GLYCOLIPIDS AND INKT-CELLS: A PROMISING MARRIAGE IN IMMUNITY

Anton DE SPIEGELEER
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Promotor: Prof. Dr. D. Elewaut
Co-promotor: Dr. S. Aspeslagh

Dissertation presented in the 2nd Master year in the programme of

Master of Medicine

Academic Year 2011 - 2012
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Date

Names
ACKNOWLEDGEMENTS

Theoretical lessons with some clinical internships were our daily activities during the bachelor years of medicine. But intrigued by the fundamental why-questions, inspired by the person of Prof. Zonnebloem, we choose to explore the immunology world between mice and test tubes for our master thesis, in the hope to attribute a small knowledge drop for the benefit of human health. It was neither the charismatic absentmindedness nor the unique moustache of Prof. Zonnebloem that made us enthusiastic, but well his drive to discover and not to give up despite of failures on the road. We are thankful for his inspiration and life lessons.

Of course, this wonderful two years would not have been possible without the person of Prof. Dr. Dirk Elewaut. We would like to express our gratitude, not only for his material support, but especially for all his advice, contributions and encouragement. His great ideas made us sometimes overconfident, but also made us dream about immunology. We could not receive a more precious gift.

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We can impossibly name all the friends that contributed to interesting discussions and funny moments throughout these 2 years, but a special word of thanks is directed to Veerle Van Lysebettens and Anne-Sophie Rowies. They brought us back to reality when Prof. Zonnebloem was too much infiltrating our brains.

Anton & Matthias
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The aim of this thesis was to explore the chemical and biological relationships of iNKT-cell activators and their potential function as adjuvant. In the introduction, we reviewed general aspects of the immune system and some more specific facts about iNKT-cells and their activators, the α-GalCer-analogs. The functional features of iNKT-cells make them interesting targets for immunotherapy and vaccine adjuvantia. An objective in iNKT-research is to design new α-GalCer-analogs with strong T-helper 1 or T-helper 2 properties. In this thesis, we gave an overview of the chemical and biological space wherein the currently known α-GalCer-analogs are moving. With this information, we developed a structure-activity model that can be used as high-throughput instrument to screen potential glycolipids, saving time and costs. Furthermore, we explored the in vitro and in vivo biological responses of some new glycolipids and their potency as adjuvant in an experimental arthritis model. Finally, we reviewed the literature about clinically used adjuvantia and the place of α-GalCer therein.


**SAMENVATTING**

**Hoofdstuk I** is een inleiding over iNKT-cellen en hun plaats in de immunologie. INKT-cellen zijn atypische T-cellen met zowel een semi-invariante T-cel receptor als Natural Killer-cel receptoren. Twee belangrijke eigenschappen zijn dat ze na activatie heel snel grote hoeveelheden cytokines produceren en ze in staat zijn met cellen te interageren van zowel het aangeboren als het specifieke immuunsysteem. Zij worden geactiveerd door glycolipiden gepresenteerd gebonden aan CD1d in plaats van peptiden op MHC, zoals bij klassieke T-cellen. Bepaalde modificaties aan de structuur van glycolipiden kunnen leiden tot een verandering in de iNKT-respons. De iNKT-cellen spelen een belangrijke rol in de pathofysiologie van diverse aandoeningen o.a. in de oncologie en reumatologie. Mogelijks kunnen zij ook gebruikt worden voor de ontwikkeling van nieuwe vaccins. Onderzoek naar dit type cellen is daarom veelbelovend en kan leiden tot een brede waaier aan klinische toepassingen.

In **hoofdstuk II** onderzoeken we het verband tussen de chemische structuur en de biologische respons van glycolipiden. Door multivariate analyse (PCA en HCA) konden we verschillende clusters van glycolipiden identificeren op basis van hun chemische eigenschappen. Daarnaast hebben we ook biologische data uit de literatuur samengebracht en met elkaar vergeleken. Hieruit bleek onder andere dat sterke T-helper 1 of 2 responsen voornamelijk in vivo gezien worden, wat bevestigt dat polarisatie van de T-helper respons door glycolipiden afhangt van secundaire activatie van andere cellen dan iNKT-cellen, zoals NK-cellen. Uiteindelijk hebben we door het samenbrengen van de chemische en biologische data een nieuw model ontwikkeld dat voor het eerst een sterk verband kon aantonen tussen de chemische eigenschappen van glycolipiden en hun biologische respons. Dit model kan gebruikt worden voor de zoektocht naar nieuwe sterkere glycolipiden door aan de hand van de chemische eigenschappen te voorspellen welk effect dit glycolipide zal hebben op de biologische respons.

Een volgende stap is de toepassing van deze kennis in een dierenmodel. In **hoofdstuk III** bekijken we de modulerende rol die iNKT-cel activatie kan hebben in collageen-geïnduceerde artritis (CIA), een auto-immuun artritis model. Na late intraperitoneale toediening van α-GalCer, een sterke maar T-helper neutrale iNKT-agonist, zagen we een hoger maximum klinische score vergeleken met de controle. Ons labo toonde in een vorige studie een gunstig effect van α-GalCer wanneer het vroeg werd toegediend. Bovendien gaf NU-α-GalCer, een sterke IFN-γ inductor, een verslechtering van het ziekteverloop, terwijl volgens de literatuur IFN-γ een verzachtende rol zou hebben in een later ziektestadium. Verder bracht de pro-inflammatoire capaciteit van α-GalCer en zijn analogen in dit artritis model ons ertoe te onderzoeken of α-GalCer bruikbaar is als adjuvans. In plaats van componenten van *M. tuberculosis* gebruikten wij α-GalCer om een immuunreactie uit te lokken tegen
het gelijktijdig toegediend collageen. Eerder onverwachts kon α-GalCer zijn verwachtingen niet inlossen: artritis werd maar in beperkte mate geïnduceerd en bovendien konden we een mogelijk effect van het solvent (DMSO) niet uitsluiten. We weten ook niet of α-GalCer goed geëmulsificeerd kon worden in Incomplete Freund Adjuvant (IFA), wat mogelijks leidt tot een gebrekkige activatie van iNKT-cellen. Een recente studie wees erop dat er mogelijk ook nog andere T-helper responsen in de pathogenese van CIA betrokken zijn. Het kan ook zijn dat α-GalCer als T-helper neutraal glycolipide niet geschikt is en er voor een goede adjuvanswerking beter een sterk T-helper 1 of 2 glycolipide gebruikt kan worden.

In hoofdstuk IV geven we een overzicht over vaccins en het gebruik van adjuvantia. De ontdekking van vaccins is een belangrijke mijlpaal in de geschiedenis van de geneeskunde. Het toedienen van een antigen stimuleert het adaptieve immuunsysteem waardoor dit sneller herkend wordt. De bacterie of het virus waarvan het antigen afkomstig kan zo sneller geneutraliseerd en vernietigd worden door het immuunsysteem. Voor sommige infectieuze aandoeningen is de respons op een vaccin onvoldoende. Dit kan problematisch zijn voor bijvoorbeeld de oudere populatie, die gevoeliger is voor ernstige complicaties op infecties en waarbij de huidige vaccins ook minder effectief zijn. Omwille van de demografische veroudering neemt aldus de vraag naar sterkere doch veilige vaccins toe. Hierbij is de rol van adjuvantia cruciaal. Zij helpen het immuunsysteem een antigen beter te herkennen en zo een sterkere immuniteit op te bouwen. Er zijn 2 manieren waarop adjuvantia werkzaam zijn: enerzijds door een betere afgifte van het antigen en anderzijds door stimulatie van het innate immuunsysteem met een inflammatoire respons. Deze inflammatie kan de specifieke respons van het adaptieve systeem versterken. Adjuvantia die iNKT-cellen kunnen activeren zijn veel belovend in de zoektocht naar nieuwe vaccins, aangezien iNKT-cellen zowel het aangeboren als het specifieke immuunsysteem kunnen stimuleren. Door de activiteit van iNKT-cellen te moduleren met gemodificeerde glycolipiden zou het mogelijk moeten zijn een betere respons tegen het antigen te helpen ontwikkelen.

Een aantal belangrijke elementen rond iNKT-cellen zijn tot nog toe onvoldoende uitgeklaard. Globaal kunnen we drie grote doelstellingen formuleren voor toekomstig onderzoek:

- Het inzicht vergroten in de rol van iNKT-cellen in de immunopathofysiologie van aandoeningen in de oncologie, bij auto-immuunziekten en infecties.
- Beter de werking van iNKT-cel agonisten begrijpen en verklaren aan de hand van hun chemische structuur.
- Nieuwe klinische toepassingen te vinden voor iNKT-cellen door het gebruik van synthetisch ontworpen glycolipiden die het immuunsysteem op een gepaste manier activeren.
CHAPTER I

INTRODUCTION

“Hope has two beautiful daughters. Their names are anger and courage; anger at the way things are, and courage to see that they do not remain the way they are.”

Augustine of Hippo (345-430)
CHAPTER I

INTRODUCTION: INKT-CELLS AS TARGETS FOR NEW IMMUNE-INTERVENTIONS

1. INNATE AND ADAPTIVE SYSTEM

The immune system is classically divided in an innate system (e.g. macrophages, natural killer cells (NK-cells), complement) and an adaptive system (e.g. B- and T-lymphocytes). Its features are displayed in Table 1.

Table 1. Features of the innate and adaptive system.

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<tr>
<th>Attributes</th>
<th>Innate system</th>
<th>Adaptive system</th>
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<tr>
<td>Cellular Components</td>
<td>Macrophages, NK-cells, NFs, mast-cells, dendritic cells</td>
<td>T-cells (CD8+cytotoxic and CD4+helper -cells), B-cells (plasmocytes)</td>
</tr>
<tr>
<td>Humoral Components</td>
<td>Complement, CRP, MBL</td>
<td>Antibodies</td>
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<tr>
<td>Receptors</td>
<td>PRR (e.g. TLRs)</td>
<td>TCR and BCR</td>
</tr>
<tr>
<td></td>
<td>Fixed in genome</td>
<td>Rearrangements necessary</td>
</tr>
<tr>
<td>Antigen Recognition</td>
<td>Conserved molecular patterns</td>
<td>Details of molecular structures</td>
</tr>
<tr>
<td>Memory</td>
<td>No</td>
<td>Yes*</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>High</td>
<td>Low*</td>
</tr>
<tr>
<td>Specificity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Time of Action</td>
<td>Immediate</td>
<td>Delayed*</td>
</tr>
<tr>
<td>Effector Function</td>
<td>First line defense &amp; inflammation; Activation of the adaptive system by costimulation and antigen processing &amp; presentation</td>
<td>Clonal expansion or anergy; Cytotoxicity &amp; T-helper responses</td>
</tr>
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PRR (PAMP-recognition receptors), TLR (Toll-like receptors), NK-cells (natural killer cells), NFs (neutrofils), TCR (T-cell Receptor), BCR (B-cell receptor), CRP (C-reactive protein), MBL (Mannose binding Lectin). *On repeated encounter with an antigen, the adaptive system becomes more efficient (i.e. higher antigen specificity and faster immune responses on subsequent encounters with the antigen) thanks to its immunologic memory. Adapted from Janeway et al. (1).
**Innate system**

The innate system is responsible for the first recognition of pathogens. This occurs through recognition of certain preserved molecular features, the Pathogen-associated Molecular Patterns (PAMPs). Alternatively, cellular components that indicate cellular damage, the Damage-associated Molecular Patterns (DAMPs) can also activate the innate immune system. PAMPs and DAMPs consist of carbohydrates, proteins, lipids, lipopolysaccharide (LPS) and DNA- or RNA-fragments and are recognized at the cell surface or in the endosomal compartment by Pattern Recognition Receptors (PRRs). To date, several classes of PRRs, such as Toll-like receptors (TLRs), have been discovered and characterized (for review see Kumar et al. (2)). Recognition results in direct cellular responses like phagocytosis and diapedesis, or in regulation of gene transcription and translation with subsequent production of cytokines, chemokines and expression of co-stimulatory molecules (3).

The main cell types that are involved in the innate system are macrophages (MFs), dendritic cells (DCs) and natural-killer cells (NK-cells). Their action triggers inflammation (dolor, calor, rubor, tumor, functio laesa), which helps to combat the invader and to initiate tissue repair, and activates the adaptive system. The most important role of this bridging has been classically attributed to the antigen presenting cells (APCs) and more specifically to dendritic cells (DCs). APCs are characterized by the presence of MHC proteins, which present antigens to the adaptive immune system.

The receptors of the innate system are evolutionary highly conserved. If a cell is activated, the receptor will not increase its specificity, in contrast to somatic gene-rearrangements and hypermutations seen in the adaptive system. This is an important feature of the innate system: it provides a rapid first line defense because there are no time consuming adaptations of receptors. On the other hand, the response can be inefficient by the lack of specificity. This is where the adaptive system comes into action. On repeated encounter, the innate system will not act faster or with more potency because there are no innate memory cells in contrast to the adaptive system (4).

**Adaptive system**

**Activation**

The adaptive system acts slowly but very specifically. The cells involved in adaptive immunity are B-cells and T-cells. T-cells are categorized into CD8+ and CD4+ cells. CD8+ T-cells mediate cytotoxicity: they recognize and kill tumor cells or virus-infected cells. CD4+ T-cells play a central role: upon activation they differentiate into T-helper cells (Th-cells) that enhance and direct antigen-specific immune responses, e.g. antigen-specific antibody production or enhanced phagocytosis by macrophages. The T-helper cells form the central intelligence of the immune system: they direct the right defense strategy against immunologic threads (see ‘T-helper-responses’). An effective T-cell mediated immune system also needs an effective intercellular communication. This is mediated through the secretion of small proteins called cytokines.
T-cells are characterized by the presence of a T-cell receptor (TCR) (Figure 1). The TCR recognizes antigens that are presented by Major Histology Complex (MHC) molecules, which are expressed at the cell surface of both antigen presenting cells and non-immune cells such as fibroblasts. MHCs are divided into two classes: MHC-I and MHC-II. When foreign proteins (e.g. bacterial or viral proteins) are taken up by an APC, they are processed to small protein fragments, peptides. Peptides originating from cytoplasmatic or lysosomal processing bind MHC-I or MHC-II, respectively. Subsequently, MHC, loaded with the peptide, is transported to the cell-surface where it is recognized by conventional T-cell by their specific TCR: CD4+ T-cells recognize peptides presented on MHC-II and CD8+ T-cells on MHC-I. Conventional T-cells recognize only peptides as antigen (5).

Figure 1. The TCR consists of two polypeptide chains: an α-chain (TCRα) and a β-chain (TCRβ). Each chain has a variable (V) and a constant (C) domain. The diversity of TCRs is a consequence of the recombination of germline-alleles in each chain. CD3 and ζ are molecules that associates with TCR and help with transducing an activation signal to the cell. After recognition of a peptide on MHC, a signal is transduced to cytoplasmic signaling pathways. On this figure, the MHC is an MHC-I molecule, which has three α-chains in association with one β2-microglobuline (β2m). In contrast to conventional T-cells, iNKT-cells are activated by glycolipids presented on CD1d. Adapted from Hennecke J and Wiley DC (6).

Beside the antigen-specific signal from the MHC-TCR-complex (“signal 1”), co-stimulatory signaling which involves other interactions between the receptors on cell membranes are necessary to provide full activation (“signal 2”). In fact, this co-stimulus can be rather defined as a “co-stimulatory balance”, since both co-stimulatory ligand-receptor interactions like CD28 to B7.1/7.2, and co-
inhibitory ligand-receptor interactions, like cytotoxic T lymphocyte associated antigen-4 (CTLA-4) to B7.1/7.2 exists and are mostly present at the cell surface at the same time (for review see Scanduzzi (7) and van den Heuvel et al.(8)).

**Effector Function**

The T-helper cells produce different cytokines dependent on the specific threat to mediate the immune response in the adequate direction, e.g. antigen-specific antibody production or enhanced phagocytosis: this response is called a T-helper response (Th-response). It is characterized by the presence of a cluster of cytokines: the Th1- and Th2-responses are the best studied, although lately alternative T-helper responses such as the Th17-response have been discovered. Th1-cytokines are mainly interferon-γ (IFN-γ) and interleukine-12 (IL-12) and induce primarily the adaptive cellular immunity. This enhances inflammation and phagocytosis, plays an essential role in the control of infections and is useful in preventing metastatic progression of tumors. Th2-cytokines are interleukine-4 (IL-4) and interleukine-13 (IL-13) and are primarily responsible for the adaptive humoral immunity (9), which enhances antibody production by activating B-cells. A Th2 response has beneficial effects on most autoimmune diseases and plays a key role in the induction of IgE-antibody-mediated allergies (10). In addition, the T-helper response is polarized: once a specific T-helper response is activated, the system becomes self-enhanced by autocrine loops and suppresses the other responses (11).

The immune response has to be adequate: a specific immunogenic threat requires a specific Th-response. If the polarization is directed wrongly, this can be the cause of an adverse progression of disease. In that case, the organism fails to eliminate the immunogenic threat and possibly damages itself. A striking illustration of the clinical importance of these cytokine responses is the progression of infections by *Mycobacterium Leprae*. An infection can progress in two different ways: a Th1-response causes tuberculoid leprosy, which carries a good prognosis. On the other hand, the Th2-response causes lepromatous leprosy, which correlates with progressive disease (12, 13). However, the T-helper-model is a general paradigm, but in reality most immune reaction are not so strictly confined to Th1 or Th2. Therefore it would be a great advancement in immunotherapy if we could deliberately modulate these cytokine responses.

After the first or ‘naïve’ encounter to a new type of pathogen it takes about 1 to 2 weeks for the adaptive system to develop its response. Following its first response, the adaptive system develops an immunologic memory. Naïve T-cells will differentiate into a distinct type of memory-T-cells. Memory T-cells can be activated by a wider range of APCs and require less co-stimulatory signals (14). The immune response on subsequent encounters of the same pathogen becomes more rapid, more powerful
and more specific than the previous one. This explains why it is sometimes useful to re-immunize in vaccination programs.

2. INKT-CELLS

**Phenotype of iNKT-cells**

iNKT-cells are a distinct type of cells that have features of both NK-cells (*e.g.* NK1.1-expression) and T-cells (TCR-expression), and their function lies between the innate and the adaptive system (15). They develop from the T-cell lineage, but are selected by CD1d (see below), a non-classical MHC molecule. This is in contrast to conventional T-cells who are dependent on MHC molecules for their development in the thymus.

The iNKTCR consists of a restricted set of α- and β-chains: Vα14-Jα18 in mice and Vα24-Jα18 in human for the α-chain and Vβ7, Vβ8.1, Vβ8.2 and Vβ2 in mice and Vβ11 in humans for the β-chain (16). Because of the restriction of α- and β-chains, the TCRs are called ‘invariant TCRs’ and the NKT-cells ‘invariant NKT-cells’ (*iNKT*-cells). These invariant TCRs recognize glycolipids instead of peptides, presented by CD1d instead of MHC.

CD1d is a non-variable MHC-I-class molecule. The structure of CD1d resembles the structure of other MHC type I-molecules: 3 α-chains associated with β2-microglobulin. The antigen, a glycolipid, is presented in the hydrophobic groove of the α1- and α2-domains (17).

As mentioned earlier, TCRs of iNKT-cells recognize glycolipid antigens in the context of CD1d. Unlike the structure of MHC molecules, the structure of CD1d structure is highly conserved among mammals. Human iNKT-cells have even shown to be able to recognize mice CD1d and vice versa (18). This untypically conserved structure implies an evolutionary important function for CD1d, which might be due to their role in anti-microbial defense (19). Thanks to these similarities, experiments with mice are likely to give similar results in human models (18).

**Activation and Effector function**

INKT-cells can be activated in a direct or an indirect way. In the direct way, a DC presents a glycolipid on CD1d to the TCR of the iNKT-cell. This mode of iNKT-cell activation is antigen-driven.

In the indirect way, iNKT-cells are activated by cytokines (IL-12, IL-18 and type I Interferon) produced by DCs. The DCs produce these cytokines after TLR-mediated activation. Signaling from the TCR-CD1d can reinforce iNKT-cell-activation but this is not necessary. The activation is cytokine-driven (20). After activation, iNKT-cells proliferate and exhibit an effector function: their principal effector functions are direct cytotoxicity by the expression of granzyme B, perforin and Fas Ligand (FasL), cytokine production and cross-regulation with other cells (*e.g.* B-cells). Because of
their broad range of effects and the variety of cells they interact with, they are also called the swiss army knives of the immune system (Figure 2)(21).

Figure 2. iNKT-cells are also called the “swiss-army knives of the immune system”. A. They exhibit 3 principal effector functions: cytotoxicity, cytokine production and cross-regulation. B. NKT-cells are known to rapidly produce high amounts of IFN-γ and IL-4 and they can interact with a spectrum of cell types. Adapted from Matsuda et al. (21).

The most striking feature of this effector function is the high and rapid production of effector cytokines after activation by α-GalCer, the prototype of glycolipid antigens. INKT-cells produce both Th1- and Th2-cytokines in a characteristic pattern (Figure 3): IL-4 is predominantly produced early after activation with a peak after 4 hours. The response then shifts toward IFN-γ production, which peaks at 16 hours. In vivo expansion of the iNKT-cells peaks after 3 days. Interleukine-2 is triggered by TCR activation and promotes iNKT-cell proliferation (22-24). When iNKT-cells are restimulated with α-GalCer within 1 month, the iNKT-cells are less responsive and produce predominantly IL-4 (25). The explanation of this rapid cytokine production lies probably in the presence of high amounts of constitutive mRNA (IFN-γ and IL-4) in iNKT-cells. Present mRNA can be readily translated once the cell is activated (26). Activation by the direct pathway results in IFN-γ and IL-4 production, whereas the cytokine-driven pathway fails to elicit high amounts of IL-4 (25).
After activation by α-GalCer, iNKT-cells first produce IL-4. Subsequently, cytokine secretion shifts toward IFN-γ. IL-2 secretion stimulates iNKT-cell proliferation. When iNKT-cells are restimulated with α-GalCer within 1 month, the iNKT-cells are less responsive and produce predominantly IL-4. These cells are then called anergic cells (27). Adapted from Parekh et al. (25).

When cytokines are measured systemically (e.g. in serum), they originate both from iNKT-cells and other bystanders cells. If a T-helper-response is skewed to Th1 or Th2, this is mainly caused by these bystander cells, like NK-cells, because iNKT-cells themselves seem to be resistant to polarization (10, 28-30).

This is observed because iNKT-cell-activation by α-GalCer causes transactivation of a variety of other cells resulting in enhanced effector functions and cytokine production: DCs release in response to IFN-γ pro-inflammatory cytokines like IL-12 and TNF-α. IL-12 on its turn reinforces iNKT-cell activation, resulting in an autocrine loop of reciprocal activation (Figure 4). NK-cells produce large amounts of IFN-γ in response to stimulation by IL-12 and IFN-γ, originating from DCs and iNKT-cells, respectively. Also T-cells (CD4+ and CD8+) and B-cells show enhanced effector functions (4, 25). Therefore, iNKT-cells are able to reinforce the innate response (e.g. NK-cell activation) and the adaptive immune response (T-cells and B-cells).
**Figure 4.** Interactions between a DC, iNKT-cell and NK-cell. A glycolipid is presented by a DC on CD1d, resulting in iTCR-activation. IL-12 secreted by the DC also contributes to iNKT-cell activation. The iNKT-cell produces IL-4 and IFN-ɤ in response. IFN-ɤ activates the DC that produces more IL-12, resulting in reciprocal activation between the iNKT-cell and DC. CD40 and CD40L are co-stimulatory molecules. Adapted from Sandrine Aspeslagh.

**Clinic**

iNKT-cells have been shown to play an important role in the pathogenesis of several auto-immune diseases in mice as well in humans, including autoimmune hepatitis (31), spondylarthritis (32) to multiple sclerosis (33) (for review see Yu et al. (34)).

### 3. GLYCOLIPIDS

The prototype of iNKT-cell antigens is alpha-galactosylceramide (α-GalCer) or (2S,3S,4R)-1-O-(alpha-D-galactosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol. This glycolipid has been isolated from the marine sponge Agelas mauritianus, near the Okinawa Islands (Japan) and was synthesized for the first time in 1993 by Kirin Brewery Co. (35). This molecule doesn’t occur in mammals, although it
has some vast immunomodulatory effects. Kawano et al. (36) discovered the CD1d-dependent activation of iNKT-cells by α-GalCer. Thereafter Kobayashi et al. discovered the strong anti-tumoral responses after injection of α-GalCer in mice, which resulted in the development of a whole new area of research (Figure 5) (37, 38).

![Figure 5](image)

**Figure 5.** Alpha-Galactoceramide. A ceramide-structure with a C18:0- and C26:0- acyl-chain. Galactose is linked with an alfa-glycosidic bound.

**Main structure**

Basically, glycolipids consist of a ceramide connected to a sugar-moiety by an alpha-glycosidic binding. A ceramide is composed of a phytosphingosine with a N-linked acyl-chain (Figure 6). The α-anomerity of the carbohydrate is considered to be an important requisite for the CD1d-specific activation of iNKT-cells (39). However, also β-anomerically linked glycolipids are able to activate iNKT-cells (40).

**Endogenous, bacterial and synthetic glycolipids**

Several endogenous glyco- and phospholipids have been found capable to accommodate in the hydrophobic groove of CD1d (41). Some of them (*e.g.* Isoglobotrihexosylceramide (iGB3)) might play a physiological role: they are possibly involved in development of iNKT-cells in the thymus and probably have a subsidiary function in the cytokine-driven pathway of iNKT-cell activation (42, 43).

Some bacterial species are known to express glycolipids that are able to activate NKT-cells as was shown for *Borrelia burgdorferi* (44-46), *Leishmania donovani* (47, 48) and *Sphingomonas* (49). Alpha-GalCer is a non-polarizing ligand, as it induces the production of both Th1- and Th2-cytokines, also designated as a Th0-response. Synthetically modified derivatives of α-GalCer (analogs of α-GalCer), like C-glycoside 13 and OCH 99 (Table S1 Supplementary Information) are able to alter cytokine production towards a more Th1- or Th2- polarized response, respectively, compared to α-GalCer (28, 50).
**Therapies**

The central idea in research for new therapies is that structurally modified glycolipids can alter iNKT-cell-dependent T-helper responses. Currently, there are two main therapeutic areas were iNKT-cells and glycolipids are involved (51).

The first area is the use of modified glycolipids in immunotherapy. Memory cells retain a certain functional plasticity in their commitment to produce cytokines of a certain T-helper response (52) and it is possible to alter T-helper responses generated by memory cells (53). These observations open new perspectives for immunotherapy against established diseases, like chronic infections, autoimmune disease and cancer (54).

As described earlier, iNKT-cells are able to interact with several types of cells of the innate and adaptive immunity. This makes them a potential target in immunotherapy. One of the first discoveries in iNKT-cell research was the surprising effectiveness in clearing metastatic melanoma noduli in the lungs upon activation of iNKT-cells by α-GalCer in mice. Also, the use of synthetically modified glycolipids can be protective in some murine disease models: OCH exerts a Th2-skewing-effect and has been shown to ameliorate disease in experimental autoimmunity models like collagen induced arthritis (CIA) (55), experimental autoimmune encephalitis (EAE) (50) and non-obese diabetes (NOD) (56). In contrast, C-glycoside is rather seen as a Th1-skewer and exhibits a protective effect against melanoma metastasis and malaria, two diseases models where an effective immune response requires a Th1-response (28, 30, 38). The first clinical trials for iNKT-cell based immunotherapy have already been performed, e.g. in the treatment of lung cancer and head- and neck cancer (57-60).

The second area is the use of glycolipids in the development of new vaccines. The first, early interaction of naïve T-cells with APCs is crucial for the adaptive response and the development of memory cells (61). Enhancing this first response could lead to a stronger and more specific response on subsequent encounters (54). This is how adjuvants in vaccines work: they help the immune system to recognize and to react against specific antigens (see chapter IV). Although current vaccines are very potent in inducing antibody responses (B-cell dependent), they are less effective in inducing T-cell specific responses (62, 63). As iNKT-cells are able to enhance both T-cell and B-cell-responses, they are a potential target for the development of new vaccines.

The functional features of iNKT-cells make them a target for immunotherapy and for development of new vaccines. The ultimate goal in research is to design new glycolipids with both strong activating and predictable Th-biasing properties.
4. STUDY AIMS

The aim of this thesis was to explore the chemical and biological relationships of α-GalCer-analogs and their function as adjuvant. We formulated 3 main objectives:

- What are the chemical and biological spaces wherein the currently known α-GalCer-analogs are moving?
- Is it possible to predict how certain modifications will affect cytokine production?
- What is the role of α-GalCer and its analogs as vaccine adjuvant, from an experimental point of view using an animal model as well as from a future clinical use point of view?

5. THESIS OUTLINE

In chapter I we review general aspects of the immune system and some more specific facts about iNKT-cells and α-GalCer-analogs as introduction on our experiments.

In chapter II we present an overview of the chemical and biological space wherein the currently used iNKT-activators are situated and we looked at structure-activity relationships of these α-GalCer-analogs. Based on the biological data, (1) the choice of biological test-system was evaluated and (2) some assumed cytokine-relationships were confirmed. The model we developed can be used for the prediction of biological responses of newly designed α-GalCer-analogs.

In Chapter III we discuss the results of experiments that explore a possible adjuvant effect of α-GalCer in a disease model of arthritis. Also some new α-GalCer analogs were tested on their cytokine-production in vitro and in vivo. In addition, we tested their modulating role in the arthritis-model.

In chapter IV we review the literature about clinically used vaccine-adjuvants as well as the potential role and position of α-GalCer herein.

Finally, in the chapter Summary and General Conclusions we reflect about the global impact and perspectives for the future.
CHAPTER II

CLUSTERING OF INKT-ACTIVATORS: A SURPRISINGLY NEW STEP TO STRUCTURE-ACTIVITY PREDICTIONS?

“Un tas de pierres cesse d’être un tas de pierres dès qu’un seul home le contemple avec en lui l’image d’une cathédrale.”

Antoine de Saint-Exupérie (1900-1944)
This chapter is a manuscript in preparation for publication.

Clustering of iNKT-activators: a surprisingly new step to structure-activity predictions?

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CHAPTER II

CLUSTERING OF INKT-ACTIVATORS: A SURPRISINGLY NEW STEP TO STRUCTURE-ACTIVITY PREDICTIONS

Main focus in this chapter:
- To give an overview of the chemical and biological space of iNKT-activators.
- To find structure-activity relationships between iNKT-activators.
- To evaluate the choice of test-system in cytokine-measuring assays.
- To confirm assumed relationships between cytokines.

1. INTRODUCTION

iNKT-cells are a regulatory type of T cells that have been involved in many different disease settings. They express a T-cell receptor that is composed of an invariant alpha-chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) and a restricted set of beta-chains (Vβ7, Vβ8.1, Vβ8.2 and Vβ2 in mice, Vβ11 in humans). These invariant T-cell receptors recognize antigens in the context of CD1d, which is a non-classical MHC molecule expressed by antigen presenting cells (APCs). In contrast to classical MHC molecules CD1d presents glycolipids instead of peptides. Upon TCR recognition, iNKT-cells are activated which results in the production of large amounts of Th1- (IFN-γ, TNF-α) and Th2-cytokines (IL-4, IL-13) (64) both by iNKT-cells themselves and activated bystander cells. As such glycolipid induced iNKT-cell activation is able to alter the outcome of several pathologies including an experimental model of rheumatoid arthritis (CIA) (55) and different cancer models (65). These multiple actions make them promising potential targets for immunomodulatory therapies (cancer, auto-immune diseases and infections) (21, 29, 66).

Alpha-galactosylceramide (α-GalCer), the synthetic prototype of glycolipids with strong iNKT-activation, consists of a galactose connected to a lipid backbone by an alpha-glycosidic binding. The lipid backbone consists of a ceramide: an N-acyl chain coupled to a phytosphingosine-chain. Alpha-galactosylceramide evokes the production of a combined Th1/Th2-cytokine response (9).
A very promising strategy in iNKT-cell research is to alter the structure of glycolipids which results in a Th1 or Th2 polarized iNKT-cell response. This should lead to more disease-specific therapies. Nowadays, hundreds of these altered glycolipids have been synthesized and tested on their ability to provoke different cytokine-responses, in mice as well as in humans, in vitro and in vivo. Despite this numerous chemical and biological data reported, no structural overview of this information is available. An overall view on the glycolipids already tested is of interest for all involved in iNKT-research in order to specify synthesis of novel iNKT-cell antigens. In this article we give an overview of the chemical and biological space wherein the currently known iNKT-activators are situated, incorporating data from the existing literature and results from our own group. The chemical space of the iNKT-antigens is defined by their chemical properties, while the biological space is based on some major cytokine-responses. Combining this chemical and biological information we report the structure-activity relations of iNKT-activators using a system biology computational approach. This novel approach resulted in some tentative structure-activity models of iNKT-activators. These models can be used to select the best molecules (strong Th1- or Th2-response) in a high-throughput screening approach, decreasing analysis time and costs for functionality analysis. As such we hope our findings will help in the transition of iNKT-cell therapy from experimental to real human therapy.

2. MATERIALS AND METHODS

Dataset
Based on recent reviews on iNKT-activators, we searched the electronic databases of Web of Science. We conducted citation searches and screened cited references of these reviews for original articles. We only included articles containing defined chemical structures accompanied with biological data. Because the reviews commented on articles published until 2010, we inserted keywords as α-GalCer-analogs and iNKT-activators in Web of Science to include articles from 2010-2012. We did not impose inclusion restrictions in terms of language or document type. After a preliminary search, we found that the most frequently used biological markers are the cytokines IL-2, IFN-γ, IL-4 and IL-13. Moreover, as we can define five test-systems for every marker (mice/in-vivo, mice/in-vitro/cell-cell, mice/in-vitro/cell-plate, human/in-vitro/cell-cell, human/in-vitro/cell-plate), maximum 20 biological responses for every compound are thus possible. Every biological response that fitted in one of these 20 responses was included in our dataset. Therefore, we used cytokine-values written in the article; if these were absent, we measured the bar heights in the corresponding graphs. To standardize the responses from different articles and experiments, we measured the response relative to α-GalCer, which is numbered as compound 1. This ratio relative to the reference compound 1 is the raw response. If different analog doses or incubation times were used in the same experiment, results are withheld corresponding to the dose and incubation time for which α-GalCer gave maximal cytokine-
response. The reference compound 1, α-GalCer, was used in almost all studies. The few studies who did use another α-GalCer compound as reference were further linked to the α-GalCer compound 1 by the ratio available from other studies. Studies which did not include a reference compound that could be linked to the reference compound α-GalCer 1 were not included in this dataset.

In total, we analyzed 311 compounds (Table S1 Supplementary information) from 53 articles (Text S1 Supplementary information) and from unpublished data of our lab. From these compounds, 300 are included in all analyses described, while 11 compounds are only used as external validation for the structure-activity models.

**Chemical space**

In order to calculate the chemical descriptors of the 300 glycolipids, the three-dimensional structure was optimized using Hyperchem 8.0 (Hypercube, Gainesville, FL, USA). Therefore, the molecular mechanics force field method was applied, using the Polak-Ribiére conjugate gradient algorithm with a root mean square gradient of 0.1 kcal/(Å×mol) as stop criterion. Next, over 3000 descriptors were calculated, using the Dragon 5.5 (Talete, Milan, Italy) and Hyperchem 8.0.8 software programs. The 3224 Dragon descriptors can be classified into 22 groups: 48 constitutional descriptors, 119 topological descriptors, 47 walk and path counts, 33 connectivity indices, 47 information indices, 96 2D autocorrelations, 107 edge adjacency indices, 64 Burden eigenvalues, 21 topological charge indices, 44 eigenvalue-based indices, 41 Randic molecular profiles, 74 geometrical descriptors, 150 RDF (radial distribution function) descriptors, 160 3D-MoRSE (3D-Molecule Representation of Structures based on Electron diffraction) descriptors, 99 WHIM (Weighted Holistic Invariant Molecular) descriptors, 197 GETAWAY (Geometry, Topology and Atom-Weights Assembly) descriptors, 154 functional group counts, 120 atom-centred fragments, 14 charge descriptors, 29 molecular properties, 780 2D binary fingerprints and 780 2D frequency fingerprints. Using Hyperchem, the surface area (Å²), volume (Å³), logP and refractivity (Å³) of all molecules was calculated using the 3D-optimized .mol-files, and these descriptors were added to the Dragon-obtained descriptor values. After removal of the constant descriptors, which are thus not discriminative, a final dataset of 1649 descriptors was retained. As each descriptor differed in the scales in which their values lie, normalization was performed by Unit Variance (UV) scaling and mean centering (67). This resulted in a 300 x 1649 matrix of z-scaled descriptor values: $z = \frac{x - \overline{x}}{SD}$, where SD is the standard deviation and $\overline{x}$ the mean of each variable.

The multivariate data-analysis of this resulting data-matrix was performed using Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) with SIMCA-P+ 12.0 (Umetrics, Sweden) and SPSS Statistics 20.0 (Illinois, USA) software programs, respectively. The dendrogram of the HCA
analysis was obtained using average-linkage clustering between groups and the Euclidean distance as the dissimilarity criterion (68).

**Biological space**

For the analysis of frequencies, variability and correlations, SPSS Statistics 20.0 was used. In order to integrate the 20 different responses into one global response, a multi-criteria decision technique was employed. To achieve this, we used the Derringer concept of desirability (69, 70). The biological responses were transformed into a dimensionless desirability (d) scale via the following linear desirability functions:

$$d(Y) = \frac{0.9 - 0.1}{Y_{\text{max}} - Y_{\text{min}}} \times (Y_i - Y_{\text{min}}) + 0.1$$

or

$$d(Y) = \frac{0.1 - 0.9}{Y_{\text{max}} - Y_{\text{min}}} \times (Y_i - Y_{\text{min}}) + 0.9$$

for parameters to be maximized or minimized respectively. In the equations above, $Y_i$ was the experimental value for the respective response, whereas $Y_{\text{min}}$ and $Y_{\text{max}}$ were the minimum and maximum response values found, respectively. $Y_{\text{min}}$ was arbitrarily set at the reporting threshold of 0.01 for every response; $Y_{\text{max}}$ is the maximal value found for each methodology in our dataset and their values are reported in Table 2. Compounds with a high Th1 desirability should have high IFN-$\gamma$ values, combined with low IL-$4$ responses. This contrasts the Th2 desirability: high IL-$4$ responses combined with low IFN-$\gamma$ values are required. After this linear d-transformation of each of the responses, all values range from 0.1 (undesirable) to 0.9 (most desirable). These standardized d-values were combined to calculate a global D-value:

$$D = \sqrt[n]{\prod_{i=1}^{n} d_i^p}$$

The compound with the highest D-value expresses the best combination of the different desired responses. In this equation, $p_i$ is the relative importance given to the respective response. Here, we weighted the responses equally, so $p_i = 1$ for each of the 20 responses.

**Table 2.** Maximally found response values $Y_{\text{max}}$.

<table>
<thead>
<tr>
<th>Test-system</th>
<th>Marker</th>
<th>$Y_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice/in-vivo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
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</tr>
<tr>
<td>IFN-$\gamma$</td>
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<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
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<td></td>
</tr>
<tr>
<td><strong>Mice/in-vitro/cell-cell</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>----------------------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>Mice/in-vitro/cell-plate</td>
<td>2.6*</td>
<td>1.0</td>
</tr>
<tr>
<td>Human/in-vitro/cell-cell</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Human/in-vitro/cell-plate</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*The α-GalCer analogs 23 and 24 showed extreme high responses (>10), considered as quantitative outliers, reported in 1 study only (referentie 51). Therefore, both analogs were excluded from the Ymax determination. In their further processing, their Yi value was assigned the Ymax value of 2.6 and thus the maximal d-value of 0.9.

**Structure-activity relationship**

To evaluate the correlation between the chemical and biological properties of the alpha-galactosylceramide molecules, PCA and linear regression (Partial Least Squares, PLS) was performed using SIMCA-P+ 12.0 software. PLS is a method for relating two data matrices, X (variables) and Y (responses), to each other by a linear multivariate model (71). For each model, the ‘goodness of fit’ is calculated and given by the parameter $R^2$ (= the explained variation), as well as the ‘goodness of prediction’, given by $Q^2$ (= the predicted variation). The models were obtained when a balance between predictive power and reasonable fit was found (67, 71).

Using the previously obtained PCA score plots, the functionalities (Th1 and Th2 response) were also visually presented based on their calculated D-value. Therefore, the low to moderate D-values (< D-value of reference molecule 1) of the individual molecules were grouped in pairs and represented in green: the darker green, the higher the desirability for that compound. The same procedure was performed for the highly desirable alpha-galactosylceramide molecules, colored in red in the same figures.

Finally, the best models were analyzed using a dataset of 16 randomly selected molecules, predicting the D-value for each individual compound.

3. RESULTS AND DISCUSSION

**Dataset**

Table 3 gives the distribution of our data set, ordered by the different test-systems and markers used. In total, 745 data-points were available, covering 300 different α-GalCer compounds. Some α-GalCer compounds were evaluated by different research groups using multiple methods. For one compound, the normalized data obtained with a specific test-system and marker were averaged to
obtain one data-point in the total set of 745 data-points. Two test-systems are almost equally and most frequently applied to obtain our dataset, *i.e.* human/in-vitro/cell-cell (35.70%) and mice/in-vitro/cell-cell (31.68%). This is followed by the mice/in-vivo method (24.16%). The two in-vitro/cell-plate methods are only marginally used, with the human/in-vitro/cell-plate method only reported in two studies (Table S2: references 4, 53).

IFN-γ and IL-4 are the markers with the broadest application over the 3 most important test-systems, *i.e.* ranging from 8.72% to 14.63%. IL-2 data were dominantly obtained from the mice/in-vitro/cell-cell method (11.28%), while IL-13 was only used to a limited extent in the human/in-vitro/cell-cell test-system (6.98%).

**Table 3.** Distribution of methodologies used in α-GalCer functional studies.

<table>
<thead>
<tr>
<th>Test-system</th>
<th>Marker</th>
<th>No</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
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<td>IL-2</td>
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<td>0.27</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>92</td>
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</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>85</td>
<td>11.41</td>
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<tr>
<td></td>
<td>IL-13</td>
<td>1</td>
<td>0.13</td>
</tr>
<tr>
<td>Mice/in-vitro/cell-cell</td>
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<td>84</td>
<td>11.28</td>
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<tr>
<td></td>
<td>IFN-γ</td>
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<tr>
<td></td>
<td>IL-4</td>
<td>1</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>1</td>
<td>0.13</td>
</tr>
<tr>
<td>Human/in-vitro/cell-cell</td>
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<td></td>
<td>IFN-γ</td>
<td>109</td>
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<td>IFN-γ</td>
<td>2</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>1</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>1</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>745</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

**Chemical space**

The calculated PCA model contains eight principal components (PCs), with 48.7% of the variability explained by the first two principal components. The eigenvalues of the covariance matrix, the total variance explained and the predictive ability of this model are given in Table 4.

**Table 4.** PCA model description.

<table>
<thead>
<tr>
<th>Principal Component</th>
<th>Eigenvalue</th>
<th>Cumulative R²</th>
<th>Cumulative Q²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>106</td>
<td>0.355</td>
<td>0.342</td>
</tr>
<tr>
<td>2</td>
<td>39.6</td>
<td>0.487</td>
<td>0.469</td>
</tr>
<tr>
<td>3</td>
<td>17.2</td>
<td>0.544</td>
<td>0.519</td>
</tr>
</tbody>
</table>
Looking at the score plots, three outlying groups can be observed, mainly based on the sugar composition: the molecules containing four (125, 126, 127 and 88), five (100 and 55) and six (104) sugar-derived moieties are classified in different groups. As the score plot of the PCA analysis only contained large groups, the in-depth study of the different subclusters was performed using the dendrogram of the HCA analysis (Figure S1 Supplementary information). This visual representation categorizes the α-GalCer derivatives containing two or three sugar molecules (56, 58, 59, 79-84, 87, 89-93, 103, and 230) in one cluster. This reflects previous crystallographic studies showing that the extra sugar groups have to be accommodated by the iNK TCR, resulting in loss of energy and thus weaker antigens (72). Another chemically distinct group are α-GalCer analogs lacking the characteristic carbohydrate moiety. In our analysis, they are organized in different subclusters. First, the substitution of the sugar group by a cyclitol results in different chemical properties, leading to a separate clustering (e.g. 50, 51, 106, 113, 114, 115, 245, 246). Second, the threitolceramide-derived molecules (255-268) and third, the hydroxyl- (101, 112a and 112b) or phosphate- (102, 105, 107, 108 and 109) substituted sugar molecules are also separately clustered. Next, the sugar moiety can be modified by the addition of different functional groups as well. One cluster includes the 3,4-dichlorophenyl (139, 142, 133 and 136) and 4-chlorophenyl (194, 21, 130 and 132) modifications, each divided into a separate subcluster of the dendrogram. The addition of a ring structure on the sugar molecule also is a common feature: phenyl (193, 20, 195, 19, 141, 145, 138, 22, 24 and 23), naphthalene(-1-sulfonamide) (140, 18, 129, 243, 135, 241 and 242), 1,2,3-triazole, with or without a phenyl ring structure (146-151), pyridine (131) and an S-containing cyclic moiety, i.e. thieno[3,4-d]imidazolone or thiophene (244 and 143). All of these modifications are grouped separately. In addition, the presence of a fluor atom, directly on the sugar molecule (96, 97, 205 and 275) or on a phenyl ring as trifluoromethyl, in combination with a chloride-substitution (134 and 137), is characteristic for a separate clustering.

Interestingly, there is no chemical difference seen based on the type of linker between the sugar and the 6-OH alteration: carbamate, ureum or amide linkers are clustered in the same group, independent of the number of linker atoms between the sugar and the modification. So, one cluster can contain molecules with an ureum linker (19) as well as an amide linker of variable length (138, 141 and 145). Consequently, the type of modification (e.g. phenyl, 3,4-dichlorophenyl) apparently influences the

<p>| | | | |</p>
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<tr>
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<tbody>
<tr>
<td>4</td>
<td>15.6</td>
<td>0.596</td>
<td>0.566</td>
</tr>
<tr>
<td>5</td>
<td>12.6</td>
<td>0.638</td>
<td>0.602</td>
</tr>
<tr>
<td>6</td>
<td>8.86</td>
<td>0.668</td>
<td>0.626</td>
</tr>
<tr>
<td>7</td>
<td>7.73</td>
<td>0.693</td>
<td>0.642</td>
</tr>
<tr>
<td>8</td>
<td>7.46</td>
<td>0.718</td>
<td>0.664</td>
</tr>
</tbody>
</table>
clustering the most. One of the hydroxyl groups on the sugar molecule can also be replaced by a sulphate or thiol group (203, 204, 202, 98 and 211), resulting in altered chemical characteristics.

Beside galactose modifications, several groups have provided analogs with ceramide modifications as well. A first group consists of glycolipids with shortened fatty acid chains (69, 14, 180 and 57). A second group is characterized by the introduction of a four-membered (216, 219, 221 and 222) or five-membered (217, 218 and 220) ring structure between the acyl chains and sugar moiety. A third alteration possibility includes the incorporation of a fluor atom into the ceramide group: a trifluoromethyl group on a phenyl ring (36, 40, 44, 189 and 213), a difluorophenyl functional group (16) or a fluorophenyl ring (11, 190, 172, 35, 43 and 39) can be attached. Next to the cyclic fluor-containing modifications, phenyl (e.g. 25-33, 169-171, 186, 291-295), biphenyl (37, 41, 45), methoxyphenyl (34, 38, 42, 181 and 187), propylphenyl (214), methylphenyl (15), chlorobenzene (182), bromobenzene (238), tricyclocdecane (239), thiophene (184), piperidine (191), 1,2,3-triazole (249-254), pyridine (183) and naphthalene (185) rings are also present on the N-acyl group. However, the phenyl and methoxyphenyl containing molecules are not clustered separately, indicating similar chemical properties for these compounds. Moreover, a phenyl ring can be present on both the N-acyl as well as on the phytosphingosine-chain (277-279 and 281-289). These molecules are again grouped in a separate branch of the dendrogram. If the phenyl (46, 47, 163, 164, 280 and 290), phtalamide (154 and 155) or cyclopropane ring (124a, 124b, 124c and 124d) is only attached to the phytosphingosine-chain, this also results in an individual clustering. Finally, the triple bond containing alkyl chains (233 and 235) are clustered in one group, while the α-GalCer molecules with a double bond are not.

**Biological space**

Besides finding structure-activity relationships of the α-GalCer analogs, the biological data were investigated with the objective of (1) evaluating the choice made to measure a specific cytokine in its specific test-system, and (2) confirming the currently assumed relations between the different cytokines. When looking at Table 3, IL-2 is measured almost only in *in-vitro* systems, and very seldom in *in-vivo* studies. This seems logical as IL-2 is rather a high-throughput estimate about the strength of iNKT-cell activation and as such, is used in the initial *in-vitro* screening systems. More surprisingly, it is the predominant choice for *mice/cell-cell* rather than *human/cell-cell* for *in-vitro* assays, having the ultimate clinical human goal in mind. This contrasts the IL-4 and IFN-γ *in-vitro* systems, which are also quite often used as marker in the *human/in-vitro/cell-cell* system. To gain more insight in these observations, we looked at the variability or range over which the values are spread, which is inversely related to the discriminating power of a test-system. No simultaneous data were available for IL-13: while some investigations used only mice, others used only human cell-systems; only for two α-GalCer analogs, IL-13 data from both systems were available. Hence, IL-13
data were not visualized, nor related to other markers/test-system. The same holds true for the cell-plate systems. From the scatterplots for IFN-γ and IL-4 (Figure 7), it is observed that the discriminating power of the mice/in-vitro/cell-cell test-system, similar to the human/in-vitro/cell-cell test-system, is less discriminating than the mice/in-vivo test-system. This implies that when similar compounds are to be fine-tuned in a differentiation study, preference should be given to the mice/in-vivo system when possible.

![Matrix scatterplots of α-GalCer methodologies (each point is an α-GalCer analogue; X and Y axes are constant from 0 to 3.5).](image)

**Figure 7.** Matrix scatterplots of α-GalCer methodologies (each point is an α-GalCer analogue; X and Y axes are constant from 0 to 3.5).

Beside the similarity of the discriminating power, it is also observed that the mice results are closely related to the human results for the in-vitro/cell-cell test-systems. The mice/in-vivo system did not show any meaningful relation with the other systems: this test-system is thus delivering new information next to the mice/in-vitro/cell-cell and human/in-vitro/cell-cell systems which are giving very similar information. This implies that if one has to make choices, the mice/in-vitro/cell-cell test-system is becoming superfluous, and the mice/in-vivo plus the human/in-vitro/cell-cell test-systems are giving the most discriminative and orthogonal information.
Moreover, the intrinsic method variation was calculated as a relative standard deviation for every compound that had identical biological responses from more than one study. The available results are shown in Figure 8. They suggest that overall IL-4 and IFN-γ variability are the lowest in the human/in-vitro/cell-cell test-system. This means that human/in-vitro/cell-cell systems are more consistent to compare IL-4 and IFN-γ biological activity of α-GalCer compounds than mice/in-vitro or mice/in-vivo systems. In conclusion, also from a global variability perspective, the human/in-vitro/cell-cell test-system is preferred above the mice/in-vitro/cell-cell system, which is thus superfluous.

![Figure 8. Box-plots of relative standard deviations.](image)

Figure 9 confirms the well-accepted association between the two Th2-cytokines IL-4 and IL-13. While in the mice system equivalent relative values (ratio’s relative to α-GalCer) for IL-4 and IL-13 are obtained (i.e. slope of approximately 1), this is clearly not the case for the human system, where the sensitivity for IL-4 is lower than for IL-13 (i.e. slope of approximately 0.6). When we plotted IFN-γ versus IL-4 in the three test-systems under evaluation (Figure 10), it was interesting to see that strong Th1 or Th2 polarized compounds were seen with the in-vivo test-system: quite some points are in the outer quarters of the graph (Figure 10A). Much less outward points are observed with the in-vitro test-systems (Figure 10B and 10C). This can be explained by the relative lack of bystander cells in the in-vitro systems, such as NK-cells which are supposed to play an important role in cytokine-polarization (9). The reason why this effect is less pronounced with the mice/in-vitro assays is probably due to the frequent use of mice spleen-extracts, where other cells beside iNKT-cells and APCs are still present.
The position of the data-points in Figure 10B also implies that there is a Th2-overestimation with the mice/in-vitro/cell-cell test-systems compared to the other methods.

![Figure 9. Relations between markers. A) IL-4 versus IL-13 for mice/in-vitro assay on cells. B) IL-4 versus IL-13 for human/in-vitro assay on cells.](image)

With the objective of finding and predicting global structure-activity relationships between the α-GalCer analogs, the multivariate biological information was reduced to two biological responses per compound, using the concept of the Derringer desirability D (69, 73). An optimal Th1 compound was defined as a molecule exhibiting a high IFN-γ response and low IL-4 response. Because most compounds had biological data from only one test-system, we calculated an in-vivo and an in-vitro Th1 desirability or D_{Th1}. The in vitro D_{Th1} consists of the well correlated human/cell-cell and mice/cell-cell responses. After calculating the two D_{Th1} values for the compounds when the necessary data were available, distributions shown in Figure S2 and S3 in supplementary information were obtained. Inherent to this desirability-concept, all D-values lie between 0.1 and 0.9. The standard compound α-GalCer gave an in-vivo D_{Th1}-value of 0.51 and an in-vitro D_{Th1}-value of 0.48. The six most desired in-vivo compounds, i.e. with a D_{Th1}-value of more than 0.6, were 8, 128, 131, 138, 195 and 240. The eleven most desired in-vitro compounds (D_{Th1}>0.6) were 18, 25, 27, 30, 35, 38, 40, 43, 44 and 190. A similar approach was used to calculate Th2-desirabilities, D_{Th2}, with high IL-4 and low IFN-γ values for in-vivo and in-vitro.
Structure-activity relationship

The results of the PLS modeling between the chemical and biological properties are given in Table 5. From this table, it can be concluded that most models give a reasonable regression, with the Th1 mice/in-vivo (n = 85), Th2 mice/in-vivo (n = 85) models showing the best regression results ($R^2$ and $Q^2$).

Table 5. PLS model description.

<table>
<thead>
<tr>
<th>Functionality</th>
<th>Principal components</th>
<th>Cumulative $R^2$</th>
<th>Cumulative $Q^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1 mice/in-vivo</td>
<td>5</td>
<td>0.930</td>
<td>0.810</td>
</tr>
<tr>
<td>Th2 mice/in-vivo</td>
<td>5</td>
<td>0.894</td>
<td>0.722</td>
</tr>
<tr>
<td>Th1 human/in-vitro/cell-cell</td>
<td>3</td>
<td>0.746</td>
<td>0.512</td>
</tr>
</tbody>
</table>

Figure 10 (A, B, C). Th1/Th2 polarization in the different test-systems. The IFNγ/IL-4 graph is divided in 4 equal angle-parts, with extremes representing the stronger polarizers. α-GalCer is shown in red on the y=x line.
From the Th1 mice/in-vivo model, it is clear that chloride-substituted (phenyl containing) molecules show high Th1 (e.g. 130, 132-134). Moreover, the presence of C-N bonds at different topological distances seems important for the Th1 functionality. Next, the Th1 in-vitro/cell-cell model is highly influenced by the occurrence of S- and F-modifications (e.g. 40, 43): the model is described by i.a. the frequency and presence/absence of [O-S], [C-S/F] and [N-S] bonds. The Th2 mice/in-vivo model is characterized by i.a. the nConj, Fx[C-N/Cl] and nCL descriptors, which is confirmed by e.g. analogs 3 and 9 (D = 0.7).

The results are also visualized on the PCA score plots (Figure 11A, 11B, 11C and 11D): the darker green (D < D1) or red (D > D1), the higher the Th1- or Th2-desirability for that compound.

**Figure 11A.** PCA score plot for Th1 mice/in vivo.
Figure 11B. PCA score plot for Th2 mice/in vivo.

Figure 11C. PCA score plot for Th1 mice/in-vitro/cell-cell.
The models were then analyzed for their ability to predict the functionality of 16 test compounds (296-311). Therefore, PCA was performed with the molecules describing the model, as well as with the test compounds. Based on the resulting score plots, the α-GalCer derivatives were found to be representative for our dataset. Next, the D-values were predicted using the models and compared with the observed functionality responses (Table 6). From these results, it can be concluded that this functionality determination may be a good tool for high-throughput screening of α-GalCer compounds: molecules with a good Th1 in-vivo (302) or Th2 in-vitro (298) response were found using these models. So, screening a large dataset of molecules with a specific D cut-off can result in an important sample reduction, drastically saving experimental work and costs for functionality analysis. However, this laboratory work can not be fully eliminated as the response-values of the most promising compounds still have to be determined and confirmed using in-vitro and in-vivo assays. These models should be further optimized using a larger dataset of compounds to predict the functionality responses of the moderate molecules as well.

Table 6. Prediction of D-values.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Th1 in-vivo</th>
<th>Th2 in-vivo</th>
<th>Th1 in-vitro</th>
<th>Th2 in-vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dpred</td>
<td>Dobs</td>
<td>Dpred</td>
<td>Dobs</td>
</tr>
<tr>
<td>296</td>
<td>0.30</td>
<td>-</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>297</td>
<td>0.12</td>
<td>0.33</td>
<td>0.10</td>
<td>0.35</td>
</tr>
<tr>
<td>298</td>
<td>0.17</td>
<td>-</td>
<td>0.19</td>
<td>-</td>
</tr>
<tr>
<td>299</td>
<td>0.25</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
</tr>
</tbody>
</table>
4. CONCLUSION

In this study, the biological cytokine responses of iNKT-activators in different test-systems were investigated based on previously described literature data. By looking at the chemical space of the α-GalCer molecules we could distinguish several groups, which indicates the diversity of the dataset: substitutions, additions and deletions on both the sugar and ceramide moiety were observed. It was clear that the biological responses obtained with the mice and human in-vitro/cell-cell test-systems did not significantly differ between each other. Therefore, the use of the human/in-vitro/cell-cell test-system is preferred, having the subsequent clinical studies in mind and the observed higher inter-laboratory variability of human test-systems. However, when similar compounds are to be fine-tuned in a differentiation study, the mice/in-vivo technique is preferred as an additional assay, because of improved discriminating properties. Another interesting aspect discussed in the biological space-part, is the need of an in-vivo environment for strong Th1- or Th2-biasing. A major difference with in-vitro assays is the presence of bystander cells like NK-cells in an in-vivo system. This confirms recent studies where bystander cells are suggested to be responsible for polarization of the cytokine-response. Based on the chemical and biological properties, a descriptive model was obtained to predict the functionality of newly synthesized α-GalCer derived molecules. These individual models can be used to select the best molecules (high Th1- and Th2-response) in a high-throughput screening approach, decreasing analysis time and costs for functionality analysis. Therefore, clustering and modeling of glycolipids should be seen as a new structure-modifying strategy to obtain selective Th1- or Th2-active compounds.
CHAPTER III

INKT-ACTIVATORS AND THEIR ROLE AS ADJUVANTIA

“Science is a great game. It is inspiring and refreshing. The playing field is the universe itself”

Isidor Isaac Rabi (1898-1988)
Main focus in this chapter:
- To evaluate the effect of *in vitro* and *in vivo* iNKT-cell activation by glycolipid analogs on cytokine levels.
- To evaluate the effect of glycolipid administration on progression of collagen-induced arthritis.
- To evaluate the potential of α-GalCer as adjuvant in collagen-induced arthritis.

1. INTRODUCTION

Collagen-induced arthritis (CIA) is an experimental rodent model of immune-mediated arthritis (RMIA) and is the most widely studied model of RMIs (74). The joint lesions of CIA display the most resemblance with rheumatoid arthritis (RA) in men. Important differences between CIA and RA are the absence of rheumatoid factor in CIA, the little differences between males and females in CIA and the monophasic progression of CIA in contrast to the intermittent exacerbations and regressions in RA (Table 7)(75). Collagen-induced arthritis is induced in DBA/1-mice by intradermal injection of an emulsion of Complete Freund’s Adjuvant (CFA) and heterologous type II collagen (CII). After 21 days the mice get a booster injection of Incomplete Freund Adjuvant (IFA) and CII (76). IFA lacks the mycobacterial component of CFA (see protocol).

Table 7. Similarities and differences between murine CIA and human RA. RA (Rheumatoid arthritis), CIA (collagen induced arthritis), RF (rheumatoid factor). Adapted from Brand et al. (74).

<table>
<thead>
<tr>
<th>SIMILARITIES</th>
<th>MICE CIA</th>
<th>HUMAN RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cell infiltration</td>
<td>RF absent</td>
<td>Related to RF</td>
</tr>
<tr>
<td>Cartilage degradation</td>
<td>Monophasic</td>
<td>Relapsing</td>
</tr>
<tr>
<td>Genetic susceptibility related to MHC-II (HLA-DR4 (human) and I-Aq (mice))</td>
<td>No sex bias</td>
<td>Women &gt; Men</td>
</tr>
</tbody>
</table>

We briefly review some important pathophysiologic features of CIA (77). When collagen, a protein, and CFA are injected intradermally the collagen is processed by a DC and presented on a specific MHC-II molecule, I-Aq. Susceptibility to develop arthritis is related to this specific type, as is seen in
RA and HLA-DR4 (78). The DC carries the antigen to the lymph nodes, where CD4+ T-cells become activated and differentiate into Th1-cells. CFA works as an adjuvant and provides a strong co-stimulatory signal. Subsequently B-cells are activated and produce anti-CII-antibodies that are carried along the bloodstream until they recognize collagen in the joints where they trigger inflammation with activation of macrophages and neutrophils which leads to tissue destruction (for review see Luross JA and Williams NA) (77). The mycobacterial component in CFA provokes predominantly a T-helper 1 response with high production of pro-inflammatory cytokines like IFN-γ, IL-12 and TNF-α and low production of IL-4 and IL-10 (77). Following the paradigm of the T-helper responses, modulation of the immune response by promoting a Th1- or Th2-response should enhance or attenuate disease severity, respectively.

As discussed in chapter I, iNKT-cells are able to exert a regulatory function and/or therapeutic effect in several autoimmune disease models (79). When CIA is induced in iNKT-cell-deficient-mice, disease onset and severity are less compared to normal mice. Also early blockade of CD1d displays an amelioration of disease compared to the control group (80-82). Additionally, Miellot et al. show that early activation of iNKT-cells contributes to the progression of CIA (82). It appears that iNKT-cells play a significant role in the pathogenesis of CIA.

Up till now, several experiments explored the capability of iNKT-cells to modulate the onset and severity of CIA. Administration of OCH (99) (Table S1 Supplementary information), a Th2-polarizing glycolipid, inhibits the onset of arthritis by the induction of IL-4 and IL-10 (55). Single, early administration of α-GalCer, which is T-helper neutral, also gives long-term protection, an effect that is IL-10 mediated (83, 84). Injection of C-glycoside (13) (Table S1 Supplementary information), a Th1-polarizer, also provides protection but this was less compared with α-GalCer (84). In addition, time-dependent effects of IFN-γ seem to play a key role: early in disease, it contributes to the development of arthritis, while it is protective later on (66, 84, 85). These observations are somewhat confusing and show that the role of IFN-γ, IL-10 and iNKT-cell-activation in the pathogenesis of CIA is more complex than only the T-helper paradigm.

In our experiments we assessed the effect on cytokine production after \textit{in vitro} and \textit{in vivo} iNKT-cell activation by new analogs compared to α-GalCer, and we examined possible modulating effects of these new analogs in the setting of CIA. Furthermore, we explored the potency of α-GalCer as an adjuvant for inducing CIA. The adjuvant function of α-GalCer has already been explored in the experiments of Hermans et al., where co-administration of α-GalCer and chicken ovalbumin (a protein) significantly enhanced the adaptive response against ovalbumin (86). Also, in a model of autoimmune diabetes, administration of CFA provides protection against disease (87, 88). A recent study from Lee et al. shows that mycobacterial components of CFA activate iNKT-cells and that this
activation is essential for the protective role of CFA in that model, an effect that was CD1d-dependent (89). Based on these observations, we looked if it was possible to induce CIA by replacing the mycobacterial component of CFA by α-GalCer, which also activates iNKT-cells in a CD1d-dependent manner. Therefore, we co-administered CII with α-GalCer in IFA and monitored the clinical progression of CIA, compared to CFA and IFA.

2. MATERIALS AND METHODS

Glycolipid analogs

Glycolipid analogs were synthesized in the Laboratory of Medicinal Chemistry (Ghent University, Faculty of Pharmaceutical Sciences). Analogs were diluted in pure dimethylsulfoxide (DMSO) at a concentration of 1mg/ml. For APC loading, 11 µl of analog in DMSO was added to 1089µl of vehicle-solution (55µl vehicle (96mg/ml sucrose, 10mg/ml sodium deoxycholate, 0.05% Tween-20) with 1045µl phosphate buffered saline (PBS)) resulting in an end concentration of 10µg/ml. For intraperitoneal injection, 1089µl of pure PBS was used. Before use, analogs were warmed with a water bath of 80°C for 20min followed by 10min of sonication in water at 80°C.

Mice

Experiments were performed with DBA/1- and C57BL/6-mice. Experiments were conducted according to the guidelines of the Ethics Committee of Laboratory Animals Welfare of Ghent University.

Reagents

Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) (GIBCO, Life Technologies™) were used as cell culture media. To make complete medium (cDMEM or cRPMI), 1% penicillin-streptomycin, 1% glutamine and 10% Fetal Bovine Serum (FBS) were added.

Antigen presenting cells (APCs)

Two types of APCs were used: CD1d-transfected A20-cells, originating from a murine B-cell lymphoma cell line. Bone marrow dendritic cells (BMDCs) were isolated from C57BL/6-mice. The used iNKT-cells were murine 2C12 iNKT-cell-hybridomas provided by L Brossay (90).

A20-cell-preparation

Frozen A20-cells were thawed and taken from their tubes. Total amount of cells was calculated. After centrifugation (5min, 1500rpm on 22°C), cells were counted with a Bürker counting chamber on 40x magnification. Before counting, cells were stained with trypan blue to differentiate between living and dead cells. Only living cells were counted. Cell concentrations were adjusted to 1x10⁶/ml by adding
cDMEM supplemented with 1/1000 β-mercaptoethanol. One ml of cell suspension was added to each well of a 24-well-microplate. Before use, diluted glycolipids (concentration 10µg/ml) were heated in a water bath at 80°C for 20min, subsequently sonicated for 10min at 80° and cooled for 5min at room temperature. A20 cells were loaded with 0,1µg of analog in vehicle-solution by adding 10µl of the solution to each well and incubated at 37°C. After 2 hours of incubation, all loaded A20-cells were collected from the wells to be washed 3 times. Therefore, the cells were transferred to 50mL tubes, centrifuged (5min, 1500rpm on 22°C) and resuspended in cDMEM supplemented with 1/1000 β-mercaptoethanol. After the last centrifugation round, cells were resuspended in exactly 0,5ml cDMEM and 1/1000 β-mercaptoethanol. Cells were counted and their concentration was adjusted to 1x10⁶ cells/ml by adding cDMEM supplemented with 1/1000 β-mercaptoethanol (Table 8).

Table 8. A20-cell protocol.

<table>
<thead>
<tr>
<th>Murine A20-hybridoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading analogs on APCs</td>
</tr>
<tr>
<td>2 hours of incubation</td>
</tr>
<tr>
<td>Co-culture with iNKT-cell hybridomas</td>
</tr>
<tr>
<td>Take of supernatant</td>
</tr>
<tr>
<td>Cytokine detection</td>
</tr>
</tbody>
</table>

**Bone marrow dendritic cell (BMDC)-preparation.**

BMDCs were isolated from tibial and femoral bone marrow of C57BL/6-mice (min. 8 weeks old) (Figure 12). Mice were bled and killed by cervical dislocation. Skin and muscle tissue were carefully dissected from the tibial and femoral bone. Tibiae and femurs were cut with scissors at the distal ends and bone marrow cells were flushed out with sterile PBS using an insulin syringe. After filtration of the bone marrow cell suspension through a 70µm cell strainer and centrifugation (5 min, 1500rpm at 4°C), cells were counted and resuspended in cRPMI supplemented with 1/1000 β-mercaptoethanol and 1/1000 GM-CSF to an end concentration of 0.1x10⁶/ml. Cells were grown at 37°C in petri dishes, containing 2x10⁶ cells each in 20ml medium. Generally 30x10⁶ cells were harvested from one mouse.
On day 3 and day 6, medium was refreshed from the petri dishes. Ten mL was taken from each petri dish and centrifuged (5min, 1500rpm at 22°C). Hereafter the pellet was gently resuspended with warm cRPMI (supplemented with 1/1000 β-mercaptoethanol and 1/1000 GM-CSF) and added back 10ml to the petri dish. On day 8 the medium was refreshed and BMDCs were transferred to new petri dishes, because macrophages and fibroblast were still present and adherent on the plate bottom. All medium was collected and plates were washed thoroughly with PBS to wash off BMDCs (macrophages and fibroblasts are more adherent). Cells were subsequently centrifuged (5min, 1500rpm at 22°C) and resuspended in cRPMI supplemented with 1/1000 β-mercaptoethanol and 1/2000 GM-CSF. Cells were counted and suspended to a concentration of 0.5x10^6 cells/mL. Ten ml was added to each petri dish. On day 9, BMDCs were loaded with 1µg of analog in vehicle-solution by adding 100µl of the solution to each petri dish and incubated at 37°C. After 20 hours all loaded BMDCs were collected from the petri dishes to be washed 3 times. Therefore, cells were transferred to 50mL tubes and centrifuged (5min, 1500rpm on 22°C) and resuspended in cDMEM supplemented with 1/1000 β-mercaptoethanol. After the last centrifugation cells were resuspended in exactly 0.5ml cDMEM and 1/1000 β-mercaptoethanol. For intravenous injection, PBS was used. Cells were counted and their concentration was adjusted for co-culture to 1x10^6 cells/mL by adding cDMEM supplemented with 1/1000 β-mercaptoethanol or for intravenous injection to 3x10^6 cells/ml by adding PBS. For intravenous injection, BMDCs were washed 3 times with PBS and resuspended in exactly 0.5ml PBS. After resuspension, the cells were counted and their concentration was adjusted to 3x10^6 cells/ml with PBS. To inject 6x10^5 cells, 200µl was injected intravenously (Table 9).

Table 9. BMDCs protocol.

<table>
<thead>
<tr>
<th>Day</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>BMDCs isolation</td>
</tr>
<tr>
<td>3</td>
<td>Medium refreshment</td>
</tr>
<tr>
<td>6</td>
<td>Medium refreshment</td>
</tr>
<tr>
<td>8</td>
<td>Changing plates</td>
</tr>
<tr>
<td>9</td>
<td>Harvesting. Loading with glycolipids. Incubation for 20 hours. In vivo injection or co-culture</td>
</tr>
<tr>
<td>10</td>
<td>Cytokine analysis</td>
</tr>
</tbody>
</table>
**In vitro activation of iNKT-cells**

100µl of BMDC- or A20-cell-suspension was added (=1x10⁵ cells/well) in each well of a 96-well microplate together with 100µl of a 0,5x10⁵ iNKT-cell hybridoma suspension (=0,5x10⁵ cells/well). The microplate was incubated at 37°C and cytokine analysis was performed on a chosen point of time (see results).

**In vivo activation of iNKT-cells**

For in vivo activation of iNKT-cells, 5µg of analog in PBS was injected intraperitoneally in C57BL/6-mice or 6x10E5 loaded BMDCs (200µl of cell suspension) were injected intravenously.

**Cytokine detection and Analysis**

Cytokine production was assessed in the supernatant of the cell culture or in serum by ELISA. Assay plates (96-wells) (Costar®) were coated with anti-mouse capture antibodies in carbonate solution. As washing solution PBS and 0.05% Tween-20 were used. Blocking was performed with 0,1% casein. After adding standards and samples, anti-murine detection antibodies were added. For detection, avidine-horseradish-peroxidase linked to the detection antibodies catalyzed a color change of TMB Substrate Reagent (A+B). Reaction was stopped with sulfuric acid and plates were scanned with a Thermo/Labsystem Multiskan RC microplate reader. Results were analyzed with Ascent software version 2.6 (Thermo Labsystem Oy).

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism (version 5.00, GraphPad Software, Inc. Free trial version) and Excel (Microsoft Office 2010).

**CIA-Protocol**

The protocol was based on the protocol of Brand et al. (Table 10) (74).

**Table 10. CIA protocol.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Preparation of IFA</td>
</tr>
<tr>
<td>2.</td>
<td>Preparation of CFA</td>
</tr>
<tr>
<td>3.</td>
<td>Preparation of emulsion</td>
</tr>
<tr>
<td>4.</td>
<td>Immunization with CFA (day 0)</td>
</tr>
<tr>
<td>5.</td>
<td>Booster with IFA (day 21)</td>
</tr>
<tr>
<td>6.</td>
<td>Monitoring arthritis</td>
</tr>
</tbody>
</table>
Heat-killed *M. tuberculosis* H37RA (150µg/mouse) was added to Incomplete Freund Adjuvant (IFA) (mineral oil (85%) and Arlacel A (15%)) to make Complete Freund Adjuvant (CFA). Chicken collagen type II (CII) was emulsified with CFA. Mice were immunized with 200µg CII at the base of the tail by intradermal injection of 50µl emulsion with a 1 ml syringe. On day 21 mice were boosted with CII and IFA. Arthritis was monitored each day from day 21 with a clinical assessment score (Table 11) (74, 91). Each mouse was identifiable with ear cuts and tail markings.

Table 11. Clinical score. Adapted from Seeuws et al. (91).

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>0.5</td>
<td>Erythema and edema in only one digit</td>
</tr>
<tr>
<td>1</td>
<td>Erythema and mild edema of the footpad, ankle or two to five digits</td>
</tr>
<tr>
<td>2</td>
<td>Erythema and moderate edema of two joints</td>
</tr>
<tr>
<td>3</td>
<td>Erythema and severe edema of the entire paw</td>
</tr>
<tr>
<td>4</td>
<td>Reduced swelling and deformation leading to incapacitated limb</td>
</tr>
</tbody>
</table>

3. RESULTS

Cytokine analysis

The analogs that were studied for cytokine analysis were, NU-α-GalCer (18), BnNH-GSL-1’ (142) and xylo-α-GalCer (17) (Table S1 Supplementary Information). Their effect was compared to α-GalCer. NU-α-GalCer, is a 6"derivative of α-GalCer (group I) where 6-OH of galactose is replaced by a napthyl-group linked by ureum to galactose. BnNH-GSL-1’ is a group II-derivative where the 6”-OH from galacturonic acid is replaced by benzyl amide. Furthermore, xylo-α-GalCer is a 3”epimere of α-GalCer (58).

In a screening of 19 structurally related analogs, NU-α-GalCer and BnNH-GSL-1’ triggered higher levels of IL-2 compared with α-GalCer, while xylo-α-GalCer induced lower IL-2 levels. As IL-2 secretion is triggered by TCR-activation (22-24)(Figure 13), NU-α-GalCer and BnNH-GSL-1’ seem to be strong iNKT-cell activators *in vitro*. 
Subsequently we performed an *in vivo* experiment to assess the effects of intraperitoneally injected glycolipids on cytokine levels. When iNKT-cells are activated by α-GalCer, levels of IL-4 and IFN-γ normally peak after 4 and 16 hours respectively (see chapter I). Levels were assessed at the moment of their presumed maximum level (25, 92). NU-α-GalCer and BnNH-GSL-1’ produced less IL-4 compared to α-GalCer. Production of IFN-γ by NU-α-GalCer was similar to α-GalCer, while BnNH-GSL-1’ produced significantly less (Figure 14). Interleukine-12 production by NU-α-GalCer was elevated compared with α-GalCer. From these *in vitro* and *in vivo* assays experiments we learn that NU-α-GalCer and BnNH-GSL-1’ are both potentially good iNKT-cell activators. Also, NU-α-GalCer displayed relatively high IFN-γ levels, suggesting a Th1-biasing response. High IL-12 levels elicited by NU-α-GalCer could indicate a strong adjuvant function for these analogs as IL-12, mainly produced by DCs, is important for iNKT-cell activation in the cytokine driven way (see chapter I), with subsequently enhanced bridging of the innate and adaptive system.
Thereupon, we measured serum levels of IL-12 and IFN-γ after intravenous administration of 6x10^5 glycolipid loaded BMDCs. Fujii et al. observed that when α-GalCer is administered to mice on loaded DCs, the response is more prolonged and marked by higher levels of IFN-γ compared with administration of free α-GalCer (93). Therefore, measurements were done after an interval of 4, 16 and 24 hours respectively to assess if there is a delay in cytokine response (Figure 15). After 4 hours, no difference was seen. After 16 hours, levels of IFN-γ induced by NU-α-GalCer were significantly higher compared with α-GalCer and this was even more pronounced after 24 hours. Assessment of IL-12 levels suggested that NU-α-GalCer induces higher levels of IL-12 compared to α-GalCer after 16 hours (Figure S4 Supplementary Information), parallel to our previous in vivo experiments.

We conclude that NU-α-GalCer provides a stronger in vivo induction of IFN-γ compared with α-GalCer after 16 and 24 hours when loaded on BMDCs. This difference is not seen after intraperitoneal injection, as α-GalCer tended to produce similar responses for IFN-γ compared with NU-α-GalCer.
For IL-12, NU-α-GalCer confirmed its ability to elicit high IL-12 production on injection of loaded-BMDCs compared with α-GalCer.

**Collagen-induced Arthritis**

In the first experiment, we applied the results of our *in vitro* and *in vivo* cytokine assays. In line with our own observations, we looked at the effect of intraperitoneal injection of NU-α-GalCer and BnNH-GSL-1’ compared with α-GalCer and two control conditions, DMSO and PBS (no CFA was injected and mice were only anesthetized (Table 12 and Table 14)). As analogs are administered late after the first encounter to CII (intraperitoneally on day 19), we assess their potency to modulate the adaptive system when it has already developed some specificity against CII.

**Table 12.** Experiment 1. Forty DBA/1-mice (8-12 weeks old) were equally distributed over 8 cages. On day 0 mice were immunized with CFA and CII. On day 19, analogs were injected intraperitoneally (5µg). On day 20 mice were boosted with IFA and CII.

<table>
<thead>
<tr>
<th>Day</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2</td>
<td>Weighting and caging</td>
</tr>
<tr>
<td>0</td>
<td>Immunization with CFA</td>
</tr>
<tr>
<td>19</td>
<td>Treatment with one of the five conditions</td>
</tr>
<tr>
<td>20</td>
<td>Boosting with IFA</td>
</tr>
</tbody>
</table>

In DMSO, our positive control, disease incidence was 63%, which is lower than the expected incidence of 80 to 100% (Table S2 Supplementary Information) (94). Also, the mean day of onset was 31 days, while the first signs of arthritis are generally seen between day 18 and 25 post-immunization (74). Figure 16 shows clinical scores of the mice that were affected with arthritis. We see higher clinical scores on administration of α-GalCer and NU-α-GalCer compared to BnNH-GSL-1’ and DMSO, our positive control condition. Disease severity for BnNH-GSL-1’ seems comparable to DMSO. However, as our positive control, DMSO, did not differ significantly from our negative control, PBS, it is difficult to make conclusions without speculating. Only NU-α-GalCer and α-GalCer were able to elicit a significant difference in clinical scores compared to PBS, and that was only late after disease onset (*e.g.* after 40 days).
Experiment 1

In the second and third CIA-experiment we focused on a possible adjuvant effect of α-GalCer. Therefore, we co-administered α-GalCer and CII. Co-administration is essential to elicit adjuvant effects, as Hermans et al. showed that simultaneous uptake of α-GalCer and ovalbumin by the same DC were essential to enhance the T-cell response (86). Therefore, it is difficult to compare results from the second and third experiment with the first, as timing (day 0 vs. day 19) and way of α-GalCer administration (intradermally vs. intraperitoneally) are different. The objective of this experiment was also different: we wanted to assess if it was possible to replace heat-killed *M. tuberculosis* in CFA as adjuvant with α-GalCer. The results of CFA and α-GalCer were compared to IFA, as IFA lacks both heat-killed *M. tuberculosis* and α-GalCer. Alpha-GalCer is dissolved in DMSO and it is not known how well it can be emulsified in IFA. To control for a possible effect from DMSO we also administered different volumes of DMSO. The third experiment is nearly similar to our second experiment, but now C57BL/6-mice are used (Table 13 and Table 14). As most of the cytokine analyses are performed with C57BL/6-mice, it’s more reliable to relate results from cytokine assays with effects in CIA in this mice-strain. However, C57BL/6 tend to be less susceptible to CIA (74).
Table 13. Experiment 2 and 3. One hundred ten DBA/1-mice (8-12 weeks old) were equally distributed over 11 cages. Eleven conditions were tested: IFA with α-GalCer (5µg, 0.5µg and 0.05µg) in DMSO, IFA with DMSO (5µl, 0.5µl and 0.05µl) alone, CFA with DMSO (5µl, 0.5µl and 0.05µl) alone, CFA alone and IFA alone. For experiment 3, C57BL/6 mice were used.

<table>
<thead>
<tr>
<th>Day</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2</td>
<td>Weighting and caging</td>
</tr>
<tr>
<td>0</td>
<td>Immunization with one of the conditions</td>
</tr>
<tr>
<td>21</td>
<td>Boosting with IFA</td>
</tr>
</tbody>
</table>

Table 14. Overview of experiment 1, 2 and 3. CFA=Complete Freund's Adjuvant; IFA=Incomplete Freund's Adjuvant; DMSO=dimethylsulfoxide. *Timing of administration differs.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>40</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>Mice Strains</td>
<td>DBA/1</td>
<td>DBA/1</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>Conditions*</td>
<td>α-GalCer</td>
<td>IFA + α-GalCer (5µg, 0.5µg and 0.05µg) in DMSO</td>
<td>IFA + α-GalCer (5µg, 0.5µg and 0.05µg) in DMSO</td>
</tr>
<tr>
<td></td>
<td>BnNH-GSL-1’</td>
<td>IFA + DMSO (5µl, 0.5µl and 0.05µl)</td>
<td>IFA + DMSO (5µl, 0.5µl and 0.05µl)</td>
</tr>
<tr>
<td></td>
<td>NU-α-GalCer</td>
<td>CFA + DMSO (5µl, 0.5µl and 0.05µl)</td>
<td>CFA + DMSO (5µl, 0.5µl and 0.05µl)</td>
</tr>
<tr>
<td>Positive control</td>
<td>DMSO</td>
<td>CFA</td>
<td>CFA</td>
</tr>
<tr>
<td>Negative control</td>
<td>PBS (no CFA)</td>
<td>IFA</td>
<td>IFA</td>
</tr>
</tbody>
</table>

Figures S5 and S6 (Supplementary Information) show the clinical scores of the mice that were affected with arthritis. The time axis indicates the number of days after immunization. Figure 17 and 18 show mean arthritis scores on the day of maximum scores. The frequency of arthritis incidence for CFA, our positive control, in experiment 2 was similar to experiment 3 (50%) (Table S3 and S4 Supplementary Information). However, the overall incidence in experiment 2 (51%) was significantly higher than in experiment 3 (19%) (Fisher’s exact test). This difference could be explained by the higher sensitivity to arthritis-induction in DBA/1 mice (74).
The arthritis-score for the conditions of IFA+α-GalCer was significantly (p<0.001) less compared with CFA, the positive control for both experiments. From the conditions that lacked the mycobacterial component, only IFA+0.05μg α-GalCer and IFA+5μl DMSO differed significantly from IFA in experiment 2. In experiment 3, these conditions showed no difference with IFA. Therefore it seems that the addition of α-GalCer to IFA had no consistent CIA-inducing effect.
To rule out a possible effect of DMSO, we included conditions of DMSO without α-GalCer. When we compared CFA+DMSO with CFA in experiment 2, this suggested a dose-related effect, although only CFA + 5µl DMSO differed significantly from CFA. In experiment 3, no dose related effect was seen, and CIA induction was rather less than CFA. In addition, in experiment 2, IFA + DMSO also displayed elevated clinical scores and higher frequencies of incidence compared with IFA. This was not seen in experiment 3. From our data we conclude that α-GalCer could not reveal a significant adjuvant effect on inducing CIA both in DBA/1 as in C57Bl/6 mice. We do not exclude DMSO to have an adjuvant effect as some significant effects were seen in the DBA/1 mice.

4. DISCUSSION AND CONCLUSION

In our first CIA-experiment we looked at the modifying effect on disease progression after administration of NU-α-GalCer, α-GalCer and BnNH-GSL-1’. In the experiments of Coppieters et al., early intraperitoneal administration (i.e. day 5 after immunization) of α-GalCer provided protection against CIA and later administration (i.e. day 20) showed no difference (84). In our observations however, administration on day 19 of α-GalCer and NU-α-GalCer seemed to have a disease promoting effect in CIA compared to DMSO. The effect of BnNH-GSL-1’ was similar to DMSO. As IFN-γ tends to have a protective role later in pathogenesis (66, 84, 85), we would rather expect α-GalCer and NU-α-GalCer to attenuate than to exacerbate disease progression, as they induce more IFN-γ production compared to BnNH-GSL-1’. However, it is possible that kinetics of IFN-γ and other cytokines after α-GalCer administration, in synovium differ from serum, but this is not well understood up till now. It could be useful to measure synovial cytokine levels after intraperitoneal administration of analogs in the setting of CIA, however this is technically very challenging. An exacerbating effect by NU-α-GalCer and α-GalCer might also be mediated by other cytokines than IFN-γ. As we did not include a condition with CFA alone, we could not exclude effects from DMSO in this experiment.

Data from the second and third CIA-experiment suggest that α-GalCer in DMSO was not capable to replace heat-killed *M. tuberculosis* as adjuvant in a CIA-model. Our experiments were based on the assumption that α-GalCer could serve as adjuvant by activating iNKT-cells and thereby enhancing the adaptive immune response against CII. However, α-GalCer did not display higher rates of CIA-incidence in our experiments. The therapeutic effect of α-GalCer in experimental tumor models is presumed to be dependent on NK-cell-mediated tumor lysis that is triggered by IFN-γ from activated iNKT-cells (95-97). For CIA however, it is still unclear if and how NK-cells are involved in pathogenesis. Recent studies claim that CIA is triggered by other T-helper responses (Th17 and follicular Th) than T-helper 1 and that mobilization of NK-cells inhibits development of collagen-induced arthritis by lysis of Th17 and follicular T-cells in lymph nodes (98, 99). It is possible that the
development of CIA by α-GalCer is more impeded by the effects of NK-cell lysis compared to CFA, given that the function of CFA as adjuvant also depends on other pathways, like classical DC and T-cell interactions (100). Another possible explanation is that α-GalCer failed to activate iNKT-cells strong enough, due to intrinsic weakness of α-GalCer or poor emulsification in IFA. It could be useful to assess in vivo iNKT-cell activation and to test more potent and more Th-biasing glycolipids. An underlying mechanism for the suggested adjuvant role of DMSO could be activation of iNKT- and NK-cells as described for hepatic iNKT- and NK-cells (101). However, also anti-inflammatory effects from DMSO have been described (101-103).

We suggest some other approaches on testing the adjuvant function of α-GalCer in CIA. Our experiments were mainly focused on co-administration of free α-GalCer with CII. It could be useful to administer glycolipid-loaded BMCDs, as our observations showed that intravenous injection of NU-α-GalCer-loaded BMDCs induced more potent Th1-skewing compared to α-GalCer. An earlier study showed a significant reduction of lung metastases on administration of α-GalCer-loaded DCs compared to treatment with free α-GalCer alone (104). In addition, administration of NU-α-GalCer-loaded BMDCs was more effective in tumor suppression compared to α-GalCer-loaded BMDCs (58). Also, administration of α-GalCer-loaded BMDCs combined with selective blockade of NK-dependent lysis of Th17 and follicular Th-cells (e.g. with anti-NKG2A antibodies (99)) could possibly reveal a higher adjuvant potency of α-GalCer.

Beside the signal from the TCR-CD1d-complex (signal 1), also co-stimulatory and co-inhibitory molecules are involved in iNKT-cell activation (signal 2). As the enhanced CD4+ and CD8+ T-cell activation against ovalbumin triggered by α-GalCer was dependent on CD40 (86, 105), a co-stimulatory molecule, we suggest the opportunity to perform experiments that combine the effects of antigen-dependent iNKT-cell-activation with additional reinforcement of co-stimulatory signals or inhibition of co-inhibitory signals (e.g. with anti-CTLA-4 antibodies) in CIA (7).
“Shall I refuse my dinner because I do not fully understand the process of digestion?”

Oliver Heaviside (1850-1925)
This chapter is submitted for publication.

Adjuvantia of de queeste naar het ultieme vaccin
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Main focus in this chapter:
- To discuss the mode of action of the currently used vaccine-adjuvantia.
- To give an overview of effectiveness and safety of the currently used adjuvant-based vaccines.
- To present the iNKT-activators as future new vaccine-adjuvantia.

1. INLEIDING

Geadjuvanteerde vaccins: effectief en veilig

Van een ideaal vaccin wordt verwacht dat het veilig, doeltreffend en goedkoop is. Daarbij is vooral veiligheid de laatste jaren een meer prominente rol gaan spelen. Terwijl de eerste vaccins die werden ontwikkeld in de 18e en 19e eeuw bestonden uit levend verzwakte of geïnactiveerde pathogenen, maakt men tegenwoordig vooral gebruik van zeer zuivere fragmenten (subunits) van pathogenen, zoals bijvoorbeeld manteleiwitten. Deze werkwijze verlaagt de kans op bijwerkingen, doch gaat ten koste van de effectiviteit. Immers bij subunit-vaccinatie is zowel de cellulaire als de humorale immuunrespons vaak onvoldoende, in het bijzonder bij immuungecompromiteerde personen, kinderen en ouderen. Dit is begrijpelijk vermits het toedienen van een volledig organisme zoals bij een vaccin op basis van geïnactiveerde pathogenen, meer op een reële infectie lijkt dan een subunit-vaccin. Daarom probeert men nu om de immuunrespons tegen subunitvaccins te versterken met het gebruik van adjuvantia. Adjuvantia zijn ‘helpers van het immuunsysteem’ (adjuvare: Lat. helpen) en brengen een kwalitatief en/of kwantitatief sterkere immuunrespons tegen de bijgevoegde antigenen op gang, zonder de bedoeling zelf immunogeen te zijn. Het gebruik van adjuvantia heeft een nieuwe generatie vaccins ingeleid, waarvan het einde nog lang niet in zicht is.

Het immuunsysteem: hoofdrolspeler bij adjuvantia

Aangeboren immuun systeem als trigger...

Een specifieke immuunrespons omvat zowel inductie van T-cellen als B-cellen. Echter voor activatie van deze cellen is de aanwezigheid van een inflammatoire milieu noodzakelijk. Inflammatoire stimuli zoals cytokines worden gemaakt na herkenning van gevaarsignalen door de cellen van het aangeboren
immuun systeem. Gevaarsignalen omvatten evolutief bewaarde pathogeenpatronen, de zogenaamde PAMPs (pathogen associated molecular patterns), en endogene cellulaire componenten die wijzen op celschade, de DAMPs (damage-associated molecular patterns). PAMPs en DAMPs worden herkend door patroonherkenende receptoren (PRR, pattern recognition receptors) op antigen-presenterende cellen (APCs). Deze herkenning leidt tot activatie van het aangeboren immuunsysteem. De PRRs omvatten verschillende receptorfamilies waarvan de Toll-like-receptoren, gekoppeld aan het MyD88-eiwit, en de NOD-like receptoren de meest gekende zijn. De ontdekking van deze receptoren en antigen-presenterende cellen is van zo’n fundamenteel belang gebleken dat in 2011 hiervoor de Nobelprijs voor fysiologie en geneeskunde werd uitgereikt aan Beutler, Hoffmann en Steinman.

Dit gehele inflammatoire proces leidt tot maturatie van APCs zoals dendritische cellen, waardoor ze costimulatoire eiwitten tot expressie brengen en migreren naar secundair lymfoid weefsel, waar de T- en B-cellen residueren.

...van het specifiek immuunsysteem (figuur 19)

Naiëve T-cell worden geactiveerd door de combinatie van MHC-gebonden antigen (signaal 1) en costimulatie (signaal 2), beide aanwezig op de mature APC (figuur 19A). Dit in contrast met de geactiveerde T-cel die genoeg heeft aan signaal 1 om haar effector-functie uit te oefenen (figuur 19B).

De klassieke T-cellen bestaan uit twee grote celtypen: de CD4+ T-cell en de CD8+ T-cell. CD4+ T-cellen worden geactiveerd door antigen opgenomen in de APC via endocytose. Dit antigen vormt een complex met MHCII op de APC. Eenmaal geactiveerd kan de CD4+ T-cel differentiële functies krijgen. Zo onderscheiden we de Thelper1- en Thelper2-cellen, die respectievelijk Th1- (IFN-γ, TNF-α) en Th2- (IL-4, IL-13) cytokines secreteren. Th1-cellen stimuleren vooral de fagocytose door macrofagen terwijl Th2-cellen noodzakelijk zijn voor een sterke antilichaamrespons door B-cel-activatie (figuur 19B).
CD8+ T-cellen herkennen antigenen die in het cytoplasma voorkomen en die vooral aanwezig zijn bij viraal-geïnfecteerde cellen, via hun presentatie op MHC I. Activatie van de CD8+ T-cel leidt tot een cytotoxische respons, waardoor viraal-geïnfecteerde cellen worden gedood via een proces dat apoptose wordt genoemd (106, 107).

### 2. WERKINGSMECHANISMEN VAN DE HUIDIGE ADJUVANTIA


#### Tabel 15. Huidige adjuvantia en gebruik.

<table>
<thead>
<tr>
<th>Adjuvans</th>
<th>Gebruik (klinisch voorbeeld)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium</td>
<td>HAV (Havrix®), HBV (HBvaxpro®), HPV (Gardasil®), Difterie-Tetanus-Kinkhoest (Boostrix®),</td>
</tr>
</tbody>
</table>
In de EU zijn er momenteel vijf verschillende adjuvantia verwerkt in vaccins die inmiddels op de markt zijn (tabel 15). Aluminiumzouten waren de eerste adjuvantia en vele decennia lang ook de enige die in klinische preparaten werden gebruikt. In het voorbije decennium werden een aantal nieuwe adjuvantia goedgekeurd door de gezondheidsinstanties in Europa. Kennis van hun werkingsmechanismen zal leiden tot meer inzicht in hun klinische effectiviteit en bijwerkingen. Figuur 20 geeft een overzicht van de belangrijkste werkingsmechanismen.
Figuur 20. Werkingsmechanismen van verschillende adjuvantia. Aluminium kan op vier verschillende mechanismen de APC activeren: (pad 1) directe opname met vervolgens lysosomale schade en vrijstelling van lysosomale DAMPs; deze activeren de PRR NLRP3, die een multi-proteïne complex of inflammasoom vormt en procytokines in cytokines omzet// (pad 2+3) onrechtstreeks door lyse van omgevende cellen en vrijstelling van urinezuur (pad 2) en DNA (pad 3); urinezuur activeert dezelfde cascade als pad 1. DNA werkt via nog onbekende PRRs// (pad 4) binding aan cholesterol (rood) en sfingomyeline (zwart) veroorzaakt proteïneclustering (blauw) in de celmembraan; via de intracellulaire proteïnestaarten wordt het signaal vertaald binnen in de cel. MF59 activeert een MyD88-afhankelijke signaalcascade. MPL, aanwezig in AS04, maakt gebruik van TLR4 en MyD88 om IL-6-secretie te stimuleren. De virosomen bootsen een virale infectie na door hun antigenen cytoplasmatisch af te geven. De werking van AS03 is nog grotendeels onbekend.
Aluminium: het oudste ‘little secret’

Het oudste en meest beschreven adjuvans, aluminium, bestaat uit onoplosbare aluminiumzouten (vooral Al(OH)₃ en AlPO₄). Deze versterken de Th2 humorale respons (110, 111). Ondanks het veelvuldig klinisch gebruik heerst nog veel controverse over het werkingsmechanisme. Historisch werd de werking van dit adjuvant volledig toegeschreven aan verhoogde presentatie van antigen aan immuuncellen (112). De theoretische verklaring hiervoor bestaat uit enerzijds haar depotfunctie en anderzijds haar verhoogde fagocytose door de APCs. Door het gebruik van weinig oplosbare zouten en de antigen-adsorptie eraan, wordt een depotfunctie met langdurige vrijstelling van antigen verondersteld. De verhoogde fagocytose door APCs kan men verklaren door de partikelgrootte en antigendensiteit die dichter bij de reëele infectie staan. Bij de mechanismen resulteren in een verhoogde antigenpresentatie door de APC aan de T-cellen (signaal 1) met een bias voor Th2-differentiatie. Recente onderzoeken echter wijzen op een directe aangeboren immuunstimulatie als belangrijkste adjuvans-werking van aluminiumzouten. Vier verschillende mechanismen werden voorgesteld (figuur 20).


Naast de aluminiumkristallen als rechtstreekse trigger, is er geopperd dat aluminium ook een onrechtstreekse trigger kan zijn van de inflammasoom-signaalcascade (pad 2, figuur 20) (114). Immers, als membraanbeschadiger brengt ze schade toe aan omgevende cellen en zorgt ze voor vrijstelling van cellulaire componenten, waaronder urinezuur. Het urinezuur wordt opgenomen in endosomen en activeert dezelfde DAMPs-inflammasoom-signaalcascade. Aluminium kan dus zowel rechtstreeks als onrechtstreeks via urinezuur, de inflammasoom-signaalcascade en daaruitvolgende cytokine-storm op gang brengen.

Een nieuw onderzoekspoor wijst op aluminium-geïnduceerde celdood en DNA-loslating als stimulator van het aangeboren immuunsysteem (pad 3, figuur 20) (115). Het vrijgestelde DNA is dan opnieuw een DAMP dat herkend kan worden door een PRR.

Tot slot is heel recent een totaal nieuwe signaalcascade ontdekt die een rol kan spelen in de aluminium-geïnduceerde immuunrespons (pad 4, figuur 20) (116). Binding van de aluminiumkristallen aan APC-membraanlipiden zoals cholesterol en sfingomyeline veroorzaakt een clustering van proteïne-receptors. Deze clustering activeert een intracellulair cascadesysteem met celactivatie als gevolg. Opvallend is dat het aluminium zelf niet wordt opgenomen door de APC, hetgeen implicaties kan hebben naar mogelijke bijwerkingen door extracellulaire aluminiumopslag.
MF59 en AS03

Een ander adjuvant dat reeds in de praktijk wordt gebruikt is MF59 (Novartis). MF59 is een olie-in-water emulsie waarvan squaleen de belangrijkste lipidecomponent is. Ook hier werd het adjuvant lang aangezien als louter antigen-presentatie verhogend. Door de antigen-bevattende micellaire partikels bootst men beter de reële infectie na en wordt er meer antigen gefagocyteerd en gepresenteerd op de MHCII moleculen (signaal 1). Echter, tegelijk met de ontdekking van de aangeboren immuunstimulus van aluminium, heeft men zo’n stimulus ook voor MF59 gevonden. Via een MyD88-afhankelijke signaalcascade recruteert en activeert de emulsie APCs (117).

Een adjuvant systeem dat grote gelijkenis vertoont met MF59 is het AS03 (GSK). Dit is net zoals MF59 een olie-in-water emulsie, waarbij in de lipide fase naast squaleen ook α-tocopherol (vitamine E) wordt gebruikt. Het α-tocopherol zorgt voor verhoogde inflammatoire cytokine-productie en antigen presentatie (118). De precieze wijze waarop α-tocopherol dit doet, is niet gekend.

Virosomen

De virosoomtechniek probeert nog beter de reële infectie na te bootsen (119, 120). Het basisprincipe is eenvoudig: detergenten toegevoegd aan een viruspopulatie zorgen voor membraandisruptie waardoor het virale genetisch materiaal vrijkomt. Dit wordt verwijderd uit het mengsel en antigen wordt eventueel toegevoegd. Na verwijdering van het detergent sluiten de membranen zich opnieuw. Het resultaat is een virusomhulsel met een lege of selectieve antigen-gevulde inhoud (figuur 20). Door deze techniek blijven vele virale eigenschappen behouden. Zo o.a. de membraanfusie van de partikels, waardoor de antigenen cytoplasmatisch terechtkomen en een virale CD8+ cellulaire respons uitlokken (121).

AS04

Recent werd het adjuvant systeem AS04 door de gezondheidsorganisaties goedgekeurd voor het gebruik in een HBV vaccin voor patiënten met nierinsufficiëntie (Fendrix®, GSK) en HPV vaccin (Cervarix®, GSK). MPL en aluminiumhydroxide zijn de twee actieve bestanddelen van AS04. MPL of monophosphoryl lipid A is een chemisch gewijzigde vorm van een lipopolysaccharide (LPS) afkomstig van een Salmonella minnesota. MPL activeert de Toll-like-receptor 4 net zoals LPS dit doet. Hierdoor worden cytokines waaronder IL-6 extracellulair vrijgesteld. Deze cytokines, in combinatie met aluminium-geïnduceerde reacties kunnen naast een sterke humorale respons ook bijdragen tot een sterke CD8+ cellulaire respons (122-124).
α-GalCer-derivaten als volgende generatie adjuvantia?

Hoe zullen de adjuvantia van de toekomst er uit zien? Om nog meer winst te boeken op effectiviteit en veiligheid wenst men het adjuvant nog specifieker te laten aangrijpen naargelang het vaccin. Ook de kosten blijven een belangrijk aandachtspunt met het oog op wereldwijde vaccinaties. Een veelbelovende groep zijn de α-GalCer-derivaten, koppelingen van een lipide en een sacharide.

**Alpha-GalCer-derivaten: NKT-activators**

De α-GalCer-analogen zijn activatoren van NKT-cellen. NKT-cellen zijn een specifieke set van regulatoire T cellen, waarvan de T-cell receptor glycolipiden herkent in plaats van peptiden. Deze glycolipiden worden gepresenteerd door de APC op CD1d, een MHC-I-achtige molecule. Bij activatie kunnen NKT-cellen, in tegenstelling tot naïeve klassieke T-cellen, binnen enkele uren hun effectorfuncties uitoefenen via cytokine-secretie en directe cel-cel-interacties. De gesecreteerde cytokines omvatten zowel Th1- (IFN-γ) als Th2- (IL-4) moleculen. Via deze cytokines en cel-cel-interacties worden omgevende immuuncellen zoals APC’s, NK-cellen, B-cellen,... geactiveerd. Op deze wijze vormen NKT-cellen een brug tussen het aangeboren en specifieke immuunsysteem.

Zowel endogene, microbiële als synthetische CD1d-liganden worden onderzocht op hun geschiktheid als NKT-activatoren. Het prototype is α-GalCer, een sfinolipide met sterke NKT-antigen activiteit, afkomstig van de spons Agelas mauritianus. Bij α-GalCer-activatie zien we een gemengd Th1/Th2-cytokinebeeld. Door modificaties aan te brengen aan de chemische structuur kan men de balans doen overhellen naar Th1 of Th2. (51, 125)

**Alpha-GalCer-derivaten als adjuvans**

Als brug tussen het aangeboren en het specifieke immuunsysteem, zijn de α-GalCer-derivaten heel geschikt om te gebruiken als vaccin-adjuvantia. Experimentele studies tonen zowel een verhoogde cellulaire als humorale respons wanneer een antigen wordt aangeboden samen met α-GalCer. Hoewel het exacte werkingsmechanisme nog niet volledig ontrafeld is geeft figuur 21 enkele mogelijke aangrijpingspunten aan (126). In de inductiefase activeren NKT-cellen APC’s, hetgeen leidt tot verhoogde presentatie van MHC en daaruitvolgend stimulatie van CD4+ en CD8+ T-cellen. Voor deze cellulaire respons lijken vooral Th1-cytokines van belang te zijn. De humorale respons wordt zowel indirect als direct geactiveerd: indirect via de gestimuleerde CD4+ Th2-cellen en direct via NKT-B-cel-interacties. Bij dit laatste nemen NKT-cellen feitelijk de functie van de CD4+ Th2-cellen over. Recent werd gesuggereerd dat IL-21 hier ook een belangrijke rol speelt (127). Ook voor het immuunantwoord op lange termijn lijken NKT-cellen een belangrijke rol te spelen. Er werd immers vastgesteld dat NKT-cellen verantwoordelijk zijn voor het langer persisteren van het humoraal immuunantwoord, hetgeen suggereert dat ze overlevingssignalen geven aan de plasmacellen (128).


Ten slotte kunnen NKT-cellen ook een humorale immuunrespons opwekken tegen T-cel-onafhankelijke antigenen (suikers) (131). Nu dienen deze suikers aan een eiwitmolecuule gekoppeld te worden om voldoende immuunrespons te krijgen, vooral bij kinderen jonger dan 2 jaar.

Een mogelijk nadeel van adjuvantia die NKT-cellen activeren is het optreden van NKT-cel ongevoeligheid na activatie (=anergie). Gedurende weken na een eerste toediening blijven de NKT-cellen anergisch tegenover een volgende stimulatie met α-GalCer (92). Dit zou de goede werking van herhalingsdosissen en andere α-GalCer-vaccins kunnen verhinderen. Ook bescherming tegen
natuurlijke infecties waar NKT-activatie een rol speelt, zou theoretisch gezien hierdoor verstoord kunnen worden. Echter, dit probleem lijkt afhankelijk te zijn van toedieningswijze, dosis en/of gebruikte derivaat. De impact op de kliniek lijkt minimaal.

Het veiligheidsprofiel en de kosten zijn tot op heden positief geëvalueerd. Humane α-GalCer-injecties geven weinig of geen algemene bijwerkingen (51) en massaproductie van glycolipiden is relatief makkelijk en goedkoop.

3. GEBRUIK IN DE KLINIEK

Het nut en de effecten van adjuvantia moeten uiteindelijk getoetst worden op basis van het veiligheidsprofiel, de effectieve protectie en de kost van de geadjuvanteerde vaccins. De ideale protectie is er één die de gevaccineerde onmiddellijk en levenslang beschermt tegen de stam van het pathogeen die in het vaccin was opgenomen en idealiter ook nog tegen alle verwante stammen van dat pathogeen.

Met de toename van zeer zuivere recombinante eiwit- of subunitvaccins, vullen de adjuvantia een belangrijk hiat in de protectie. Zeker bij mensen die een verminderde immunité hebben zijn adjuvantia beloftevol. Hierbij denken we aan de ouderen, jonge kinderen en immuungecompromiteerde personen. Hierna worden enkele vaccins besproken waarvan reeds verschillende adjuvansformulaties commercieel beschikbaar zijn.

**Influenza-vaccin**

Zowel het seizoensgriepvaccin als het pandemische griepvaccin bestaan in verschillende geadjuvanteerde en/of niet-geadjuvanteerde formulaties. Van de trivalente geïnactiveerde seizoensgriepvaccins die intramusculair worden toegediend blijken de vaccins die geformuleerd zijn in een olie-in-water-emulsie een sterker immuunantwoord te induceren. Fluad® (MF59 geadjuvanteerd seizoensgriepvaccin van Novartis, geïndiceerd voor gebruik bij de oudere volwassene) wekt hogere antilichaam-, B-cel en T-cel antwoorden op dan niet-geadjuvanteerde griepvaccins (132). Ook bij de monovalente geïnactiveerde pandemische griepvaccins scoren de olie-in-water-emulsies hoger wat betreft protectie dan de niet-geadjuvanteerde vaccins. Het pandemisch vaccin Pandemrix® (GSK) tegen het H1N1 2009 griepvirus, waar AS03 voor het eerst grootschalig werd gebruikt, is sterk immunogen (133). Het verhoogde protectief effect van geadjuvanteerde vaccins met een olie-in-water-emulsie is voornamelijk zichtbaar in de hogergenoemde immuungecompromiteerde populatie of wanneer het rondgaande virus door drifts verschilt van de vaccinstam (134, 135). Echter bij volledig nieuwe influenzastammen die ontstaan door shift, de oorzaak van pandemieën, kan men door toevoeging van adjuvant alleen het probleem niet helemaal oplossen (136). Omdat een populatie doorgaans volkomen immunologisch naïef is tegenover een pandemisch griepvirus zal men een sterker
immuunantwoord moeten uitlokken om tot adequate bescherming te komen. Toediening van een herhalingsdosis van een sterk geadjuvanteerde griepvaccin kan hier een oplossing bieden (137). Voor de influenzavaccins op basis van virosoomtechnologie (Inflexal®, Crucell) is de protectie minder overtuigend. Een gelijkwaardige tot licht verminderde humorale en cellulaire immuunrespons wordt gezien in vergelijking met de niet-geadjuvanteerde vaccins.


Wat betreft de beschikbaarheid en kosten is het toedienen van adjuvantia dosis-sparend voor het antigen. Dit kan van cruciaal belang zijn voor het pandemisch griepvaccin, waarvan op korte tijd massale dosissen nodig zijn.

**HBV-vaccin**

De klassieke en meest gebruikte HBV-vaccins zijn geadsorbeerd aan aluminiumhydroxide als adjuvant (Engerix-B® GSK, HBVaxPro® Sanofi Pasteur). Meer dan twintig jaar ervaring met deze vaccins heeft ons geleerd dat deze vaccins bij de meeste gezonde personen (zuigelingen, kinderen, volwassenen) een beschermend immuunantwoord uitlokken na toediening van het aanbevolen schema dat bestaat uit drie dosissen (gegeven volgens een 0, 1, 6 of 0, 1, 12 maandenschema). Aan patiënten met eind-stadium renaal falen, bij immuunonderdrukte personen (na transplantatie) en bij non-responders op de klassieke HBV-vaccins kan men een HBV vaccin gebruiken dat wordt geformuleerd met het adjuvant systeem AS04 (139, 140). Dit vaccin wordt verkocht onder de merknaam Fendrix® (GSK). Dit vaccin induceert een beter immuunantwoord maar ook significant meer lokale reacties dan aluminium-geadjuvanteerde HBV vaccins. Zo melden 41% van de Fendrix-gevaccineerden pijn op de injectieplaats tov 15% van de Engerix-gevaccineerden. Alhoewel Fendrix® kan beschouwd worden als een mogelijke optie voor de inductie van een beschermende HBV response bij non-responders op de klassieke HBV-vaccinatie, wordt het product heden enkel terugbetaald voor patiënten met een nierinsufficiëntie.

**HAV-vaccin**

Naast het klassieke aluminium-geadjuvante HAV vaccin (Havrix®, GSK) is er ook HAV vaccin gebaseerd op virosoomtechnologie (Epaxal®, Crucell) op de markt. De protectie die beide vaccins
inducereren is gelijkaardig, maar het veiligheidsprofiel is in het voordeel van Epaxal®, met minder lokale reacties (141). Aan kinderen tussen 1 en 15 jaar kan dezelfde dosis Epaxal® gegeven worden als aan volwassenen. Van Havrix® daarentegen bestaan twee verschillende formulaties: één voor kinderen en adolescenten, gekend als Havrix Junior® (720 EIU HAV in 0.5 ml) en één voor volwassenen (Havrix®: 1440 EIU HAV in 1 ml).

**HPV-vaccin**

Het HPV-vaccin bestaat in twee formulaties die haast tegelijk op de markt gekomen zijn: Gardasil® (Merck & Co) en Cervarix® (GSK). Gardasil® heeft een aluminiumzout als adjuvant en bevat de oncogene HPV-16 en HPV-18, en de niet-oncogene HPV-6 en 11, oorzaak van genitale wratten, als vaccinantigenen. Cervarix® is geformuleerd in het adjuvant systeem AS04 en bevat enkel de oncogenen HPV types 16 en 18 als vaccinantigenen. Cervarix® geeft een betere immuunprotectie tegen zowel HPV types 16 en 18 als verwante oncogene HPV stammen niet opgenomen in het vaccin (142, 143). Daar tegenover staat dat Cervarix® meer lokale reacties geeft dan Gardasil® (143). In Vlaanderen zijn zowel Gardasil® als Cervarix® terugbetaald voor meisjes tussen 12 en 18 jaar.

**Zeldzame bijwerkingen**

De mogelijkheid dat adjuvantia, vooral de nieuwere producten zoals MF59, AS03 en AS04, auto-immuunziekten zouden uitlokken is vandaag een belangrijk discussie-onderwerp en een bron van bezorgdheid van de regulerende instanties zoals de FDA in US en het EMA in Europa. Door de zeldzaamheid waar mee ze optreden en de lange termijn die kan verstreiken tussen vaccinatie en het optreden van “vaccin-gerelateerde” immuunziekten, is het bijzonder moeilijk om een causaal verband aan te tonen of te ontkrachten. Recent is de term ASIA (auto-immune/inflammatory syndrome induced by adjuvantia) door verschillende auteurs geïntroduceerd (144, 145). Het omvat verschillende immunologische aandoeningen en syndromen waarvan men meent dat ze geassocieerd zijn aan adjuvans-toediening. MMF of macrofage myofasciitis is hiervan het best gekende. Het wordt geassocieerd met de blijvende aanwezigheid van aluminium in de spieren. De diagnose wordt gesteld op basis van de anatomopathologische bevindingen in een biopsie van de geïnjecteerde spier. Tabel 16 geeft een overzicht en de frequentie van optreden van de belangrijkste ziektesymptomen die voorkomen bij MMF. Met de gegevens waarover we vandaag beschikken wegen de mogelijke zeldzame ernstige bijwerkingen van vaccins niet op tegen de voordelen van een effectieve vaccinatie (44).
Het toevoegen van een adjuvant aan een entstof (vaccinantigen) leidt tot een beter immuunantwoord en als gevolg daarvan ook tot een betere protectie. De werking van adjuvantia berust voornamelijk op stimulatie van het aangeboren immuunsysteem. Zeker bij kinderen, ouderen en immuungecompromitteerde personen bieden adjuvantia een belangrijke meerwaarde. Ook in het kader van grieppandemiën zijn adjuvantia nuttig. Toevoeging van adjuvantia aan een vaccin leidt tot meer frequent optreden van lokale reacties en heel zelden van systemische immuunziekten. In de toekomst worden veel nieuwe adjuvantia verwacht, waaronder de α-GalCer-derivaten.

**Tabel 16. Belangrijkste MMF-symptomen en frequentie (enkel symptomen met frequentie ≥25% zijn weergegeven) (42).**

<table>
<thead>
<tr>
<th>Symptoom</th>
<th>Percentage van de patienten</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myalgie</td>
<td>88-91</td>
</tr>
<tr>
<td>Arthralgie</td>
<td>57-68</td>
</tr>
<tr>
<td>Ernstige asthenie</td>
<td>55</td>
</tr>
<tr>
<td>Spierzwakte</td>
<td>45</td>
</tr>
<tr>
<td>Koorts</td>
<td>20-32</td>
</tr>
<tr>
<td>Verhoogde CK waarden</td>
<td>29-50</td>
</tr>
<tr>
<td>Gestegen ESR</td>
<td>37</td>
</tr>
<tr>
<td>Myopathisch EMG</td>
<td>35</td>
</tr>
<tr>
<td>Multiple Sclerosis</td>
<td>33</td>
</tr>
<tr>
<td>Chronische vermoeidheid</td>
<td>50-93</td>
</tr>
</tbody>
</table>

**4. BESLUIT**

Het toevoegen van een adjuvant aan een entstof (vaccinantigen) leidt tot een beter immuunantwoord en als gevolg daarvan ook tot een betere protectie. De werking van adjuvantia berust voornamelijk op stimulatie van het aangeboren immuunsysteem. Zeker bij kinderen, ouderen en immuungecompromitteerde personen bieden adjuvantia een belangrijke meerwaarde. Ook in het kader van grieppandemiën zijn adjuvantia nuttig. Toevoeging van adjuvantia aan een vaccin leidt tot meer frequent optreden van lokale reacties en heel zelden van systemische immuunziekten. In de toekomst worden veel nieuwe adjuvantia verwacht, waaronder de α-GalCer-derivaten.
Chapter I summarizes the current knowledge about iNKT-cells in fundamental and clinical immunology. They are considered as atypical T-cells with semi-invariant T-cell and NK-cell receptors. As such, they play an important role in many pathophysiological processes with specific clinical applications in oncology, autoimmunity and vaccine development currently being heavily researched. This broad spectrum of potential clinical applications is partly due to their versatile functional nature: a selective cytokine secretion and co-stimulatory expression of iNKT-cells is observed dependent on their environment. This initially non-directed behaviour assigns strong innate-like functions to these iNKT-cells.

The most straightforward environmental co-players are the iNKT-agonists. In contrast to the peptide-agonists on MHC for classical T-cells, iNKT-agonists are glycolipids presented on CD1d by dendritic cells or other APCs. They activate iNKT-cells by interaction with their T-cell receptors. Simplified, two biological variables are tuned on the chosen glycolipid: the strength of iNKT-activation and the Th1/Th2 functional outcome balance. Chapter II gives an important overview of the chemical and biological space wherein the currently known iNKT-activators are moving. With the multivariate PCA and HCA techniques, different clusters of glycolipids are observed on the basis of their chemical structure similarity. A look at the biological space firstly suggests a complementary role for the mice/in-vivo and human/in-vitro test-system, contrasting the more superfluous role for the mice/in-vitro test-system, which does not seem to give much extra new information. Also the inter-laboratory variability is higher for the mice/in-vitro test-system when compared to the other two test-systems. A second interesting aspect discussed in the biological space-part, is the need of an in-vivo environment for strong Th1 or Th2 biasing. A major difference with in-vitro assays is the presence of bystander cells like NK-cells in an in-vivo system. This confirms recent studies where bystander cells are suggested to be responsible for polarization of the cytokine-response.

Finally, for the first time, a relationship was established between the chemical structure and the above mentioned biological responses. This structure-activity relationship model makes it possible to make functionality predictions for future glycolipids. As such, it is a promising new tool in the iNKT-research for a more efficient development of new iNKT-activators.

A next step in the translational process towards clinical use of iNKT-activators, is the implementation of our fundamental chemical and biological knowledge to appropriate disease animal models. Chapter III confirms the modulating role of iNKT-activation in an auto-immune arthritis mice model. Late intraperitoneal administration of α-GalCer, the prototype of a strong unbiased iNKT-agonist, in a
collagen induced arthritis-model, shows a higher maximum clinical arthritis-severity compared to controls. Previous studies in our lab showed a reversed, beneficial effect on disease when α-GalCer is administered early. Although IFN-γ tends to have an attenuating role later in disease, NU-α-GalCer, a strong IFN-γ inducer, surprisingly exacerbated disease progression in the same experiment. The pro-inflammatory capacity of α-GalCer and its analogues in an arthritis model made us investigate the potential of α-GalCer as adjuvant in this collagen induced arthritis-model. Instead of using the standard heat-killed Mycobacterium as adjuvant to build a strong immune response against the co-administered collagen, we used α-GalCer solved in DMSO. Unexpectedly, the use of α-GalCer in DMSO could not induce arthritis sufficiently compared with heat-killed Mycobacterium. Induction of CIA by α-GalCer was very limited and a possible effect of DMSO could not be excluded. In addition, it is not known if α-GalCer can be well emulsified in IFA. This might lead to ineffective iNKT-cell activation. As recent studies indicated a role of other Th-responses than Th1- and Th2-response, it could be useful to further investigate the role of Th-responses in the pathogenesis of CIA. Another possibility is that Th1- and Th2-cytokines counteract each other with the unbiased α-GalCer glycolipid. In this case, a more Th1- or Th2-biasing glycolipid is needed for use as adjuvant.

Finally, in chapter IV, we review the current clinically used vaccine-adjuvantia. Vaccinations are one of the most important discoveries that have been made in medical sciences. The administration of pathogenic antigens stimulates the immune system to raise antigen specific responses. Subsequently, a later infection will rapidly be recognized and defeated. Unfortunately, for many infectious diseases, vaccine induced-protection is not strong enough. This problem holds true especially for the elderly because of their diminished cellular immunity. Due to global population ageing, this is becoming an increasingly important issue. In the quest for more effective and safe vaccines, the adjuvantia are playing a neglected but very much important role. They help setting up an adaptive immune-response against the antigen. Two modes of action are nowadays being distinguished for the currently used adjuvantia: an enhanced delivery of the antigen and stimulation of an innate, inflammatory environment. This inflammatory environment stimulates the adaptive antigen-specific immune system. iNKT-activating adjuvantia are promising new vaccine-helpers because of their mixed innate-adaptive nature and their versatile nature. By tuning iNKT-cells, e.g. with specific agonists, a vaccine- and person-specific antigen-response can be elicited. Chapter IV thus wants to make a contribution to a rational use of adjuvantia in vaccines with an eye-opener to the new group of iNKT-agonists being in development.

In the future, several challenges in iNKT-research are waiting to be resolved, where three main domains can be distinguished:
• to understand better and more thoroughly the biological mode of action of iNKT-agonists, based on knowledge of their chemical properties;

• to improve our knowledge of the immune pathophysiology of specific diseases and hence to find new disease targets;

• to develop clinical applications where the known biological mode of action fits the known immune pathophysiology.
Prof. Zonnebloem couldn’t stop reading about iNKT cells...


LIST OF ABBREVIATIONS AND SYMBOLS

Alpha-GalCer: α-galactosylceramide
APC: Antigen Presenting Cell
ASIA: Auto-immune/inflammatory Syndrome induced by Adjuvantia
BMDC: Bone Marrow Dendritic Cell
CD: Cluster of Differentiation
CFA: Complete Freund Adjuvant
CIA: Collagen-induced Arthritis
CII: Collagen type II
CTLA-4: Cytotoxic T lymphocyte associated Antigen-4
D: Derringer Desirability
DAMP: Damage-associated Molecular Pattern
DC: Dendritic Cell
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: Dimethylsulfoxide
ELISA: Enzyme-linked Immunosorbent Assay
EMA: European Medicines Agency
FBS: Fetal Bovine Serum
FDA: Food and Drug Administration
HAV: Hepatitis A Virus
HBV: Hepatitis B Virus
HCA: Hierarchical Cluster Analysis
HPV: Human Papilloma Virus
IFA: Incomplete Freund Adjuvant
IFN: Interferon
IL: Interleukine
iNKT: invariant Natural Killer T-cell
LPS: Lipopolysaccharide
MHC: Major Histocompatibility Complex
MMF: Macrophage Myofasciitis
MPL: Mono Phosphoryl Lipid A
MyD88: Myeloid Differentiation Primary Response Gene 88
NK-cell: Natural Killer Cell
NLR: Nucleotide-binding oligomerization domain, Leucine rich Repeat (see also NOD-receptor)
NOD-receptor: Nucleotide Oligomerization Domain Receptor (see also NLR)
PAMP: Pathogen-associated Molecular Pattern
PBS: Phosphate Buffered Saline
PCA: Principal Component Analysis
PLS: Partial Least Squares
PRR: Pattern Recognition Receptor
Q²: Predicted Variation
R²: Explained Variation
RA: Rheumatoid Arthritis
RF: Rheumatoid Factor
RMIA: Rodent Model of Immune-mediated Arthritis
RPMI: Roswell Park Memorial Institute
TCR: T-cell Receptor
Th-response: T-helper Response
TLR: Toll-like Receptor
TNF: Tumor Necrosis Factor
**SUPPLEMENTARY INFORMATION**

**Table S1.** α-GalCer analogue structures with corresponding molecule ID number.

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Text S1


Figure S1. Dendrogram using Average Linkage (between groups).
Figure S2. Distribution of Th1-Desirabilities (D-Values; $D_{Th1} = 0.51$ for α-GalCer) for in vivo data (total N=85).
Figure S3. Distribution of Th1-Desirabilities (D-Values; $D_{Th1}=0.48$ for $\alpha$-GalCer) for murine and human *in vitro* data (total N=137).
**Figure S4.** IL-12 detection on blood serum 4, 16 or 24 hours after intravenous injection of $6 \times 10^5$ BMDCs loaded with $5 \mu g$ glycolipid. Graphs represent mean with s.e.m. for 6 mice. *P*-values compared to α-GalCer (*p<0.05, **p<0.01, ***p<0.001) (two-tailed Mann–Whitney U-test) (two-tailed Mann–Whitney U-test).

**Table S2.** Experiment 1. Frequency of incidence and mean time of onset.

<table>
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<tr>
<th>Treatment group</th>
<th>Total N</th>
<th>Number of Arthritis</th>
<th>Frequency</th>
<th>Time to Onset of Arthritis</th>
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<td></td>
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<td>Mean (days)</td>
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<tr>
<td>BnNH-GSL-1'</td>
<td>8</td>
<td>4</td>
<td>50%</td>
<td>32.8</td>
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<tr>
<td>Alpha-GalCer</td>
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<td>5</td>
<td>63%</td>
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<tr>
<td>NU-α-GalCer</td>
<td>8</td>
<td>6</td>
<td>75%</td>
<td>31.8</td>
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<tr>
<td>DMSO</td>
<td>8</td>
<td>5</td>
<td>63%</td>
<td>32.6</td>
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<tr>
<td>PBS</td>
<td>8</td>
<td>2</td>
<td>25%</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>40</strong></td>
<td><strong>22</strong></td>
<td><strong>55%</strong></td>
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**Figure S5.** Experiment 2. Mean arthritis score. Time axis indicates the number of days after immunization. Data represent mean with s.e.m. for 10 mice per condition.

**Table S3.** Experiment 2. Frequency of incidence and mean time of onset. *p<0.0001, total frequency experiment 2 vs. experiment 3 (Fisher’s exact test).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total N</th>
<th>Number of Arthritis</th>
<th>Frequency</th>
<th>Time to Onset of Arthritis Mean (days)</th>
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<tr>
<td>IFA + 5µg α-GC in DMSO</td>
<td>10</td>
<td>8</td>
<td>80%</td>
<td>24.9</td>
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<tr>
<td>IFA + 0.5µg α-GC in DMSO</td>
<td>10</td>
<td>3</td>
<td>30%</td>
<td>27.0</td>
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<tr>
<td>IFA + 0.05µg α-GC in DMSO</td>
<td>10</td>
<td>6</td>
<td>60%</td>
<td>37.3</td>
</tr>
<tr>
<td>IFA + 5µL DMSO</td>
<td>10</td>
<td>6</td>
<td>60%</td>
<td>28.0</td>
</tr>
<tr>
<td>IFA + 0.5µL DMSO</td>
<td>10</td>
<td>3</td>
<td>30%</td>
<td>37.2</td>
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<tr>
<td>IFA + 0.05µL DMSO</td>
<td>10</td>
<td>1</td>
<td>10%</td>
<td>35.4</td>
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<tr>
<td>CFA + 5µL DMSO</td>
<td>10</td>
<td>10</td>
<td>100%</td>
<td>32.4</td>
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<tr>
<td>CFA + 0.5µL DMSO</td>
<td>10</td>
<td>7</td>
<td>70%</td>
<td>31.3</td>
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<tr>
<td>CFA + 0.05µL DMSO</td>
<td>10</td>
<td>6</td>
<td>60%</td>
<td>30.3</td>
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<tr>
<td>CFA</td>
<td>10</td>
<td>5</td>
<td>50%</td>
<td>28.8</td>
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<tr>
<td>IFA</td>
<td>10</td>
<td>1</td>
<td>10%</td>
<td>25.0</td>
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<tr>
<td>Total</td>
<td><strong>110</strong></td>
<td><strong>56</strong></td>
<td><strong>51%</strong></td>
<td><strong>30.7</strong></td>
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**Supplementary Information**

**Figure S6.** Experiment 3. Mean arthritis score. Data represent mean with s.e.m. for 10 mice per condition. Time axis indicates the number of days after immunization.

**Table S4.** Experiment 3. Frequency of incidence and mean time of onset. *p<0.0001, total frequency experiment 2 vs. experiment 3 (Fisher’s exact test).

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<th>Number of Arthritis</th>
<th>Frequency</th>
<th>Time to Onset of Arthritis Mean (days)</th>
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<tr>
<td>IFA + 5µg α-GC in DMSO</td>
<td>10</td>
<td>0</td>
<td>0%</td>
<td>28.0</td>
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<tr>
<td>IFA + 0.5µg α-GC in DMSO</td>
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<td>0%</td>
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<td><strong>21</strong></td>
<td><strong>19%</strong></td>
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