:e113.
141. Germanov, E., L. Veinotte, R. Cullen, E. Chamberlain, E. C. Butcher, and B. Johnston. 2008. Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. J Immunol 181:81-91.
142. Ohteki, T., C. Maki, S. Koyasu, T. W. Mak, and P. S. Ohashi. 1999. Cutting edge: LFA-1 is required for liver NK1.1+TCR alpha beta+ cell development: evidence that liver NK1.1+TCR alpha beta+ cells originate from multiple pathways. J Immunol 162:3753-3756.
143. Emoto, M., H. W. Mittrucker, R. Schmits, T. W. Mak, and S. H. Kaufmann. 1999. Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. J Immunol 162:5094-5098.

144. Monticelli, L. A., Y. Yang, J. Knell, L. M. D'Cruz, M. A. Cannarile, I. Engel, M. Kronenberg, and A. W. Goldrath. 2009. Transcriptional regulator Id2 controls survival of hepatic NKT cells. Proc Natl Acad Sci U S A 106:19461-19466.
145. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 278:1626-1629.
146. Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J Exp Med 188:1521-1528.
147. van den Heuvel, M. J., N. Garg, L. Van Kaer, and S. M. M. Haeryfar. 2011. NKT cell costimulation: experimental progress and therapeutic promise. Trends Mol. Med 17:65-77.
148. Borg, N. A., K. S. Wun, L. Kjer-Nielsen, M. C. Wilce, D. G. Pellicci, R. Koh, G. S. Besra, M. Bharadwaj, D. I. Godfrey, J. McCluskey, and J. Rossjohn. 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature 448:44-49.
149. McCarthy, C., D. Shepherd, S. Fleire, V. S. Stronge, M. Koch, P. A. Illarionov, G. Bossi, M. Salio, G. Denkberg, F. Reddington, A. Tarlton, B. G. Reddy, R. R. Schmidt, Y. Reiter, G. M. Griffiths, P. A. van der Merwe, G. S. Besra, E. Y. Jones, F. D. Batista, and V. Cerundolo. 2007. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. J Exp Med 204:1131-1144.
150. Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, M. Taniguchi, and T. Nishimura. 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J Exp Med 189:1121-1128.
151. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, H. Sato, E. Kondo, M. Harada, H. Koseki, T. Nakayama, Y. Tanaka, and M. Taniguchi. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. Proc Natl Acad Sci U S A 95:5690-5693.

152. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W. E. Paul. 1995. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. Science 270:1845-1847.
153. Takahashi, T., K. Haraguchi, S. Chiba, M. Yasukawa, Y. Shibata, and H. Hirai. 2003. Valpha24+ natural killer T-cell responses against T-acute lymphoblastic leukaemia cells: implications for immunotherapy. Br J Haematol 122:231-239.
154. Metelitsa, L. S., K. I. Weinberg, P. D. Emanuel, and R. C. Seeger. 2003. Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. Leukemia 17:1068-1077.
155. Berzofsky, J. A., and M. Terabe. 2009. The contrasting roles of NKT cells in tumor immunity. Curr Mol Med 9:667-672.
156. Stetson, D. B., M. Mohrs, R. L. Reinhardt, J. L. Baron, Z. E. Wang, L. Gapin, M. Kronenberg, and R. M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J Exp Med 198:1069-1076.
157. Matsuda, J. L., L. Gapin, J. L. Baron, S. Sidobre, D. B. Stetson, M. Mohrs, R. M. Locksley, and M. Kronenberg. 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. Proc Natl Acad Sci U S A 100:8395-8400.
158. Pellicci, D. G., O. Patel, L. Kjer-Nielsen, S. S. Pang, L. C. Sullivan, K. Kyparissoudis, A. G. Brooks, H. H. Reid, S. Gras, I. S. Lucet, R. Koh, M. J. Smyth, T. Mallevaey, J. L. Matsuda, L. Gapin, J. McCluskey, D. I. Godfrey, and J. Rossjohn. 2009. Differential recognition of CD1d-alpha-galactosyl ceramide by the V beta 8.2 and V beta 7 semi-invariant NKT T cell receptors. Immunity 31:47-59.
159. Gadue, P., N. Morton, and P. L. Stein. 1999. The Src family tyrosine kinase Fyn regulates natural killer T cell development. J Exp Med 190:1189-1196.
160. Arase, H., S. Ono, N. Arase, S. Y. Park, K. Wakizaka, H. Watanabe, H. Ohno, and T. Saito. 1995. Developmental arrest of NK1.1+ T cell antigen receptor (TCR)-alpha/beta+ T cells and expansion of NK1.1+ TCR-gamma/delta+ T cell development in CD3 zeta-deficient mice. J Exp Med 182:891-895.
161. Akbari, O., J. L. Faul, E. G. Hoyte, G. J. Berry, J. Wahlstrom, M. Kronenberg, R. H. DeKruyff, and D. T. Umetsu. 2006. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. N Engl J Med 354:1117-1129.
162. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of

NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat Med 9:582-588.

163. Meyer, E. H., S. Goya, O. Akbari, G. J. Berry, P. B. Savage, M. Kronenberg, T. Nakayama, R. H. DeKruyff, and D. T. Umetsu. 2006. Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells. Proc Natl Acad Sci U S A 103:2782-2787.

164. Cardell, S., S. Tangri, S. Chan, M. Kronenberg, C. Benoist, and D. Mathis. 1995. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. J Exp Med 182:993-1004.

165. Behar, S. M., T. A. Podrebarac, C. J. Roy, C. R. Wang, and M. B. Brenner. 1999. Diverse TCRs recognize murine CD1. J Immunol 162:161-167.

166. Jahng, A., I. Maricic, C. Aguilera, S. Cardell, R. C. Halder, and V. Kumar. 2004. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. J Exp Med 199:947-957.

167. Duarte, N., M. Stenstrom, S. Campino, M. L. Bergman, M. Lundholm, D. Holmberg, and S. L. Cardell. 2004. Prevention of diabetes in nonobese diabetic mice mediated by CD1d-restricted nonclassical NKT cells. J Immunol 173:3112-3118.

168. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. Oncol Res 7:529-534.

169. Morita, M., K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, and H. Fukushima. 1995. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. J Med Chem 38:2176-2187.

170. Motoki, K., M. Morita, E. Kobayashi, T. Uchida, K. Akimoto, H. Fukushima, and Y. Koezuka. 1995. Immunostimulatory and antitumor activities of monoglycosylceramides having various sugar moieties. Biol Pharm Bull 18:1487-1491.

171. Crowe, N. Y., M. J. Smyth, and D. I. Godfrey. 2002. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. J Exp Med 196:119-127.

172. Smyth, M. J., N. Y. Crowe, and D. I. Godfrey. 2001. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol 13:459-463.

173. Smyth, M. J., N. Y. Crowe, D. G. Pellicci, K. Kyparissoudis, J. M. Kelly, K. Takeda, H. Yagita, and D. I. Godfrey. 2002. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. Blood 99:1259-1266.
174. Crowe, N. Y., J. M. Coquet, S. P. Berzins, K. Kyparissoudis, R. Keating, D. G. Pellicci, Y. Hayakawa, D. I. Godfrey, and M. J. Smyth. 2005. Differential antitumor immunity mediated by NKT cell subsets in vivo. J Exp Med 202:1279-1288.
175. Fujii, S., K. Shimizu, M. Kronenberg, and R. M. Steinman. 2002. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. Nat Immunol 3:867-874.
176. Toura, I., T. Kawano, Y. Akutsu, T. Nakayama, T. Ochiai, and M. Taniguchi. 1999. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. J Immunol 163:2387-2391.
177. Giaccone, G., C. J. Punt, Y. Ando, R. Ruijter, N. Nishi, M. Peters, B. M. von Blomberg, R. J. Scheper, H. J. van der Vliet, A. J. van den Eertwegh, M. Roelvink, J. Beijnen, H. Zwierzina, and H. M. Pinedo. 2002. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res 8:3702-3709.
178. Crul, M., R. A. Mathot, G. Giaccone, C. A. Punt, H. Rosing, M. X. Hillebrand, Y. Ando, N. Nishi, H. Tanaka, J. M. Schellens, and J. H. Beijnen. 2002. Population pharmacokinetics of the novel anticancer agent KRN7000. Cancer Chemother Pharmacol 49:287-293.
179. Okai, M., M. Nieda, A. Tazbirkova, D. Horley, A. Kikuchi, S. Durrant, T. Takahashi, A. Boyd, R. Abraham, H. Yagita, T. Juji, and A. Nicol. 2002. Human peripheral blood Valpha24+ Vbeta11+ NKT cells expand following administration of alpha-galactosylceramide-pulsed dendritic cells. Vox Sang 83:250-253.
180. Nieda, M., M. Okai, A. Tazbirkova, H. Lin, A. Yamaura, K. Ide, R. Abraham, T. Juji, D. J. Macfarlane, and A. J. Nicol. 2004. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. Blood 103:383-389.
181. Chang, D. H., K. Osman, J. Connolly, A. Kukreja, J. Krasovsky, M. Pack, A. Hutchinson, M. Geller, N. Liu, R. Annable, J. Shay, K. Kirchhoff, N. Nishi, Y. Ando, K. Hayashi, H. Hassoun, R. M. Steinman, and M. V. Dhodapkar. 2005. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-

galactosyl-ceramide loaded mature dendritic cells in cancer patients. J Exp Med 201:1503-1517.

182. Ishikawa, A., S. Motohashi, E. Ishikawa, H. Fuchida, K. Higashino, M. Otsuji, T. Iizasa, T. Nakayama, M. Taniguchi, and T. Fujisawa. 2005. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res 11:1910-1917.

183. Motohashi, S., A. Ishikawa, E. Ishikawa, M. Otsuji, T. Iizasa, H. Hanaoka, N. Shimizu, S. Horiguchi, Y. Okamoto, S. Fujii, M. Taniguchi, T. Fujisawa, and T. Nakayama. 2006. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res 12:6079-6086.

184. Dhodapkar, K. M., B. Cirignano, F. Chamian, D. Zagzag, D. C. Miller, J. L. Finlay, and R. M. Steinman. 2004. Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion. Int J Cancer 109:893-899.

185. Park, J. M., M. Terabe, L. T. van den Broeke, D. D. Donaldson, and J. A. Berzofsky. 2005. Unmasking immunosurveillance against a syngeneic colon cancer by elimination of CD4+ NKT regulatory cells and IL-13. Int J Cancer 114:80-87.

186. Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. D. Donaldson, D. P. Carbone, W. E. Paul, and J. A. Berzofsky. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol 1:515-520.

187. Terabe, M., J. M. Park, and J. A. Berzofsky. 2004. Role of IL-13 in regulation of anti-tumor immunity and tumor growth. Cancer Immunol Immunother 53:79-85.

188. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 413:531-534.

189. Ambrosino, E., M. Terabe, R. C. Halder, J. Peng, S. Takaku, S. Miyake, T. Yamamura, V. Kumar, and J. A. Berzofsky. 2007. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. J Immunol 179:5126-5136.

190. Peggs, K. S., and J. P. Allison. 2005. Co-stimulatory pathways in lymphocyte regulation: the immunoglobulin superfamily. British Journal of Haematology 130:809-824.

191. Chen, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 4:336-347.
192. Park, S. H., K. Benlagha, D. Lee, E. Balish, and A. Bendelac. 2000. Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice. Eur J Immunol 30:620-625.
193. Parekh, V. V., M. T. Wilson, D. Olivares-Villagomez, A. K. Singh, L. Wu, C. R. Wang, S. Joyce, and L. Van Kaer. 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. J Clin Invest 115:2572-2583.
194. Williams, J. A., J. M. Lumsden, X. Yu, L. Feigenbaum, J. Zhang, S. M. Steinberg, and R. J. Hodes. 2008. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. J Immunol 181:907-917.
195. Chung, Y., R. Nurieva, E. Esashi, Y. H. Wang, D. Zhou, L. Gapin, and C. Dong. 2008. A critical role of costimulation during intrathymic development of invariant NK T cells. J Immunol 180:2276-2283.
196. Zheng, X., H. Zhang, L. Yin, C. R. Wang, Y. Liu, and P. Zheng. 2008. Modulation of NKT cell development by B7-CD28 interaction: an expanding horizon for costimulation. PLoS One 3:e2703.
197. Pal, E., T. Tabira, T. Kawano, M. Taniguchi, S. Miyake, and T. Yamamura. 2001. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of V alpha 14 NK T cells. J Immunol 166:662-668.
198. Pentcheva-Hoang, T., E. Corse, and J. P. Allison. 2009. Negative regulators of T-cell activation: potential targets for therapeutic intervention in cancer, autoimmune disease, and persistent infections. Immunol Rev 229:67-87.
199. Parekh, V. V., S. Lalani, S. Kim, R. Halder, M. Azuma, H. Yagita, V. Kumar, L. Wu, and L. V. Kaer. 2009. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. J Immunol 182:2816-2826.
200. Hayakawa, Y., K. Takeda, H. Yagita, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. J Immunol 166:6012-6018.
201. Tomura, M., W. G. Yu, H. J. Ahn, M. Yamashita, Y. F. Yang, S. Ono, T. Hamaoka, T. Kawano, M. Taniguchi, Y. Koezuka, and H. Fujiwara. 1999. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. J Immunol 163:93-101.

202. Hermans, I. F., J. D. Silk, U. Gileadi, M. Salio, B. Mathew, G. Ritter, R. Schmidt, A. L. Harris, L. Old, and V. Cerundolo. 2003. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. J Immunol 171:5140-5147.
203. Cerundolo, V., J. D. Silk, S. H. Masri, and M. Salio. 2009. Harnessing invariant NKT cells in vaccination strategies. Nat Rev Immunol 9:28-38.
204. Denkberg, G., V. S. Stronge, E. Zahavi, P. Pittoni, R. Oren, D. Shepherd, M. Salio, C. McCarthy, P. A. Illarionov, A. van der Merwe, G. S. Besra, P. Dellabona, G. Casorati, V. Cerundolo, and Y. Reiter. 2008. Phage display-derived recombinant antibodies with TCR-like specificity against alpha-galactosylceramide and its analogues in complex with human CD1d molecules. Eur J Immunol 38:829-840.
205. Yu, K. O., J. S. Im, P. A. Illarionov, R. M. Ndonye, A. R. Howell, G. S. Besra, and S. A. Porcelli. 2007. Production and characterization of monoclonal antibodies against complexes of the NKT cell ligand alpha-galactosylceramide bound to mouse CD1d. J Immunol Methods 323:11-23.
206. Yu, K. O., J. S. Im, A. Molano, Y. Dutronc, P. A. Illarionov, C. Forestier, N. Fujiwara, I. Arias, S. Miyake, T. Yamamura, Y. T. Chang, G. S. Besra, and S. A. Porcelli. 2005. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. Proc Natl Acad Sci U S A 102:3383-3388.
207. Miyake, S., and T. Yamamura. 2007. NKT cells and autoimmune diseases: unraveling the complexity. Curr Top Microbiol Immunol 314:251-267.
208. Savage, P. B., L. Teyton, and A. Bendelac. 2006. Glycolipids for natural killer T cells. Chem Soc Rev 35:771-779.
209. Chiba, A., S. Oki, K. Miyamoto, H. Hashimoto, T. Yamamura, and S. Miyake. 2004. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide. Arthritis Rheum 50:305-313.
210. Mizuno, M., M. Masumura, C. Tomi, A. Chiba, S. Oki, T. Yamamura, and S. Miyake. 2004. Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice. J Autoimmun 23:293-300.
211. Zajonc, D. M., C. Cantu, 3rd, J. Mattner, D. Zhou, P. B. Savage, A. Bendelac, I. A. Wilson, and L. Teyton. 2005. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. Nat Immunol 6:810-818.

212. Koch, M., V. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Besra, R. R. Schmidt, E. Y. Jones, and V. Cerundolo. 2005. The crystal structure of human CD1d with and without alpha-galactosylceramide. Nat Immunol 6:819-826.
213. Schmieg, J., G. Yang, R. W. Franck, and M. Tsuji. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. J Exp Med 198:1631-1641.
214. Silk, J. D., M. Salio, B. G. Reddy, D. Shepherd, U. Gileadi, J. Brown, S. H. Masri, P. Polzella, G. Ritter, G. S. Besra, E. Y. Jones, R. R. Schmidt, and V. Cerundolo. 2008. Cutting edge: nonglycosidic CD1d lipid ligands activate human and murine invariant NKT cells. J Immunol 180:6452-6456.
215. Brigl, M., L. Bry, S. C. Kent, J. E. Gumperz, and M. B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. Nat Immunol 4:1230-1237.
216. Kinjo, Y., and M. Kronenberg. 2005. V alpha14 i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes. J Clin Immunol 25:522-533.
217. Fischer, K., E. Scotet, M. Niemeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S. H. Kaufmann, and U. E. Schaible. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. Proc Natl Acad Sci U S A 101:10685-10690.
218. Courtney, A. N., P. Thapa, S. Singh, A. M. Wishahy, D. Zhou, and J. Sastry. 2011. Intranasal but not intravenous delivery of the adjuvant alpha-galactosylceramide permits repeated stimulation of natural killer T cells in the lung. Eur J Immunol 41:3312-3322.
219. Lin, K. Y., F. G. Guarnieri, K. F. Staveley-O'Carroll, H. I. Levitsky, J. T. August, D. M. Pardoll, and T. C. Wu. 1996. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 56:21-26.
220. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell 54:777-785.
221. Courtney, A. N., P. N. Nehete, B. P. Nehete, P. Thapa, D. Zhou, and K. J. Sastry. 2009. Alpha-galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens. Vaccine 27:3335-3341.

222. Hayakawa, Y., K. Takeda, H. Yagita, S. Kakuta, Y. Iwakura, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. Eur J Immunol 31:1720-1727.
223. Singh, N., S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. J Immunol 163:2373-2377.
224. Burdin, N., L. Brossay, and M. Kronenberg. 1999. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. Eur J Immunol 29:2014-2025.
225. Ko, S. Y., H. J. Ko, W. S. Chang, S. H. Park, M. N. Kweon, and C. Y. Kang. 2005. alpha-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. J Immunol 175:3309-3317.
226. Ko, S. Y., K. A. Lee, H. J. Youn, Y. J. Kim, H. J. Ko, T. H. Heo, M. N. Kweon, and C. Y. Kang. 2007. Mediastinal lymph node CD8alpha- DC initiate antigen presentation following intranasal coadministration of alpha-GalCer. Eur J Immunol 37:2127-2137.
227. Hildner, K., B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, R. D. Schreiber, T. L. Murphy, and K. M. Murphy. 2008. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science 322:1097-1100.
228. Ciabattini, A., E. Pettini, F. Fiorino, G. Prota, G. Pozzi, and D. Medaglini. 2011. Distribution of primed T cells and antigen-loaded antigen presenting cells following intranasal immunization in mice. PLoS One 6:e19346.
229. Rescigno, M. 2006. CCR6(+) dendritic cells: the gut tactical-response unit. Immunity 24:508-510.
230. Salazar-Gonzalez, R. M., J. H. Niess, D. J. Zammit, R. Ravindran, A. Srinivasan, J. R. Maxwell, T. Stoklasek, R. Yadav, I. R. Williams, X. Gu, B. A. McCormick, M. A. Pazos, A. T. Vella, L. Lefrancois, H. C. Reinecker, and S. J. McSorley. 2006. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. Immunity 24:623-632.
231. Varol, C., A. Vallon-Eberhard, E. Elinav, T. Aychek, Y. Shapira, H. Luche, H. J. Fehling, W. D. Hardt, G. Shakhar, and S. Jung. 2009. Intestinal lamina propria dendritic cell subsets have different origin and functions. Immunity 31:502-512.

232. Jiang, X., T. Shimaoka, S. Kojo, M. Harada, H. Watarai, H. Wakao, N. Ohkohchi, S. Yonehara, M. Taniguchi, and K. Seino. 2005. Cutting edge: critical role of CXCL16/CXCR6 in NKT cell trafficking in allograft tolerance. J Immunol 175:2051-2055.
233. Johnston, B., C. H. Kim, D. Soler, M. Emoto, and E. C. Butcher. 2003. Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. J Immunol 171:2960-2969.
234. Meyer, E. H., M. A. Wurbel, T. L. Staton, M. Pichavant, M. J. Kan, P. B. Savage, R. H. DeKruyff, E. C. Butcher, J. J. Campbell, and D. T. Umetsu. 2007. iNKT cells require CCR4 to localize to the airways and to induce airway hyperreactivity. J Immunol 179:4661-4671.
235. Chang, W. S., J. Y. Kim, Y. J. Kim, Y. S. Kim, J. M. Lee, M. Azuma, H. Yagita, and C. Y. Kang. 2008. Cutting edge: Programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. J Immunol 181:6707-6710.
236. Uldrich, A. P., N. Y. Crowe, K. Kyparissoudis, D. G. Pellicci, Y. Zhan, A. M. Lew, P. Bouillet, A. Strasser, M. J. Smyth, and D. I. Godfrey. 2005. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. J Immunol 175:3092-3101.
237. Ota, T., K. Takeda, H. Akiba, Y. Hayakawa, K. Ogasawara, Y. Ikarashi, S. Miyake, H. Wakasugi, T. Yamamura, M. Kronenberg, D. H. Raulet, K. Kinoshita, H. Yagita, M. J. Smyth, and K. Okumura. 2005. IFN-gamma-mediated negative feedback regulation of NKT-cell function by CD94/NKG2. Blood 106:184-192.
238. Powell, J. D. 2006. The induction and maintenance of T cell anergy. Clin Immunol 120:239-246.
239. Clavijo, P. E., and K. A. Frauwirth. 2012. Anergic CD8+ T lymphocytes have impaired NF-kappaB activation with defects in p65 phosphorylation and acetylation. J Immunol 188:1213-1221.
240. Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102:15545-15550.

241. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity 27:670-684.

242. Coquet, J. M., K. Kyparissoudis, D. G. Pellicci, G. Besra, S. P. Berzins, M. J. Smyth, and D. I. Godfrey. 2007. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J Immunol 178:2827-2834.

243. Brandt, K., S. Bulfone-Paus, D. C. Foster, and R. Ruckert. 2003. Interleukin-21 inhibits dendritic cell activation and maturation. Blood 102:4090-4098.

CHAPTER 9: VITA

Ameerah Wishahy was born in St.John's, Newfoundland, Canada and moved to Houston, Texas in August of 1997. After graduating from Cinco Ranch High School, Katy, Texas in 2004, she went on to pursue her undergraduate studies at The University of Houston. She received her Bachelor of Science in Biochemical and Biophysical Sciences as well as her Bachelor of Arts in French Studies in December 2008. In April 2009, she began working as a Research Intern and moved on to Research Assistant under the mentorship of Dr.Jagan Sastry at UT MD Anderson Cancer Center. She began her graduate immunology studies at The University of Texas Health Science Center at Houston, Graduate School of Biomedical Sciences in May 2010.