T cell recognition of unconventional antigens

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Summary/abstract

CD1 proteins, expressed on many antigen presenting cells, are capable of binding lipids to form antigen complexes that contact T cell receptors and activate T cells. Invariant T cell populations exist in mycobacterium infected humans that recognize mycobacterial lipids. Other bacteria might also possess lipid antigens that activate T cells. Using an ELISPOT IFN-γ assay, we determined that lipids from pathogens other than mycobacteria can stimulate human IFN-γ responses. We generated T cell lines based on binding of CD1b loaded with lipid antigens. Analysis of T cell lines using ELISPOT revealed not only reactivity to CD1b loaded with *S. aureus* and *B. melitensis* lipid antigens, but also to untreated CD1b. A shared phospholipid between bacteria and mammalian cells, phosphatidyl glycerol, was determined to be the stimulating antigen. We sequenced the T cell receptor (TCR) from T cell lines using single cell PCR. Our data show that the identified TCR α and β chain genes are TRAV 9-2 in combination with TRBV 6-2. The identified TCR might play an important role in CD1 mediated immunity and potentially also in CD1 mediated autoimmunity, as phosphatidyl glycerol is also recognized as an antigen.

INDEX

Introduction

Many studies on T cells focus on the model that highly diverse T cell receptors (TCR) interact with the Major Histocompatibility Complex (MHC) class I and class II molecules loaded with antigenic peptides derived from pathogens. The discovery of the ability of the group of CD1 proteins to present antigenic lipids to T cells broadens the biochemical range of T-cell antigens to include lipids [6]. Over two decades ago, CD1 molecules were first shown to mediate T-cell responses to lipid antigens [6]. Since then, the range of known lipid antigens that are presented by CD1 molecules has expanded [5,19,25,27,37,40]. The isolation of diverse antigens from mammalian and bacterial sources, together with the finding that individual CD1 proteins selectively acquire antigens in separate cellular subcompartments, has led to the emerging picture of the CD1 pathway as an antigen-presentation system that surveys cells for changes in their lipid content [8,13,21,41]. This includes surveying the presence of foreign antigenic lipids, produced by human and animal pathogens [3]. Including lipids as potential activating antigens for T cells can create new possibilities for the development of vaccines as well as for new diagnostic approaches.

CD1 molecules, closely related to the MHC class I molecules, are well conserved among many mammalian species, including humans, canines and cattle [9,27,43]. Based on sequence homology and coordinate gene regulation, CD1 molecules are divided in group 1 (CD1a, CD1b, CD1c), group 2 (CD1d) and group 3 (CD1e) [3]. Each of the CD1 isoforms contain distinct antigen binding grooves and show different subcellular localizations and expression patterns, which suggests distinct immunological functions. Humans express all CD1isoforms, whereas muroid rodents express only CD1d [9]. All other well-studied mammalian species express multiple CD1 isoforms. In the guinea pig, evolutionary homologs of human CD1b, CD1c, CD1d and CD1e proteins have been found [10]. Bovines only express CD1a, CD1d, CD1e and multiple CD1b proteins [43]. Rabbits, canines, porcines and equines possess at least one or multiple homologs of CD1a, CD1b, CD1c, CD1d and CD1e proteins [14,15,18,27]. Although CD1 genes have also been found in chickens, they differ from mammalian CD1 proteins. The evolutionary relationship between mammalian and avian CD1 remains to be clarified [29].

CD1 proteins are synthesized in the endoplasmic reticulum (ER) and loaded with endogenous lipids, including phospholipids [14,34]. After loading, CD1 proteins follow the secretory pathway and end up in the plasma membrane [7,13,22,34]. After trafficking to the plasma membrane, CD1 proteins are internalized into early or sorting endosomes. After internalization, the different CD1 isoforms follow distinct pathways [3]. Nevertheless, all CD1 isoforms can undergo the process of lipid exchange between loaded endogenous lipids and foreign lipids. After being loaded with foreign lipids, CD1 proteins traffic back to the plasma membrane for recognition by TCR [3]. Although many

components of the CD1 pathway are still poorly understood, the general view is that CD1 proteins survey the endocytic system for changes in lipid content [3].

The group 2 CD1 protein CD1d presents lipid antigens that are recognized by type I and type II NKT cells [3]. The CD1d presented lipid antigens include synthetic, self and microbial lipids. So far, research on the group 1 CD1 proteins has mainly focused on antigenic lipids from mycobacterial origin [23,44]. These lipids are presented by CD1a, CD1b and CD1c molecules (group 1 CD1). In mycobacterium infected humans, an invariant T cell population exists that recognizes mycobacterial lipids. This was demonstrated by using human CD1b tetramers [42]. Other pathogens might also be used to stimulate the immune system via the group 1 CD1 proteins, but this has not yet been demonstrated.

CD1 restricted T cells in blood from healthy donors are rare and the frequency is generally well below 0,1 percent [2]. Dextramers provide a novel tool to identify and isolate rare antigen specific T cells. They consist of a dextran backbone fluorescently labeled with streptavidin and can be loaded with antigen presenting molecules. A dextramer can be loaded with 10-14 CD1 molecules which increases avidity of the TCR interaction as compared to tetramers. By treating CD1 molecules with lipid antigens and assembling them into dextramers, CD1 restricted T cells can be isolated and expanded [24].

We set out to demonstrate group 1 CD1-restricted T cell responses to the following pathogens: *Staphylococcus aureus*, *Brucella melitensis*, *Campylobacter jejuni*, *Coxiella burnettii*, *Listeria monocytogenes* and *Trypanosoma brucei brucei*. Together, these pathogens carry a broad range of lipids that could potentially activate T cells via the CD1 pathway. This broad range of lipids includes lipids that are shared between mammalian and bacterial cells such as phospolipids. There are several approaches possible to study lipid reactive T cells. One approach is to test lipid reactivity directly using ELISPOT IFN-γ. If lipid reactive T cells are present in random blood bank donors, adding lipid should increase spot count in this assay. Another approach is by using dextramers to sort and expand T cells. As the frequency of lipid reactive T cells is well below 0,1 percent in a random blood bank donor, the percentage is expected to increase after expansion. An increased percentage implies the presence of one or several expanded T cells with TCRs specific for CD1 treated with lipids. Expanded cells can then be expanded and analyzed to determine the specific TCR. This might lead to the discovery of new invariant T cells in unrelated human blood bank donors.

The identification of an invariant T cell population recognizing mycobacterial lipids presented by CD1b is in sharp contrast with the general view that group 1 CD1 reactive TCRs are clonally diverse. The diverse nature of group 1 CD1 reactive TCRs is based on a small panel of clones. Possibly, interdonor TCR conservation is more common in

the CD1 repertoire. By generating more T cell lines with a group 1 CD1 restriction, more information on the diversity or invariant nature of group 1 CD1 restricted T cells can be gathered. Here we have identified a TCR specific for phosphatidyl glycerol, a shared phospholipid between mammalian and bacterial cells.

Research at the Faculty of Veterinary Medicine at Utrecht University is concentrated in five thematic, interdisciplinary programs. This study is part of the focus area of Infection & Immunity and part of the research programme Strategic Infection Biology. One of the key objectives is the modulation of the innate and adaptive immune responses towards pathogens. Emerging infections and antimicrobial resistance are becoming a major burden in human and animal health. Alternatives for antimicrobials are scarce. The use of vaccines in humans and animals provides protection to many diseases, although many vaccines are directed against viruses. For many other pathogens, such as bacteria and parasites, vaccine development is often challenging, for example due to evasion strategies, antigenic variation or the presence of many different virulent strains. In this study, we have selected human and animal pathogens pathogens to which no effective vaccines have been developed so far. By identifying group 1 CD1 restricted T cells responding to lipid antigens, we hope to contribute to the key objective of the research programme of Strategic Infection Biology and provide new insights into the interaction between pathogens and the immune system.

Materials & methods

Antigens.

Coxiella burnetii and *Brucella melitensis* were cultured at the Centraal Veterinair Instituut, Lelystad and lipid extraction was performed according to protocols provided by us. *Staphylococcus aureus* and *Campylobacter jejuni* were obtained from the department of infectious diseases and immunology at Utrecht University and cultured according to protocol.(appendix I) *Tryponasoma brucei brucei* was obtained from the department of biochemistry and cell biology at Utrecht university and cultured according to protocol.(appendix I).

All cultured pathogens were pelleted by centrifugation for 30 minutes at 5100rpm. Next, the wet pellets were extracted for 2 hours at room temperature in chloroform:methanol 1:2 (V:V) and 2:1 (V:V) consecutively. The total lipid extracts were dried in a rotating evaporator at room temperature, re-dissolved in chloroform:acetone 1:1 (V:V) and quantification and analysis was done by TLC. Fractionation of the total lipid extracts was performed by loading on a silica solid phase extraction column with a bed weight of 2 g (Supelco) and consecutive elution with three bed volumes (12 mL) of the following eluents: chloroform, acetone, methanol. Quantification and analysis was done by TLC. All TLC plates (200 μm) were resolved in 60:16:2 v:v chloroform:methanol:water, dried and sprayed with cupricacetate phosphoric acid and heated at 160^0C to visualize bands.

Phosphotidyl glycerol was obtained from Avanti polar lipids inc.

Acquiring polymorphous blood mononuclear cells (PBMC), T cells and antigen presenting cells

Buffy coats were obtained from random blood bank donors (Sanquin). PBMC were isolated using Ficollpaque (protocol in appendix I). For acquiring autologous G4 cells, isolated PBMC were aliquotted into a 645cc corning flask with a surface area of 225cm^2 and incubated 30 minutes at 37^0C 5% CO_2 in medium with granulocyte monocyte colony stimulating factor (GM-CSF). After incubation, PBMC were aliquotted into centrifuge tube and frozen according to protocol (appendix I) Adherent cells were incubated for 3 to 5 days in GM-CSF medium with IL-4. After incubation, acquired G4 cells were counted and frozen in 20% DMSO in T cell medium (TCM).

Flow cytometry.

CD1b monomers (NIH Tetramer Core Facility) were incubated with lipid extracts and assembled into dextramers (appendix I). Dextramers were incubated with human PBMCs for 20 minutes at 37^0C. Flow cytometry data were

pregated for lymphocytes based on forward and side scatter. T cells were sorted after staining with anti-CD3. Cells were analysed on a Beckton Dickinson FACS Canto or sorted on a FACS Cytopeia II cell sorter.

T cell assays.

Sorted cells were expanded polyclonal with 5000-10000 cells per well in round-bottomed 96-well plates containing 2 × 105 irradiated allogeneic PBMC, 4 × 104 irradiated Epstein-Barr virus–transformed B cells and 30 ng/ml anti-CD3 (OKT3), with 1 ng/ml of IL-2 added on day 2 of the culture. After 2 weeks, wells with visible growth were restimulated and tested for binding of lipid-extract-loaded CD1b-dextramer by flow cytometry and for antigen specificity by ELISPOT assay.

For ELISPOT assays, cocultures of antigen-presenting cells and T cell were incubated for 16 h in a Multiscreen-IP filter Plate (96 wells; Millipore) coated according to the manufacturer's guidelines. We added 20.000 K562 cells or 40.000 G4 cells per well as antigen-presenting cells. Lipid extracts were dried and redissolved in TCM at a concentration of 15μg/ml. When using PBMC from a donor with a low population of responsive cells, we added 400.000 cells per well. For generated T cell lines, up to 10.000 cells per well were added. ELISPOT plates were analysed using Automated ImmunoSpot Image Analyzer Software (AELVIS Tecnologies, TEMA-Ricerca, Italy). Stimulation indices were calculated as follows: Stimulation index = mean spot number in triplicate antigen-containing wells divided by the mean spot number in triplicate medium containing control wells. Overall stimulation index to lipid extracts was calculated as the mean stimulation index of all donors.

PCR and molecular cloning.

From expanding T cell clones and sorted T cell populations, RNA was isolated with an RNeasy kit (Qiagen), and cDNA was synthesized with a Quantitect reverse-transcription kit (Qiagen), including a genomic DNA–removal step.

For single cell TCR sequencing, cells were suspended in a 2.5 ml cDNA synthesis reaction mix consisting of 0.1 % Triton X-100 (Sigma-Aldrich) and 0.2 μl iScript reverse transcriptase, and incubated 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Subsequently, a pre-amplification targeting all TRAV and all TRBV segments (TCR primer sequences in appendix II) is performed in GoTaq Flexi (Promega) reaction mixture containing 2.5 mM MgCl, 0.2 mM each dNTP, 0.025 U/ml GoTaq polymerase. The pre-amplification mixture was aliquoted into individual PCR mixtures for each TRAV and TRBV segment. Positive reactions were detected by electrophoresis through agarose gels and the amplicons were sequenced.

Results

Autologous antigen presenting cells treated with pathogen derived lipid extracts induce IFN-gamma production in polymorphous blood mononuclear cells.

To study lipid reactive T cells, we isolated PBMC from 10 different human blood donors and tested IFN- γ production using ELISPOT. Lipids were isolated from a broad range of pathogens and quantified and visualized using thin layer chromatography (TLC) (figure 1). Figure 1 shows that lipids were successfully extracted. Even though an estimated amount of 30μg standard lipid (phosphatidyl inositol (PI), phosphatidyl choline (PC), phosphatidyl glycerol (PG) or 30μg of pathogen derived lipid extract was applied in each lane, the amount of *Brucella melitensis* (Bruc.) total lipid seemed higher and the amount of *Trypanosoma brucei brucei* (Tryp.) total lipid seemed lower, so we adjusted the estimated concentrations of these lipids.

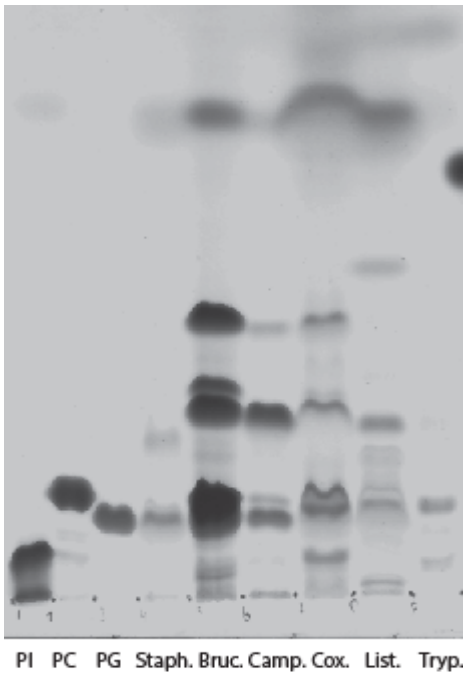

Figure 1: Thin layer chromatography of pathogen derived lipid extracts

An estimated amount of 30μg of pathogen derived lipid extracts were applied. PI: Phosphatidyl inositol; PC: Phosphatidyl choline; PG: Phosphatidyl glycerol; Staph.: *Staphylococcus aureus*; Bruc.: *Brucella melitensis*; Camp.: *Campylobacter jejuni*: Cox.: *Coxiella burnetii*; List.: *Listeria monocytogenes*; Tryp.: *Trypanosoma brucei brucei*

Stimulation of random blood bank donor PBMC in an IFN-γ ELISPOT assay showed that stimulation with *Brucella*, *Campylobacter*, *Coxiella* and *Listeria* total lipids caused an increase in spot count as compared to stimulation with medium only. Stimulation with these pathogen derived lipids had an average stimulation index of >2 (figure 2). A stimulation index of 2 has often been considered a positive response [16,26,36]. Even though the error bars within donors are very small (data not shown), the relatively large error bars in figure 2 are caused by the fact that not

all donors respond to the same set of pathogen derived lipid extracts. For example, donor BC 3 has a stimulation index of 0.98 for total lipid extract from *Campylobacter jejuni* whereas donor BC 5 has a stimulation index of 10.81. On average, the stimulation index for total lipid extract from *C. jejuni* is 4.70. Table 1 shows the stimulation indices for each of the pathogen derived lipid extracts for each of the human blood donors used. Although large variation between donors exists, we conclude that lipids from pathogens other than mycobacterium can stimulate human IFN-γ responses.

Table 1: Responses of individual donors to pathogen derived lipid extracts

Stimulation indices from donors BC 3- 10 to pathogen derived lipid extracts as compared to medium alone. MET = methanol fraction.

Donor	BC 3	BC 4	BC 5	BC 6	BC 7	BC 8	BC 9	BC 10	BC 11	BC 12
S. aureus	1.17	1.68	1.57	0.82	1.55	2.15	3.31	1.95	0.96	1.15
B. melitensis	1.63	4.21	3.95	1.22	1.56	2.28	4.06	2.26	1.45	1.5
C. jejuni	0.98	3.23	10.81	3.96	3.21	9.85	5.46	4.49	2.43	2.58
C. burnetii	1.24	4.03	2.05	1.21	1.55	2.67	3.97	2.1	1.5	1.39
L. monocytogenes	1.9	5.23	4.14	2.14	1.44	4.31	4.28	1.92	1.75	1.17
T. brucei brucei	1.24	1.33	2.33	1.42	1.48	1.18	1.84	1.56	1.1	0.83
S. aureus MET	0.57	0.77	1.29	0.83	1.51	2.23	1.06	1.45	0.97	0.93
B. melitensis MET	1.12	2.83	3.38	1.06	1.59	2.51	2.26	1.87	1.02	1.07

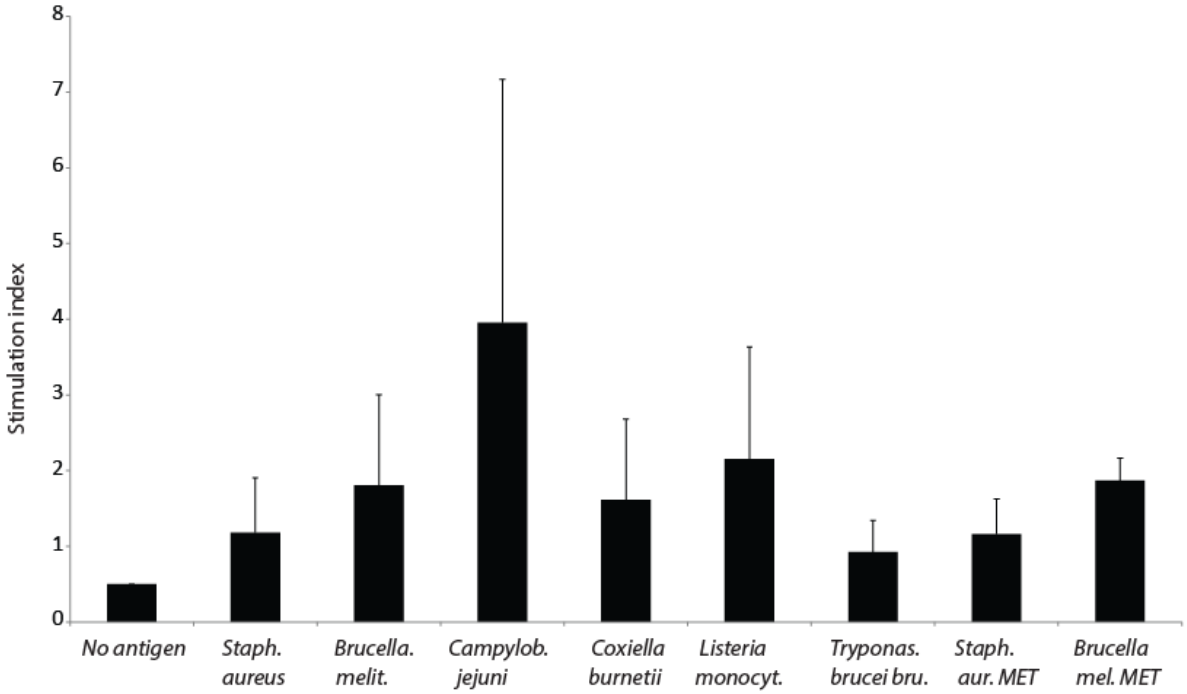

Figure 2: ELISPOT stimulation index

PBMC and monocyte-derived dendritic cells were stimulated with pathogen derived lipid extracts or medium alone in an IFN-γ ELISPOT assay. Stimulation indices were calculated. Average stimulation index of 10 donors + SD are shown. MET = methanol fraction

CD1b dextramers loaded with antigenic lipids to generate antigen specific cellines

Because our initial results suggested that lipids from other bacteria can be recognized by human T cells, we set out to study this possibility in more detail. We chose to load fluorescently labeled CD1 dextramers with bacterial lipids to identify lipid specific T cells. The advantage of this approach is that we can study CD1 restriction directly. There are no known T cell lines that recognize defined CD1-presented lipids from our panel of bacteria, so we lacked confirmed antigen-specific cell lines for validation of our dextramers.

Total lipid extracts from *Staphylococcus aureus* and *Brucella melitensis* were partially purified using solid phase lipid extraction. The methanol fractions, containing charged lipids, among which the phospholipids, were loaded into CD1b molecules and assembled into dextramers (Sta-met, Bru-met). We chose to work with the CD1b protein, as it has the largest antigen binding groove and is likely capable of presenting a wide array of lipid antigens to T cells. CD1b is known to bind lipids with a total of 80 carbon atoms, as well as much smaller lipids, in which case the remaining space is filled with so-called spacer lipids [4,21,31].

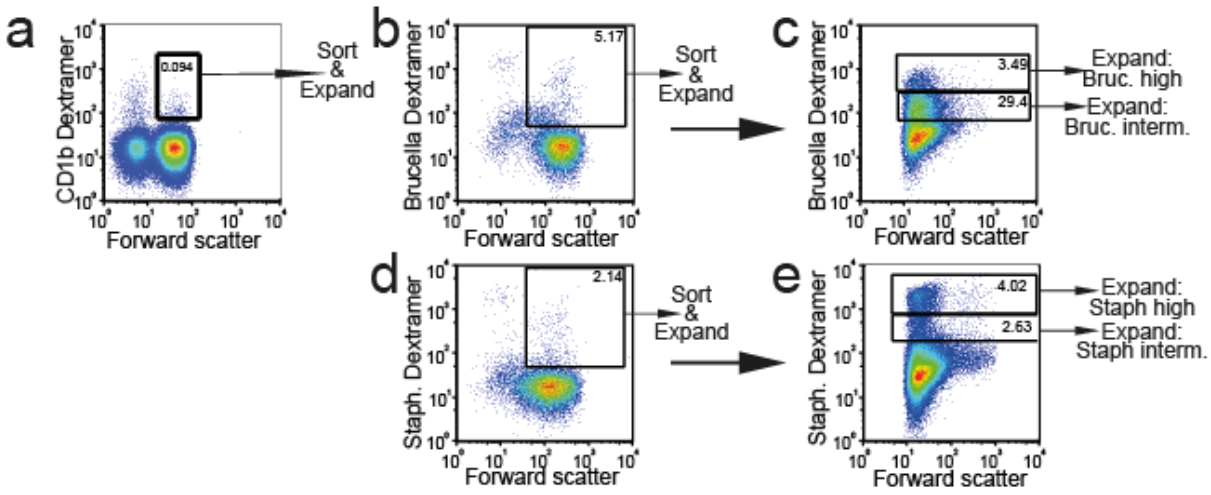

Figure 3: Generation of T cell lines

Dextramer-based approach can generate CD1 restricted antigen specific T cell lines. (a) The initial sort yielded 0.094 percent dextramer positive cells. Both *Brucella* and *Staph*. dextramer were used to sort positive T cells. Sorted T cells were divided into two different populations (b) (c) After one round of expansion, we observed an increase in dextramer positive cells. Percentages of gated cells are shown in the gates. (d) (e) After a second round of expansion cells were sorted based on staining intensity in dextramer high and intermediate populations. Bru high, Bru interm., Staph high and Staph interm. T cell populations were each expanded to generate a homogenous T cell line.

Dextramers are able to stain T cells which occur in low frequency. PBMC from donor BC 8 had a stimulation index >2 to methanol fractions from *S. aureus* and *B. melitensis*. We used PBMC from BC 8 to sort cells based on CD1b dextramer staining (fig 3a) and expanded them polyclonally. In our initial sort, approximately 1000 cells were sorted. After expansion, the amount of cells increased to approximately $5 * 10^5$. The frequency of dextramer positive

cells after expansion was raised over 100-fold. Expanded cells were resorted and expanded polyclonally twice to further increase the fraction of dextramer binding cells (Fig 3 b to e). Based on dextramer staining intensity, cell populations were divided into high and intermediate cell lines. Cells sorted with *Brucella* dextramer were named Bru high or Bru intermediate cells, according to the dextramer staining intensity, cells sorted with *Staphylococcus* dextramer were named Staph high or Staph intermediate cells,. After another round of expansion, we obtained cell lines that stained homogenously with antigen loaded dextramer (figure 4 a-d).

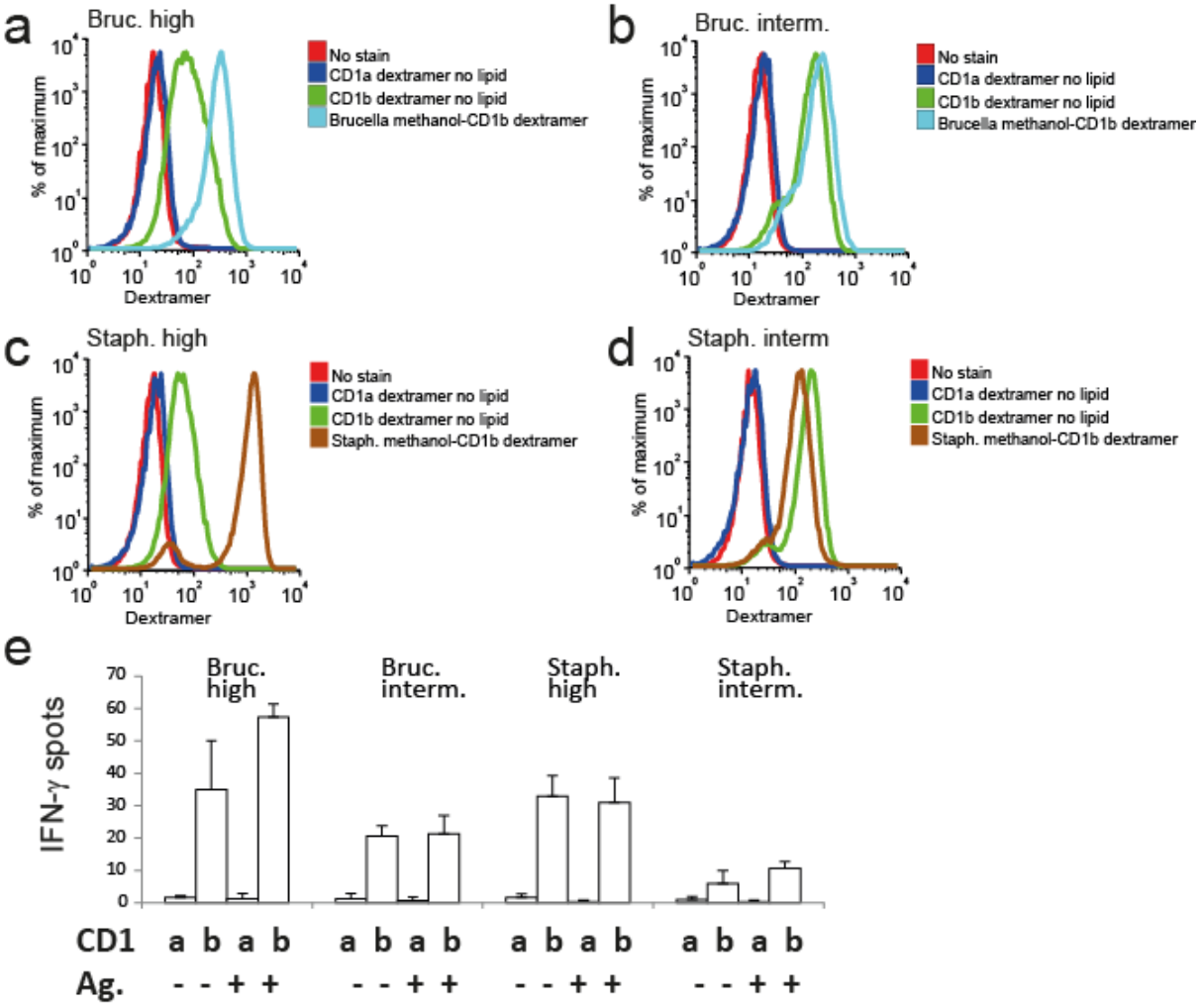

Figure 4: Antigen specificity of T cell lines

T cell lines were analysed for antigenspecificity using flow cytometry (a-d) and ELISPOT IFN- γ assay (e). (a) (c) Respective histograms of flow cytometry data. both Bruc. high and Staph. high T cell lines were antigen specific for antigen treated CD1b as compared to untreated CD1b. CD1a dextramers were included as a negative control. (b) (d) Bruc. interm. and Staph. interm. histograms failed to show marked differences between treated and untreated CD1b. (e) Results of ELISPOT IFN-γ assay using generated T cell lines. K562 cells transfected with CD1a and CD1b were used. CD1a was included as a negative control. Responses to untreated and treated CD1a were comparable for all T cell lines. Responses to untreated and treated CD1b showed little differences for both intermediate T cell lines and Staph. high T cell line. Bruc. high T cell line showed an increase in IFN-γ spots when CD1b was treated with antigen.

Antigen specific T-cell lines respond to untreated CD1b

To assess antigen specificity, all generated cell lines were analysed using FACS and ELISPOT. Bru high and Staph high cell lines appeared to be antigen specific in FACS staining, as staining intensity using an antigen loaded dextramer was higher compared to unloaded CD1b dextramers (Fig 4 a and c). Because of their antigen specificity in FACS, we expected that Bru high and Staph high cell lines would also be antigen specific in ELISPOT IFN-γ. We assessed whether our cell lines produce IFN- γ in response to CD1b transfected K562 cells treated with and without antigenic lipids. K562 cells are derived from a myelogenous leukemia cell line and lack MHC complexes. These cells can be transfected with CD1 proteins to assess antigen specificity. Because the T cells were selected for binding to CD1b, we used K562 cells transfected with CD1a as a negative control. Surprisingly, only the Bruc. high celline showed an increase in IFN- γ spots in response to antigen treated CD1b K562 cells as compared to untreated CD1b K562 cells. Other T cell lines did not show marked differences between antigen treated and untreated CD1b K562 cells. K562 cells transfected with CD1a generated no response at all (fig 4 e). From this we concluded that the Bru inter, Staph. high and Staph. interm. T cell lines are CD1b specific and we considered the possibility that CD1b presents an antigenic bacterial lipid that is shared or cross-reacts with a mammalian lipid that is present in K562 cells.

Bruc high and Staph high cell lines recognize phosphatidyl glycerol as an antigen.

Bacterial and mammalian cells have several shared phospholipids. We hypothesized that a shared phospholipid was the specific antigen causing the apparent discrepancy between FACS staining and ELISPOT IFN-γ in the Staph. high T cell line. Using dextramers, we tested a group of phospholipids, including phosphatidyl glycerol. Both Bru high and Staph high cell lines recognize CD1b treated with phosphatidyl glycerol (figure 5). Other phospholipids (data not shown) did not show marked differences in dextramer staining intensity as compared to untreated CD1b.

Our initial sort was performed using both *Brucella* and *Staphylococcus* dextramer (figure 1a) which were used seperately after one round of expansion to generate antigen specific T cell lines. The observation that both T cell lines recognize PG is possibly due to the same antigen being present in the methanol fraction of the lipid extracts of *S. aureus* and *B. melitensis*. The methanol fraction contains charged lipids, among which the phospholipid PG (figure 1) [38]. PG is a major phospholipid in nearly all bacteria, especially Gram positive bacteria. The amount of PG in bacteria depends on growth conditions, but can be as high as 90% [1].

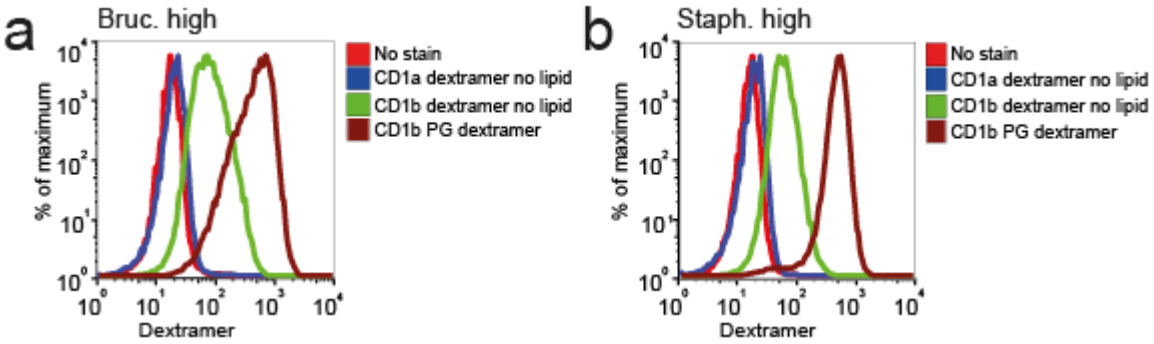

Figure 5: Recognition of phosphatidyl glycerol by Bruc. high and Staph. high T-cell lines.

T-cell lines were analysed for phosphatidyl glycerol (PG) specificity using flow cytometry. Respective histograms of flow cytometry data for Bruc. high (a) and Staph. high (b) T cell lines. Both T cell lines show increased staining intensity compared to negative controls (no stain and untreated CD1a) and to untreated CD1b.

Identifying the TCR using single cell PCR

A PCR based approach was chosen for sequencing of TCR We amplified the cDNA targeting all TRAV and TRBV gene segments in separate PCR reactions. Our initial results revealed that our T cell lines possibly had several TCRs which could be responsible for recognizing PG, as multiple α and β chains were visible in both T cell lines (figure 6). This was not unexpected since all T cell lines had not gone through T cell cloning at limiting dilution. Sequencing data from the PCR products showed several α and β chains, including some out of frame α chains. The presence of out of frame α chains is common in many mature lymphocytes. During the V(D)J recombination reaction, the random gain and loss of nucleotides, which is essential for antigen receptor gene diversification, can also lead to reading frame shifts and premature stop codons. These out-of-frame antigen-receptor alleles are actively transcribed in mature lymphocytes [28].

To determine the actual combination of α and β chains that forms the TCR in our T cell lines, we decided to run a single cell PCR. Single cells from our T cell lines were subjected to RNA isolation and cDNA was synthesized. The cDNA was amplified by PCR using the α and β chains identified in previous experiments. Figure 7 shows the results of the gel electrophoresis from our single cell PCR on one single cell. One α-β chain pair was unequivocally identified. The TCR consisted of TRAV 9-2 and TRBV 6-2 (figure 7).

α CHAINS

TRAV3

NUCL: TGT GCT GTG AGA GAC ATG AGG TAC AGC AGT GCT TCC AAG ATA ATC TTT GGA TCA GGG

AA: C A V R D M R Y S S A S K I I F G S G

TRAV5 (OUT OF FRAME)

NUCL: ACT TCT GTG CAG AGA GTG GAC AGC AGT GCT TCC AAG ATA ATC TTT GGA

AA: T S V Q R V D S S A S K I I F G

TRAV9-2

NUCL: TGT GCT CTG ACT CCC TCT GGG GGT TAC CAG AAA GTT ACC TTT GGA ACT GGA

AA: C A L T P S G G Y Q K V T F G T G

TRAV38-1/2

NUCL: TGT GCT TTC ATG AAG TCC TCC TCC TGG ACC AAA GCT GCA GGC AAC AAG CTA ACT TTT GGA GGA GGA

AA: C A F M K S S S W T K A A G N K L T F G G G

β CHAINS

TRBV3-1

NUCL: TGT GCC AGC AGA AGA CTA GCG GGA GAG GTT GAG CAG TTC TTC GGG CCA GGG

AA: C A S R R L A G E V E Q F F G P G

TRBV6-2

NUCL: TGT GCC AGC AGC ATG CCG GGA CTC AGG AGC TCC TAC GAG CAG TTC TTC GGG CCG GGC

AA: C A S S M P G L R S S Y E Q Y F G P G

Figure 6: Sequencing results PCR Bruc. high and Staph. high T cell lines

Sequencing results from Bruc. high and Staph. high T cell lines revealed multiple α and β chains. Nucleotide and amino acid sequence for TRAV 5 was out of frame with joining and constant α gene segment. NUCL: nucleotides, AA: amino acids

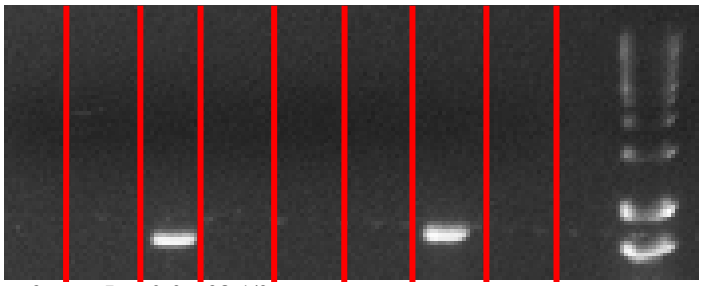

Figure 7: Results of electropheresis of single cell PCR of one single cell

Single cells were isolated from Bruc. high T cell line. RNA was isolated and cDNA was synthesized and amplified by PCR using α and β chains previously identified. Two PCR products were found, corresponding with TRAV 9-2 and TRBV 6-2.

Discussion

Here we have shown that lipids from bacteria other than mycobacteria can stimulate human T cells via CD1b. Unexpectedly, these T cells also recognize CD1b in the absence of a bacterial lipid. The immunodominant lipid for our T cell lines was phosphatidylglycerol, a lipid that is abundant in bacteria and present in low amounts in mammalian cells. In mammalian cells, PG is synthesized by mitochondria and functions as a precursor for cardiolipin, an important lipid of the inner mitochondrial membrane [20]. PG is also a minor phospolipid component of lung surfactant and can act as a potent regulator of innate immunity and respiratory viral infections [32]. In most bacteria, PG is one of the major anionic membrane lipids and is required for protein translocation across the membrane [30].

In the CD1 pathway, endogenous and exogenous lipids can be presented to T cells [3]. There are several mechanisms of exogenous lipid uptake by antigen presenting cells, including phagocytosis and via certain cell surface receptors (e.g. ApoE receptor). The exogenous lipids are predominantly loaded into CD1 molecules in late endosomal or lysosomal compartments. Once loaded with lipid antigens, CD1 molecules traffic back to the plasma membrane for recognition by a TCR [3]. The CD1 restricted lipid antigens identified thus far are either derived from mycobacteria, self-lipids or presented by the group 2 CD1 protein CD1d [3,5,19,25,27,37,40]. Among the lipids presented by CD1d are several common phospholipids, including PG, that are recognized by NKT cells [17]. However, whereas CD1d restricted NKT cells are considered as part of innate immunity, group 1 CD1 restricted T cells are part of adaptive immunity [3]. The presentation of PG by CD1b that we discovered and report here for the first time, provides new vantage points in the role of group 1 CD1 restricted T cells in adaptive immunity.

Recent work has discovered a germline encoded mycolyl reactive T cell recognizing CD1b loaded with mycobacterial lipids [42]. Although the present views still consider high diversity in group 1 CD1 reactive TCR, the GEM T cell was shown to be present in many unrelated donors. Possibly, interdonor TCR conservation in the CD1 repertoire might be more common [42]. Whether the PG-specific TCR identified in this study is conserved between unrelated donors remains to be determined and will be subject of further studies. However, if the identified TCR is conserved and present in unrelated donors, it might be used as a diagnostic tool in diseases associated with T cell reactivity against PG. Alternatively, it might be targeted by inteventional therapies.

We can imagine two normal physiological roles for PG-specific T cells that are beneficial for human health, and one pathogenic scenario.

1. PG functions as a proper bacterial antigen and PG-specific T cells have an anti-bacterial function. In this case the immunophenotype of the PG-specific T cells would include antibacterial, inflammatory mediators.

2. PG recognition functions to dampen the response against stressed or dying cells that have lost the integrity of their mitochondria, the only source of PG in a cell. In this case the immunophenotype of the T cells should be immunoregulatory.
3. Pathogenic: It is possible that bacterial infection breaks tolerance to the self antigen PG. Autoimmunity may ensue.

A cytokine profile can be used to gain insights in the role of PG-specific T cells and to assess its helper or effector functions. Although our PG-specific T cell line produces IFN-γ in ELISPOT, its complete cytokine profile remains to be determined. Cytokines such as interleukin-17 (IL-17), IL-13 and IL-2 would suggest helper functions, whereas IL-10 and TGF-β would suggest regulatory functions.

As suggested in possibility 3, the presence of a PG-specific T cell might be relevant in autoimmune diseases related to PG or during severe bacterial infections. In antiphospholipid syndrome (APS), anticardiolipin antibodies are associated with thrombosis in systemic lupus erythematosus. Sera from these patients also react against other phospholipids [33]. Possibly, these patients have an increase in PG-specific T cells which could be studied using PG-dextramers. Our work might also be relevant for patients suffering from severe bacterial infections, in which PG might be released from killed bacteria and presented via the CD1 pathway in antigen presenting cells. This might break tolerance to the self antigen PG and lead to clonal expansion of PG-specific T cells and possibly autoimmune diseases. Possible interventions in aberrant T cell reactivity against PG can be designed when the structure of the PG-specific TCR and its interaction with CD1b-PG has been solved by crystallography. A structure would give general insights in the molecular basis of the antigen specificity and the interaction of TCRα and TCRβ and this can help to identify stimulatory and inhibitory molecules, which could lead to the development of drugs that intervene with TCR recognition of PG. [42]

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APPENDIX I: Protocols

1. Culturing cp + dg cells

Method: (In biosafety cabinet)

1. Prepare CP/DG medium by mixing RPMI+glutamax with FCS and 5mL of PenStrep
2. Defrost CP + DG containing freezing vials by holding them midstream in lukewarm tap water. Keep doing so until nearly liquid (keep some ice inside).
3. Take 10ml of basis culture medium and suspend part of it in vial containing CP cells and refill all the contents back into the pipet
4. Empty the contents of the pipet in a centrifuge tube (mark tube with contents)
5. Repeat steps 2 and 3 for DG cells
6. Centrifuge both tubes for 10 minutes at 1200rpm
7. Pour the supernatant of both tubes in Quat
8. Take 10ml of culture medium and suspend it several times into the CP cells containing centrifuge tube. Make sure no more cells are attached to the bottom of the tube. Transfer all the contents back into the pipet.
9. Transfer all the contents in a 25 ml corning flask (mark flask with contents and date)
10. Repeat steps 7 and 8 for DG cells
11. Put the corning flasks in a cell incubator at 37^{o}C in an upright position
12. Check regularly if cells are still viable
13. When medium is changing color, resuspend a small volume (1:20) into a new corning flask with CP/DG medium and put it back into the incubator.

When starting culture with hybridoma's, use same procedure. For step 7, take 11ml of CP/DG culture medium and transfer 10mL into 30mL corning flask. Add 9mL CPDG medium to the centrifuge tube and transfer these 10 mL into a different 30mL corning flask. Mark down contents on flask!

For T cell cloning and polyclonal expansion, CP/DG cells have to be irradiated at 7500 rad, PBMC cells have to be irradiated at 5000 rad

2. T-cell medium (TCM)

Method: (in biosafety cabinet)

1. Open the bottle of RPMI and check for addition of HEPES
2. Add 5 ml(or 10ml) of HEPES to the RPMI
3. Add 3ml of AA (essential AA) and 3ml of NEAA (non essential AA)
4. Add 2ml of Glutamax
5. Add approximately 50ml of FCS, resuspend several times
6. Add 2 ml of 1M NaOH, the medium might get a little darker, this is to be expected
7. Add 5 ml of Penstrep
8. To check if medium is sterile add some medium in a 12-wells plate and leave in an incubator overnight.

Add IL-2 to TCM for usage in polyclonal wells (see protocol 6)

3. Cell isolation/ freezing protocol

Methods:

BEFORE STARTING PROTOCOL:

Look for mr Frosty's and check if they are filled with isopropanol, put them in fridge
Check if we have FCS in good condition (otherwise defrost from freezer)
Check if there is enough Il-15 medium

1. Remove sticker from buffycoat bag and mark material needed for future reference!
2. Spray buffycoat bag with 70% alcohol in biosafety cabinet
3. Fill a bucket with some Quat for disposable fluids
4. Open 50 ml centrifuge tubes (3 for every 50ml of buffy coat)
5. Divide the 50ml of buffycoat blood over 3 centrifuge tubes
6. Pour in PBS until centrifuge tube is filled to a maximum of 35ml
7. Mix the contents well by shaking gently (keep the cap clean)
8. Fill a 10ml pipet with Histopaque (completely full just under the filter)

9. Empty pipet with Histopaque in centrifuge tube. Keep tip of pipet just onder de border of histopaque and PBS/buffycoat mix
10. When air bubbles appear remove pipet from tube.
11. Repeat steps 5-9 for other centrifuge tubes

12. When all centrifuge tubes are filled, put them in the centrifuge, make sure it's well balanced
13. Spin tubes for 25min at 2800rpm, acc 2, brake 2

14. After centrifuge, remove serum from centrifuge tubes (into quat)
15. Use a pipet to remove the interphase from all tubes and put it in a new 50ml centrifuge tube
16. Divide the interphases over two 50ml tubes and add PBS to reach a volume of approximately 45ml
17. Put both tubes in the centrifuge and spin 10min at 850rpm, acc 4, brake 4

18. After spin, pour the supernatant of both tubes in two new 50ml centrifuge tubes
19. Directly after removing the supernatant, resuspend the pellet by ticking at the bottom of the tube (gently) to prevent clotting (if clotting occurs, only use supernatant and pipet into a new tube)
20. Wash pellet again with PBS and spin supernatant as well(repeat step 16)
21. Wash all tubes again with PBS for 10min at 850rpm (step 17)
22. After spin, remove supernatant and pool all pellets.

FOR ACQUIRING G4 CELLS CONTINUE PROTOCOL FOR ACQUIRING G4 CELLS

23. Use a pipet to empty all tubes into one centrifuge tube
24. Count the cells by extracting 10µL into an eppendorf cup and adding tryptan-blue (use amount related to expected amount of cells (usually $1 * 10^9$ per donor), between 1:1 (10 µL) and 1:20 (190 µL)).
25. Count 16 squares and calculate number of cells (Nr. Counted * 10.000 * dilution * total volume (50ml)

26. Determine amount of cells needed for sorting and REM mix and split up pooled pellets (suspended in TCM or GM-CSF TCM) in two tubes per donor.
    a. For example, 10 ml for REM and sort, the other 35 for freezing
27. Spin cells for 10min at 850rpm
28. Add FCS to cells to be frozen (250µL per vial - 200µL) and put on ice.

29. Cells to be stored overnight in incubator => add IL-15 medium
30. If not present, take 40ml of TCM and add 8 µL of IL-15 from stock containing 1 µg IL-15/ml (dilution 1:5000 0,2 ng IL-15/ml) or make double concentrated IL-15 TCM (0,4ngIL-15/ml)

31. Extract volume of IL-15 medium needed into centrifuge tube with pellet and suspend well (see step 24), then aliquot into corning flask (for big volumes use bigger flask). When using double concentrated IL-15 medium, suspend pellet with 50% TCM of total end volume and aliquot into corning flask and add the other 50% of IL-15 medium. Keep amount of cells in corning flask between 5 and $10 * 10^6$ cells/ml

32. Volume not needed for sorting and cloning is frozen at -140^0C
33. Per vial, suspend $20 * 10^6$ cells (max 15 eps per donor)
34. Spin centrifuge tube for 10min at 850rpm, acc 4, brake 4
35. After spin, pour supernatant into quat
36. See step 28, add 250 µL of pure Fetal Calf Serum per vial to be frozen. Note, after removing most of the supernatant, tube will still contain 200 µL of supernatant (for 6 tubes, add 1ml of pure FCS)
37. After adding FCS, resuspend several times with 1ml pipet (set volume at 600 µL)
38. After resuspending, put tube on ice.

39. Prepare DMSO medium at 20% by mixing with TCM/RPMI
40. Make sure DMSO medium and cells with FCS are ice cold before mixing (put on ice)

41. Print out labels for vials (Initials, Date, Sample, Non-Irr, amount of cells etc.)
42. Get plenty of vials and put labels on vials

43. When ice cold, mix DMSO medium with FCS/cells at an amount of 1:1
    a. Add DMSO medium by slowly dripping DMSO into tube with FCS/cells
    b. While adding DMSO, gently shake tube with FCS/cells
44. Quickly dispense contents into vials (400 µL per vial) and put in mr Frosty's.
45. Take mr Frosty's and put in -80^0C

1. Prepare 50mL of GM-CSF medium (get 50ml of TCM and add 150µL of GM-CSF
2. Get two special 645cc corning flasks and add 20mL of GM-CSF medium to each
3. Put corning flask into incubator
4. Prepare 50mL of GM-CSF + IL-4 medium (50ml of TCM, 150µL of GM-CSF and 100µL of IL-4)
5. Follow protocol for isolation of PBMC's
6. After step 22 (removal of supernatant), take the remaining 10mL of GM-CSF medium and add 5mL to each donor (usually two donors, if different make more or less medium)
7. Resuspend well in centrifuge tube
8. Get special 645cc (225cm^2 surface area) corning flasks and add contents of centrifuge tube into flask (mark flask with donor number)
9. Put corning flasks into incubator for half an hour
10. In the meanwhile, make sure TCM is at room temperature

11. After half an hour, get corning flasks from incubator and gently tilt the flask (adherent cells should not be resuspended)
12. After tilting several times, take a pipet and extract contents into 50ml centrifuge tube
13. Get TCM and add 7ml TCM and gently tilt again and remove into centrifuge tube. Repeat until most non-adherent cells have been removed from flask
14. Add 17.5mL of GM-CSF + IL-4 medium into corning flask (for each donor) and put into incubator for at least 2,5 days (harvest 3, 4th or 5th day
15. Count the cells that have been removed from flask into centrifuge tube
16. Ccontinue with cell isolation for sort (step 23) or cell freezing protocol (step 34)
17. Harvest cells on third, fourth or fifth day, preferably third day
18. For freezing G4 cells, collect them and wash with PBS, then follow normal cell freezing protocol (app. 1*10^6 per vial)

4. Preparing PBS 1% BSA medium

Take 500 ml of PBS (storage, make sure it's not 10X PBS!) and add 5g (1%) of BSA (from fridge).
Mix the contents. Get a filtering system and attach it to a vacuum system. Put the mixture into the filtering system and turn the vacuum system on. Try to set it at low speed so little foaming occurs. After filtering, take it to a biosafety cabinet and change the cap. Put the PBS-1%BSA medium in the fridge.

5. REM-mix double concentrated and FACS sort preparations

Materials/contents:	for 12,5 ml	(*5.6)	for 70 ml
CP/DG mix	5 * 10^6 cells	=>	28 * 10^6
PBMC	25 * 10^6 cells	=>	140 * 10^6
Okt3 (0.1 mg/ml)	7.5 µL	=>	42 µL
IL-2 (10µM)	2.5 µL	=>	14 µL
Culture medium T-cel	12.5 ml	=>	70 ml
Gentamycine if necessary	20µL	=>	112 µL

- If using PBMC from stock (frozen -140^{0}C) thaw 1 day in advance of REM-mix preparation. When thawed, dispense contents in centrifuge tube with 10ml of TCM. Spin for 10 min at 1200rpm. Remove supernatant, resuspend pellet and add IL-15 TCM before dispensing into small corning flask to leave o/n. (do not go over 5 * 10^6 cells per ml!)

Method for REM-mix preparation:

1. Count number of cells per ml for PBMC and CP/DG mix. Resuspend the contents of the corning flasks well and extract a small amount into a well. Extract 10 µL into new well and add 10 µL of tryptan blue. Mix well and extract 10 µL onto a counting plate. Count cells and calculate number of cells per ml.
2. Extract volume needed for 15*10^6 PBMC cells which will be used for FACS sorting into centrifuge tube
3. Spin tube for 7 minutes at 1200 rpm

4. Prepare (mark with expected contents) 4 FACS collection tubes by adding 1,5ml of IL-15 containing medium into each tube
5. Vortex contents by slowly increasing speed. Make sure entire FACS tube is coated but not the cap.
6. Put the FACS tubes in the incubator

FOR TRAV 1-2 PE antibody

7. Remove the supernatant and resuspend pellet (PBS 1%BSA)
8. Add 1:20 TRAV1-2 PE labeled antibody and put tube in fridge
9. Check regularly for sedimentation
10. After approximately 25 minutes add about 8ml of PBS 1% BSA
11. Spin tube for 7 minutes at 1200 rpm
12. Remove the supernatant and suspend pellet with PBS 1% BSA

13. Extract contents into a FACS filtertube
14. Change filtercap of FACS tube to normal cap
15. Put tube on ice until FACS sorting
16. Take FACS tubes from incubator and put on ice

FOR DEXTRAMERE PE AND FITC CD3 or CD4 (use 1:20) antibody

7. Count number of cells in corning flasks (per ml)
9. Extract volume for FACS sort, but leave enough cells for preparing REM-mix double concentrated (see protocol)
10. Spin extracted volume (two tubes for each donor (e.g. Staph BC 3 en Staph BC 4, Bruc BC 3, Bruc BC 4) at 1200 rpm for 7 min, acc 7 br 7
11. Wash with PBS 1% BSA and spin again with same speed etc.
12. Remove supernatant and make sure no cells are wasted
13. Add 20µL of dex-PE-loaded with CD1b-antigen (staph, bruc) in each tube (1 per donor per lipid antigen) and put in dark cabinet for 20 minutes
14. After 20 minutes, add 20µL of CD3 FITC to each tube and put in fridge for approximately 30 minutes
15. In the meanwhile, prepare plenty (1 for each sort + negative controls) of FACS BD Falcon 5ml polypropylene tubes by adding 1mL of IL-15 medium
16. Vortex so wall of tube is coated
17. Put tubes in the incubator
18. Get FACS filtertubes
19. When DEX-PE + CD3 FITC + antigen lipids are ready, wash them with PBS 1% BSA (app. 4ml per tube) and spin at 1200rpm for 7 minutes acc+br 7
20. After spin, remove supernatant and resuspend cells in app. 1ml of PBS 1% BSA (the more cells, the more PBS 1% BSA to suspend with)
21. Get FACS filtertubes and extract suspended cells through filter
22. Wash centrifuge tubes with PBS 1% BSA (small amount) and extract again through filter
23. Change filtercap to normal cap and put tubes on ice until sorting starts

23. FACS sorting using electronic gates and sort gates

24. (After sorting, do a rerun for control)
25. Mark number of sorted events per tube.
26. Put sorted cells on ice or in incubator with cap loosely on top

27. For the REM-mix, the CP/DG mix and PBMC will have to be irradiated at 7500 rad and 5000 rad respectively
28. Extract volume needed for $14*10^6$ CP cells from CP flask (in incubator) into a centrifuge tube and do the same for DG (into the same centrifuge tube).
29. Extract volume needed for $70*10^6$ PBMC from 1 donor into a centrifuge tube and use a new tube for different donors. (use smallest centrifuge tube possible)
30. Take tubes in special transport can and take to UMC for irradiation.
31. After irradiation, spin the tubes for 7 minutes at 1200 rpm
32. Remove the supernatant and resuspend in TCM. Amount can be calculated from basic double concentrated REM-mix (see above)
33. Divide the CP/DG mix by extracting it into the tube with PBMC's (usually 2 donors)
34. Add okt3 (amounts calculated using basic double concentrated REM-mix)
35. Add IL-2 the day after cells have been plated out (suspended in TCM)

36. Mark plates with planned contents (number of T-cells per well, donor, date and intitials)
37. Fill the outer ring of the plate with 190 µL of MiliQ (sterile water)
38. Transfer 6 ml of REM-mix into 96wells plate. In total only 60 wells will be filled with 0.1 ml of REM-mix per well. When using 2 donors, PBMC from one donor will be suspended into a well with T-cells for the other donor!!!!
39. Fill 9 more 96 wells plates with REM-mix.
40. Put the plates into the incubator

When using Dasatinib:

PREPARE DASATINIB FROM STOCK (1mM) => 1µL in 10mL PBS = 100nM

1. Spin cells for 7min at 850rpm
2. Wash pellet with PBS (dispense into epps) (app. 500µL)
3. Resuspend pellet in 100µL PBS
4. Add 100µL 10µM Dasa (stock = 100mM, 1µL in 10ml)
5. Incubate for 20minutes at 37^0C
6. Spin (no wash) 30 sec at 1900 rpm
7. Remove supernatant, leave 20-30µL in epp
8. Resuspend pellet and add app. 1µL Dextramere (mock CD1b, AG-CD1b

9. Incubate for 20minutes at 37^0C
10. Add 1µL of αCD3 and incubate for 30minutes at 4^0C
11. Have enough FACS tubes and get ice
12. Wash with 500µL cold PBS1%BSA Azide (30 sec, 1900rpm)
13. Remove supernatant and dispense in 230µL PBS1%BSA Azide and aliquot into FACS polystyrene tubes (glass sound)

6. Transferring T-cells for cloning

Cells have been sorted based on PE en FSC. Per donor, 5 plates will be used to clone T-cells, 2 plates will contain 1 cell/well, 3 plates will contain 3 cells/well, therefor, 2 different dilutions per donor will be made to transfer T-cells onto 96wells plate containing REM.

For the 1 cell/well plates 120 cells will have to be suspended in 12 ml medium for each donor.
For the 3 cell/well plates 540 cells will have to be suspended in 18 ml medium for each donor.

Thus, for donor A, we will have 12ml of mixture containing 120 cells and 18 ml of mixture containing 540 cells. The same goes for donor B. These mixtures will be transferred into the appropriate wells containing the REM-mix at 0.1 ml per well.

After sorting, number of cells sorted into 1,5ml of medium is known. Calculate number of cells needed to fill all plates with number of cells per well. Before extracting, make sure the contents of the FACS tubes are suspended well so no cells are attached to the inner surface of the tube. Extract volume needed for 1 cell/well plate and 3 cells/well plate for each donor into a new tube.

Another 96 wells plate can be used for polyclonal T-cell cloning. The plate is prepared in the same manner as is used for 1cell/well (or 3 cells/well). 100 µL of sorted cells (from FACS tube) is extracted into each well.

After all wells are filled, seal the plates with foil (put marked napkin on top of lid) and put all plates into the incubator for at least 2 weeks. Check regularly for contamination and after two weeks for results.

For polyclonal cells, check if cells are viable and growing after +/- 5 days
Remove 50 µL from wells without touching wells, don't hit the bottom! Make sure all pipets are filled when extracting. After extraction, add 100 µL of ***IL-2 medium*** to all wells from which 50 µL were extracted. When finished, put wells back into incubator.

Five days after adding new IL-2 TCM, get new 96 wells plate, mark contents on lid and tissue. Fill the outer ring with sterile MiliQ and the inner 60 wells with 100µL of IL-2 TCM. Resuspend contents from old 96 wells plate several times, then extract 125µL per well into new 96 wells plate. Polyclonal cells are now diluted so expansion can continue.

If lid or foil gets wet, dry very thoroughly or get new lid and foil!

For plates with 1 or 3 cells/well the cycle will take app. 17-21 days. After two weeks, check which wells are growing. Plate out growing clones into new plate (3 rows per plate). After 17 days, RNA can be isolated from growing clone0073

7. Lipid extraction

Method

1. After culturing in liquid medium (OD should be high) , divide it over two plastic centrifuge bottles
2. Spin bottles for 30min at max speed (5100rpm), use scale to balance out opposite bottles
3. After spin, remove supernatant in big erlenmeyers (with quat)
4. Fill erlenmeyers with water until nearly full, leave for at least 30min and wash down
5. Dissolve pellets in centrifuge bottles using 10ml PBS and aliquot into 50ml centrifuge tubes
6. Wash bottles again with 10ml PBS (wash one bottle, then use these 10ml to wash other bottle)
7. Spin centrifuge tube at max speed for 30min
8. After spin, remove supernatant in Erlenmeyer and start lipid extraction

9. Mark origin of lipids on paper (Listeria monocytogenes LM 104035 AJS 07/09/13)
10. Clean glass centrifuge tubes with acetone and methanol
11. **Clean distilling bottle with a double rinse with 1:1 Chl:meth, then chloroform, acetone and finish with 0.05 M HCl???**
12. Add 20ml of methanol to pellet and mix with vortex and by shaking
13. Add 20ml of methanol again and mix again
14. Divide contents of centrifuge tube over 5 or 6 centrifuge tubes (pipet)

15. Add 4ml of chloroform to every centrifuge tube (amount should be methanol:chloroform 2:1)
16. Mix contents well and put the tubes in a rotator at slow speed for at least an hour
17. After rotating, spin centrifuge tubes for 15 minutes at 2000rpm
18. Pipet supernatant into glass bottle located in cabinet (mark bottle with contents)
19. If pellet does not move when pouring, other centrifuge tubes can be poured into bottle, otherwise use pipet

20. Mix 40mL of chloroform with 20 mL of methanol (2:1)
21. Pipet a small amount (app. 2mL) into centrifuge tubes containing pellets\
22. Mix and vortex well until pellet is resuspended
23. Add another 7.5 mL of C:M 2:1 and mix/vortex again
24. Put centrifuge tubes in rotator for at least an hour at slow speed
25. After rotating, spin centrifuge tubes for 15 minutes at 2000rpm
26. Repeat steps 9 and 10. Leave the remaining pellet to dry without caps (waste)
27. All the supernatant collected in this way is distilled in a distilling bottle
28. Distill lipids by turning on vacuum, put plenty of alcohol (….%) in cooling and set temp at 4oC and turn on. Get ice to cool the waste collection bottle…
29. Apply distilling bottle under distilling apparatus and turn on rotation. When rotating get a small bucket of hot water to speed up distilling.
30. If water was present in pellet, use acetonitrille to remove the water (binds water in a 3:1 ratio Water: Acetonitrille)
31. Continue distilling until ….. is dry

8. Preparing TLC plate

Method:

1. Prepare mixture of 1:1 chloroform:acetone
2. Suspend all lipids (not already suspended) in C:A 1:1
3. Try to get as much as possible in solution in glass centrifuge tube
4. Pellet should sink to bottom, use supernatant for running TLC

5. Get a big enough TLC plate for the number of samples
6. Razor the sides (not top and bottom!) gently
7. Mark the direction of the TLC on the plate with pencil
8. Mark sample numbers by putting dots just above the bottom of the plate on one line (app. 1,5 cm between each dot)
9. Clean the TLC box with Chl:met:water solution by tilting
10. Make sure there is plenty of paper present in TLC ….
11. Prepare 60:30:5 vv chloroform:methanol:water (hydrophile) or 60:16:2 (hydrophobic)
12. Add this to the TLC box (just a small layer, level should not suspend the lipids)
13. Wet the paper inside the TLC box by tilting in different ways
14. Run a test TLC by putting the plate in the TLC …..
15. After app. one hour test should be finished (app. 1,5 cm below top of plate)
16. Remove the TLC plate and leave to dry

17. Get reference lipids from stock and make sure concentrations are all below 10 mg/mL, if not dilute from stock in chloroform:acetone
18. Mark concentrations of stock reference on paper and mark down which lipid corresponds with which sample number on plate
19. Turn on nitrogen blower (for drying)
20. Clean 20 µL pipet with chloroform:methanol by refilling 3 times and emptying in waste
21. For sample 1, fill pipet with 20 µL and carefully empty on TLC between dots in a small band (as small as possible). Dry regularlySample should run between dot 1 and dot 2.
22. Repeat step 20+21 for sample 2, 3, 4 etc.
23. Put the TLC plate in the TLC ……
24. Turn on oven at 150-1600C
25. Wait until solution is app. 2.5 cm below top of plate
26. Remove TLC plate and leave to dry
27. If not enough, prepare new CupricAcetate-Phosphoric Acid-MiliQ solution (3% g/v, 8% v/v, rest is miliQ). For 150 mL add 4,5 g CuAc, 16mL of 85% H3PO4, 134mL of MiliQ.
28. Spray plate after it is dry with CuAc H3PO4MiliQ. Make sure plate is evenly wet (no drops) and leave to dry.
29. After drying, put plate in oven at 150-1600C
30. Wait until lipids turn black and plate starts turning brownish (5-10 minutes)
31. Read out plate
32. For next TLC plate, adjust amounts so estimates are more accurate!

9. Lipid purification (Brucella m. and Staphylococcus a.)

Method:

1. Calculate amount of lipid needed and extract volume needed from stock into glass tubes
2. Dry in glass tubes by blowing nitrogen-air into tube
3. When dry, add ½ mL of chloroform and use sonicator to make sure all lipid is suspended, put cap on tube and seal with parafilm before sonicating

4. Get *Statief* and hang in the Supelco LC silica , put a waste jar under the drip
5. Fill with methanol and wait until empty (don't wait until dry!!!)
6. Refill again with methanol and wait
7. Fill with mixture of acetone and methanol (and wait) 50:50
8. Fill with acetone…refill…..
9. Fill with chloroform:acetone 50:50
10. Fill with chloroform
11. Keep refilling with chloroform until start of purification (if running more purifications, keep filling one tube while purifying with the other (steps 12-16)

12. Get plenty of supelco 2mL amber vials and print out labels for each eluaat: chloroform (first), acetone (second) and methanol (third) (mark date and lipid origin too)
13. Keep Supelco LC silica tube over the waste jar and empty the resuspended lipids (step 3) into the tube
14. When dripping stops, move the tube over the chloroform eluaat amber vial and add 1mL of chloroform and wait until dripping stops
15. Move tube over the acetone amber vial and add 1mL of acetone to tube and wait…
16. Move tube over the methanol amber vial and add 1mL of methanol to tube and wait…
17. Repeat steps 13 to 16 for other lipids to be purified
18. Put amber vials into freezer when finished

10. Loading CD1 monomers

Method:

1. Get a bottle of MiliQ (1 L)and pour out 200mL
2. Add 50mM $Na_3Citraat.2H_2O$ (for 1L app. 14,7 g)
3. Calibrate pH meter (follow instructions present)
4. Use HCl (17%) to lower pH to 4.5
5. Fill the bottle to 1L with MiliQ
6. Add 1% Azide (10mL for 1L)
7. Buffer is ready for use (put in fridge)

8. Get CHAPS
9. Weigh small amount on accurate scale (app. 1.2g)
10. Calculate amount of Buffer to be added for 0,5% CHAPS (for 50mg it should be 10mL)

11. Turn on heat baths (one at 56^0C and one at 37^0C)
12. Get glass FACS like tubes
13. Extract volume needed for app. 32µg of lipid and put into FACS-like tubes for every sample (*Brucella*, GMM => 32µg, Staph 16µg, control empty)
14. Dry tubes using nitrogen-air blower
15. When all tubes are dry, add 90µL (45µL for Staph, adjust amounts to make concentration similar) of buffer to tubes
16. SEAL TUBES WITH PARAFILM
17. Sonicate in hot water (at least 37^0C) for 10 minutes (use a floater)
18. When sonication is finished, put tubes in heat bath 56^0C (use a floater) for at least one hour
19. Sonicate again for 10 minutes in hot water (at least 37^0C)
20. Use a small basket to transport tubes in hot water (from bath)
21. Add CD1 (a,b,c) to tubes (in biosafety cabinet) and put in heat bath 37^0C for several hours (one hour is minimum) 10µL of 2mg/ml stock

22. Prepare 1M Tris at pH 8.5 using the pH meter. Add 1% Azide and put in fridge when ready using.
23. Mix 90µL of CHAPS+buffer with app. 9µL of TRIS and check if pH is at 7-7.5 using pH paper. Adjust amount of TRIS to be added to get pH at desired level
    a. FOR TRIS PREPARED ON 27-09-13 => 90µL CHAPS + 9µL TRIS

24. After CD1 (a,b,c)-lipid incubation is ready get enough eppendorf cups (1.5mL) and mark with contents to be added
25. Get tubes with CD1 (a,b,c) and put them in biosafety cabinet

26. Empty tubes in eppendorf cups
27. Get Tris and add calculated amounts to tubes
28. Get PBS, put 10mL into centrifuge tube and add 1% azide to tube
29. Extract 80µL from PBS-azide solution and wash sides of tubes, resuspend several times and extract all from tube into eppendorf cups. Adjust amounts to keep concentration the same (so for Staph use only 40µL
30. Close epps and put in fridge in small basket

31. Get dextramere backbone and to loaded CD1b monomers (for every sample)
    a. for PE: 1dex:4CD1b
    b. for FITC 3dex:16mono
32. Put in dark cabinet for half an hour
33. When ready, put epps in fridge

11. Analysing PBMC's/NAC using FACS

Method:

1. Hybridoma's (clone 18 and 42) have been cultured 3 days before usage and continued the day before (clone 18 reacts to mycolic acid, clone 42 to GMM)
2. Count number of cells, use culture containing app. 50.000 cells/mL
3. Get 10 eppendorf cups and mark 1 to 10
    a. 1) clone 18 empty
    b. 2) clone 18 TRAV1-2
    c. 3) clone 18 CD1b unloaded
    d. 4) clone 18 CD1b-GMM
    e. 5) clone 42 empty
    f. 6) clone 42 TRAV1-2
    g. 7) clone 42 CD1b unloaded
    h. 8) clone 42 CD1b-GMM
    i. 9) clone 42 CD1b-emby CONTROL
    j. 10) clone 42 CD1b-GMM CONTROL
4. Mix 4µL of PE-dextrameres with 13,2µL CD1b monomers and store for one hour at room temperature in dark cabinet
5. Prepare PBS 1% BSA and add 1% azide (500ml PBS, 5g BSA, 5ml azide
6. Extract 1ml of hybridoma culture into marked eppendorf cups
7. Spin cups for 30 seconds at 2000rpm, keep hinge at top in centrifuge
8. Remove supernatant using a small-tipped pasteurs pipette (in storage room or lab), leave small amount (20µL) in cups, don't hit the bottom, keep tip of pipet at top of fluid
9. Wash cells with 0,5ml of PBS 1% BSA 0.01% Azide using a 5mL pipet (add 0,5ml to each cup)
10. Spin cups for 30 sec at 2000rpm
11. Remove supernatant again using a small-tipped pasteurs pipet
12. Resuspend contents using a 20µL pipet (repeat 3 times)
13. Add 1µL staining (TRAV1-2, dextrameres) using a 0,2 - 2µL pipet
14. Incubate eppendorf cups at room temperature for 30min in a dark cabinet
15. If necessary, add other antibody's and incubate
16. After 30min, put eppendorf cups in fridge.

17. Get 10 FACS tubes
18. Add 0,5mL PBS and spin again for 30 sec at 2000rpm
19. Remove supernatant (leave app. 20µL in cup)
20. Add 100-150µL PBS1%BSA to cups and extract contents into small FACS tubes (plastic)
21. Cover FACS tubes and put in fridge until start of FACS

12. RNA isolation of clones

General:

Work as aseptic as possible, frequently change gloves, keep tubes closed if possible, keep isolated RNA on ice when aliquots are pipetted for downstream applications

Use disposable polypropylene tubes, non disposable plasticware + glassware should be made RNase free. Solutions used should be treated with 0.1% DEPC

Method:

1. Prepare buffer RLT by adding 10µL β-mercaptoethanol per 1ml buffer RLT (stable for 1 month after preparation.
2. Prepare buffer RPE if not present (marked with X on cap). Add 4 volumes of ethanol (96-100%), see information on bottle.

3. Number all wells 101, 102, 103etc for BC1 and 201, 202, 203 for BC2
4. Start with 4-6 wells
5. Mark eps with contents to be added
6. Remove 25% of volume in 96wells plate (25% of 225µL) and extract into eps
7. Add 400µL of PBS
8. Spin cells for 30sec at 2500rom
9. Carefully remove all supernatant by aspiration (pipet)
10. Resuspend pelleted cells and add 350µL of Buffer RLT per epp
11. Suspend well by vortex or pipet
12. Get a 20 gauge needle (0,9mm diameter) and fit to RNase free syringe. Pass the lysate at least 5 times through the needle (homogenization)
13. Add 350µL of 70% ethanol to the homogenized lysate and suspend
14. Get an RNeasy column placed in a 2ml collection tube, resuspend the sample and extract (700µL) to the column
15. Close the tube gently and centrifuge for 15s at >10.000rpm. Discard the flowthrough in the collection tubes
16. Add 700µL of Buffer RW1 to the RNeasy column, close the tube gently and centrifuge again for 15sec at >10.000rpm. Discard the flowthrough
17. Pipet 500µL Buffer RPE onto the column, close gently and spin for 15s at > 10.000rpm. Discard the flowthrough, reuse collection tube.
18. Repeat step 17
19. Spin for ***2 min*** at >10.000rpm to dry RNeasy silica-gel membrane. Directly start elution (step 17) (get enough epps ready for next steps, double the nr of samples)
20. To elute, transfer the RNeasy column to a new 1.5ml epp with cap cut off and pipet ***22µL*** of RNase-free water directly onto the RNeasy silica gel membrane, wait 1 min and then close tube gently and spin for ***1 min*** at >10.000rpm
21. Transfer the flowthrough in new epps, marked with sample nr and RNA
22. Put tubes on ice when elution is finished!
23. Continue until RNA from all wells has been isolated

13. cDNA synthese

Method:

1. Get iScript cDNA synthesis kit from freezer (in central freezer/cooling room)
2. Get 2µL of 5x iScript reaction mix, 0.5µL of iScript reverse transcriptase and 7.5µL of RNA template (from RNA isolation protocol)
3. Mix reaction mix, reverse transcriptase and RNA and incubate for 5 minutes at 25^0C, 30 minutes at 42^0C and 5 minutes at 85^0C (use right PCR machine, user Rachel, protocol cDNA iscript)
4. When incubation is ready, store cDNA at 4^0C.

14. PCR protocol and gel electrophoresis

Method:

1. Defrost Gotaq buffer, $MgCl_2$,, Primers and gNTP's
2. Get plenty of ice
3. Get cDNA samples (or PCR product => dilute 1:50 or 1:100)
4. When mixture components have defrosted, put on ice
5. Prepare mixture for 25µL samples (count 1 or 2 extra samples when preparing mixture). Mixture for 25µL consists of:
    a. 5µL Gotaq buffer
    b. 2.5µL $MgCl_2$
    c. 1.25µL dNTP's
    d. 0.625µL primer For
    e. 0.625µL primer Rev
    f. 0.125µL Gotaq polymerase
    g. 12.875µL H_2O
    h. 2µL of cDNA sample (or diluted PCR product)
6. When all samples are ready and marked put them in PCR machine, program normal (user Ildiko)
7. Program should run for approximately 2.5 hours
8. In the meanwhine prepare gel
9. Start with using tape to surround the edges of the box
10. Depending on the number of samples, prepare agarose gel by mixing 1.5% agarose powder with 10x TBE buffer and mix well
11. Microwave the mixture, keep doing so until solution is clear
12. When clear, cool down the agarose gel (viscosity should go up) and add 1 drop of ethylium bromide (TOXIC)
13. Mix well and pour gel into box
14. Put in the KAM for the holes in the gel (big enough slots for sample)

15. Wait for the gel to cool down and solidify
16. Remove KAM and tape and fill electrophoresis box with 10x TBE buffer to a level just over the gel
17. Prepare loading buffer, TBE buffer + 10% glycerol + blue color
18. Get gene ruler (1kb usually)
19. Get 96 wells plate and mix PCR samples with loading buffer (1:1) in wells 7µL of sample + 7 µL of loading buffer
20. Pipet 14µL into the holes (made by the KAM)
21. Turn on voltage at 100V
22. Wait until bands are clearly visible
23. Photograph results using UV-light

24. If results of gel are good, then use DNA concentration kit to isolate DNA for sequencing
25. Prepare order at Baseclear.com, (103Rhijn) quick shot remix, use data from earlier orders.
26. Get sample numbers and put them in the order so you know which number is assigned to which sample
27. Use Sanger Quickshot method, add 50µL binding buffer to 18µL of PCR product
28. Follow protocol for DNA concentration and collect in 30µl MiliQ
29. For sequencing, send DNA to baseclear in basebox
    a. Measure DNA concentration with spectrophotometer
    b. Combine volume for 40ng PCR product + 7.5picomole primer (F or Rev) and add MiliQ to a total of 30µL
30. Send samples in baseclearbox in envelope before 15:00
31. When results are in, analyse them using translate tool at expasy.org and chromas
    a. Look at right frame: SASYL CAV FGXF or WGXG
    b. Copy frame in word and add nucleotides
    c. Mark CDR-3 and compare to other sequences

15. ELISPOT

Method:
Do not let the plate stand dry, always make sure liquid is covering wells. Make solutions to use before flicking and flick and fill directly afterwards.

Write layout on paper before starting ELISPOT…use for future reference and put in labjournal
Prepare 35% Ethanol in H20 (sterile)
Prepare PBS by adding H20 to 10xPBS

Day 1 (coating)

1. Prewet Elipost plate (Millipore S2EM004M99) with 40 ul 35% ethanol in H2O (sterile).
2. Flick and wash 2x with sterile PBS using multi channel (180µL washes)
3. Coat with 2.5 ug/ml coating antibody in PBS. Use 100 ul/well.
    a. For IFNg this is 25 ul of mAB 1 Dik in 10 ml PBS
    b. For IL-2 this is 50 ul of IL-2-I/249 in 10 ml PBS
4. Leave overnight at 4 C (store in plastic (original wrapping)+ foil around)

Day 2 (the cellular assay)

1. Flick and wash 2x with PBS using multi channel
2. Block with 150 ul T cell medium/well for 1.5 hours or more in incubator
3. Total volume of cells is going to be 150 ul/well, so 50 APC/50 ag/50 T cells is recommended
4. Add 20,000 K562 cells or 40,000 G4 per well
5. Add 50 ul of 15 ug/ml lipid. The final concentration is going to be 5 ug/ml. In elispot toxicity is not a big issue, and dose/response curves are usually not very good.
6. Add T cells. I use 500-1000 cells/well of a healthy clone because you expect 100% of the cells to be antigen specific. If you test non adherent cells from a patient with a very small population of responsive cells you can go up till 200,000 cells/well. In case of JRT3 transfectants, do not forget to use 10 ng/ml PMA, which I add to the JRT3 in 3x concentrated form.

(T-cells from donor (fresh or defrost on day 1) G4 from same donor (fresh or from defrosting) lipids redissolve in ½ ml of 15µg/ml (7.5µg)

Day 3 (develop the plate)

1. Make fresh PBS/tween. I poor some tween in a squeeze bottle and add PBS and mix well. (PBS from 10X PBS + 9x demiwater)
2. Work at a sink: flick plate, fill the plate with PBS/tween and flick.

3. Take the bottom of the plate and fill the plate with cold tap water (not demi) and flick; also rinse the back of the plate. Do this 3 times
4. Wash with PBS/tween the wells, the back of the plate, and the bottom. Do this 3 times and put the plate back together. Click well. Make sure plate bottom is dry!
5. Add 100 ul of 0.4 ug/ml biotinylated antibody in PBS/0.5% FCS (50 ul FCS in 10 ml PBS)
6. For IFNg this is 4 ul of 7-B6-1 bio per 10 ml
7. For IL-2 this is 8 ul of IL-2-II bio per 10 ml
8. Incubate 2 hours at room temperature.
9. Wash plate, back, and bottom 3x with PBS/tween
10. Make Extravidin ALP 1:1000 in 10 ml PBS//0.5%FCS. Add 100 ul /well. CS box in 4 C refrigerator. Incubate for 1 hour at room temperature.
11. Wash plate, back, and bottom 5x with PBS/tween and 3x with PBS (made from 10X PBS)
12. Dissolve (sonicate) one tablet of BCIP/NBT (located in freezer 15) substrate in 10 ml milliQ. Box in -20. Do not touch anything with your hands. Add 100 ul/well
13. Incubate in dark till you see spots or grey/brown background. It will lighten up when you dry it, so go for dark.
14. Flick and wash with tap water (not demi).
15. Dry the plate and count on the 4th floor of the Jimmy Fund building, 1st lab on the left.

APPENDIX II: TCR primer sequences

CTGGATGCAGACACAAAGCAAAGC	TRAV10
GCAACATGATGGCGGAGCACCCAC	TRAV1-1
GCAACATGCTGGCGAAGCACCCAC	TRAV1-2
GGAGCCACTGTCGCTTTCAACTGTAC	TRAV12-1
CAGTGTTCCAGAGGGAGCCATTG	TRAV12-2/3
GGAGAGAATGTGGAGCAGCATCC	TRAV13-1
CATTCGAGCTTTATTTATGTACTTGTGG	TRAV13-2
TCCGCCAACCTTGTCATCTCCGCT	TRAV14/DV4
CCAGTACTCCAGACAACGCCTCCA	TRAV16
CCGGGCAGCAGACACTGCTTCTTA	TRAV17
CCAGGAGACGGACAGCAGAGG	TRAV18
TCGTCGGAACTCTTTTGATGAGCA	TRAV19
GCAGAGTCCCGAGGCCCTGAG	TRAV20
TGCCTCGCTGGATAAATCATCAGG	TRAV21
CGCTGCTCATCCTCCAGGTGCGGG	TRAV2
TTCATCAAAACCCTTGGGGACAGC	TRAV22
TGGGAAAGGCCCTGCATTATTGAT	TRAV23/DV6
CAGCACCAATTTCACCTGCAGCTT	TRAV24
GATACAGTTAGTGAAGAGTGGAGAAGTG	TRAV25
GGCTGGTGGCAAGAGTAACTG	TRAV26-1
TTGGTATCGACAGCTTCCCTCCCA	TRAV26-2
CAGCTGCTGGAGCAGAGCC	TRAV27
AGCAAAATTCACCATCCCTGAGCG	TRAV29/DV5
TCAACGTTGCTGAAGGGAATCCTC	TRAV3
GATATTACTGAAGGGTGGAGAACAGAAG	TRAV30
AGCCCAGCCATGCAGGCATCTAC	TRAV34
CAGCTGAATCAGAGTCCTCAATCT	TRAV35
CTCTGGTTGTCCACGAGGGAGAC	TRAV36/DV7
CCCAGCAGGCAGATGATTCTCGTT	TRAV38-1/2
AAGCCCGTCTCAGCACCCTCC	TRAV39
TCCCTGTTTATCCCTGCCGACAGA	TRAV4
CCAGCAGCAATTCAGTCAAGCAGAC	TRAV40
ACACTGGCTGCAACAGCATCCAGG	TRAV41
TTGCAGACACCCAGACTGGGGACT	TRAV5
CGGCCACCCTGACCTGCAACTA	TRAV6
GTGGAGCACAGCCCTCATTTTCTGG	TRAV7
ATGCTCCTGTTGCTCATACCAGTGC	TRAV8-1
GGCATCAACGGTTTTGAGGCTGAA	TRAV8-2/4/6*02
GTCTCTGAAGGAGCCTCACTGG	TRAV8-3
CGGTTTTGAGGCTGAATTTAACAAGAGTC	TRAV8-6*01*02
GAGCGAAACCTCCTTCTACCTGAG	TRAV8-7
CCTGATTGTGAACTGCTCCTATGAAACC	TRAV9-1
CCTGAAAGCCACGAAGGCTGATGA	TRAV9-2
CTGGGACATGGGCTGAGGCTG	TRBV10-1
TGTCACCAGACTGAGAACCACCGC	TRBV10-3
GGATCGATTTTCTGCAGAGAGGCTC	TRBV11-2/3/4
CCATGATGCGGGGACTGGAGTTGC	TRBV12-3/4
GCTCCTCTAGATGATTCGGGGATGC	TRBV12-5
GCAGGGTCCAGGTCAGGACCCCCA	TRBV13
GCCTGCAGAACTGGAGGATTCTGG	TRBV14
CCCAGTTTGGAAAGCCAGTGACCC	TRBV15
TGAAAATGTCTTTGATGAAACAG	TRBV16
GTTGATTCAGGGATGCCCAAGGAACG	TRBV17

CTGCTGAATTTCCCAAAGAGGGCC	TRBV18
TCCTCTCACTGTGACATCGGCCCA	TRBV19
AAGTGATCTTGCGCTGTGTCCCCA	TRBV2
GGCCACATACGAGCAAGGCGTCGA	TRBV20-1*01*02
CCACGGAGTCAGGGGACACAGCAC	TRBV21-1
TCTCAATGCCCCAAGAACGCACCC	TRBV23-1
CAGGCACAGGCTAAATTCTCCCTG	TRBV24-1
TGCCAGGCCCTCACATACCTCTCA	TRBV25-1
GGGCTGGGCTTAAGGCAGATCTAC	TRBV27
CGCTTCTCCCTGATTCTGGAGTCC	TRBV28
TTCCCATCAGCCGCCCAAACCTAA	TRBV29-1
TGCCCCAGAATCTCTCAGCCTCCA	TRBV30
TTCCCTGGAGCTTGGTGACTCTGC	TRBV3-1
CACCTGAATGCCCCAACAGCTCTC	TRBV4-1/2/3
CTGATCAAAACGAGAGGACAGCAAGTGA	TRBV5-1/3/4/5/6
AGCTCTGAGCTGAATGTGAACGCC	TRBV5-4/6/7/8
GGCCTTTTCTCTCCTGTGGGCA	TRBV6-1/2/3/4
TGAATGCTGGTGTCACTCAGACCCC	TRBV6-5/1/2/3/5/6/7/8/9
CAGGTGCTGGAGTCTCCCAGTC	TRBV7-1/4/6/7/8
CCAGTAACAAGGTCACAGAGAAGGG	TRBV7-2/3
GGGGACAGAATGTAACTTTCAGGTGTG	TRBV7-9
CCGCACAACAGTTCCCTGACTTGC	TRBV9

APPENDIX III: Summary of courses attended during Honours Program

During my Honours Program, I have only attended the Advanced Course Infection Biology. Due to time shortage, I was unable to attend Infection & Immunity Summer School. Other courses were not relevant for my research, did not fit in my schedule or did not have my interest. The Advanced Course Infection Biology was an interesting course in which we reviewed several aspects of infectious diseases and their interaction with the immune system. I attended this course with very much interest and learned a lot about the many challenges facing researchers in developing new vaccines, combating infectious agents and eradicating diseases. Although it was a course developed for PhD students, I had no trouble understanding the information that was presented.

I am somewhat disappointed that I was not able to attend more courses. Speaking with other HP-students, I noticed that many of them attended a course on statistics, presentation skills and scientific writing. However, after I consulted my supervisor, I decided that these courses did not really apply to my research or did have my interest. I have tried to find other courses to attend, but most of the courses which had my interest had already finished. I applied for Infection & Immunology Summer School, but due to a shortage of time, I had to cancel my application.

I do consider the many different techniques which I was taught by my supervisor very valuable and possibly even more valuable than attending some of the theoretical courses that were available. I have learned to culture pathogens, clone T cells and expand T cells polyclonally, isolate lipids from pathogens, load CD1 monomers and assemble them into dextramers, sorting T cells and analyse T cells on FACS, isolate RNA, synthesize cDNA, PCR and last but not least, analyzing and interpreting all the data I have gathered. With this information, I was able to come up with new ideas to study. At a certain point, I was offered a PhD position at my supervisors' research group. After a lot of thought, I decided to decline as becoming a veterinarian is still my primary objective.

I have learned many things during my HP. Not only all the different techniques and the theory of the Advanced Course Infection Biology, but also about research in general. I now know that I enjoy working in a laboratory setting and I have the capacity to do research. For now, I am focusing on obtaining my masters degree in Veterinary Medicine. Possibly, after working at least one or several years in the field, I might consider returning as a PhD in the field of Veterinary Medicine.

APPENDIX IIII: Flow chart research Honours Program