particular, what percentage of γδ T cells phagocytose and how does this compare to other lymphocytes? Is phagocytosis a feature of a particular type of γδ TCR+ cell? Is phagocytosis in γδ T cells followed by the processes that normally follow phagocytosis in professional phagocytes such as the release of microbicidal products? A number of experimental approaches were taken to answer these questions and they are novel in comparison to previous γδ T cell literature in 3 ways. Firstly, whenever possible, the assays used were adapted from well-characterised or clinically relevant assays. This includes the gentamicin protection assay which is a standard microbiology technique used to test for bacterial invasion and the whole blood phagocytosis assay which was adapted from an *in vitro* diagnostic (IVD) test used clinically to diagnose neutrophil disorders. Relevant clinical haematology stains were also employed to phenotype cells, such as NBT in the ROS production assay which is used clinically to diagnose CGD and Diff-Quick staining which is used by pathologists to describe the appearance of blood cells. Secondly, classical professional phagocytes (either monocytes or neutrophils) were included as a positive control alongside γδ T cells to contextualise the magnitude of γδ T cell phagocyte responses. Lastly, care was taken to score microscopy data blindly in order to eliminate the possibility of observer bias.

In this chapter it was shown that phagocytosis of IgG-opsonised *E. coli* can be observed in freshly isolated γδ T cells using confocal microscopy. However, the quantification of this phenomenon revealed that the proportion of γδ T cells undergoing phagocytosis was low and not significantly different from either αβ T cells or NK cells when these cells were isolated from the same donor PBMCs and tested in parallel. An attempt at distinguishing between attached and engulfed bacteria was pursued with a dual labelling flow cytometry phagocytosis assay. The dual labelling test did not show significant phagocytosis by γδ T cells but lacked a positive control to validate the assay. The gentamicin protection assay showed that phagocytosis in the γδ T cell fractions was substantially lower than that of the positive control tested (monocytes) but that viable colonies could be recovered from lysates from γδ T cells cultured in the presence of gentamicin which suggests that bacteria were phagocytosed and hence protected from the gentamicin by cells in the γδ T cell fractions. The microscopy, the dual labelling and the gentamicin experiments required significant cell processing which could potentially affect cell function and also inevitably results in a small

number of contaminating cells in the isolated fractions. Therefore, γδ T cells were also tested fresh, in whole blood, and compared to other leukocytes in the blood.

The whole blood phagocytosis assay was adapted from a test which is used to diagnose and study neutrophil function in the institute in immune disorders such as Wiskott-Aldrich syndrome (21;279). The original assay involves incubating whole blood samples with opsonised fluorescent *E. coli* either in a 37°C waterbath or on ice, and then fixing the samples in a fixative that preserves the light scatter properties of the different cell populations by flow cytometry. The adapted assay includes a staining step on ice after the incubation with bacteria, and uses the same GFP-*E. coli* bacteria and opsonising reagent as the other tests described in the chapter. Using whole blood samples, phagocytosis could be detected in 5 to 21% of cells double positive for CD3 and γδ TCR in 5 different donors, each tested in an independent experiment. Neither αβ T cells nor NK cells were found to significantly phagocytose in the same conditions.

To study phagocytic γδ T cells, the whole blood assay was repeated followed by FACS sorting of the phagocytic and non-phagocytic γδ T cell fractions. However, the phagocytic γδ T cells could not be isolated to a satisfactory purity. The phagocytic γδ T cell fraction produced reactive oxygen species but contained a large number of polymorphonucleated cells, as indicated by Diff-Quick staining. The only cells described in the blood with this nuclear morphology are granulocytes: eosinophils (bi-lobed), basophils (bi-lobed or tri-lobed) and neutrophils (multilobed). The multilobed nuclei seen did not have the typical "C-shape" found in mature neutrophils and no red-orange or dark purple granules typically found in eosinophils and basophils could be seen. However, since FACS sorting is post-phagocytosis, degranulation is likely to have already taken place. Curiously, two different groups had previously described that a rare population of CD3+ γδ TCR+ cells can be found in the granulocyte fraction of whole blood (291;292). However, in my own investigations I did not find any evidence of γδ TCR-expressing cells in the granulocyte fraction of blood by western blot or in neutrophils and eosinophils by flow cytometry.

The fact that so many of the cells in the phagocytic γδ T cell fraction had the nuclear morphology of a granulocyte adds uncertainty to the quantification results of the whole blood assay, which showed that a significant proportion of γδ T cells in whole blood

phagocytosed. In this assay, phagocytic γδ T cells also segregated entirely to a typical granulocyte light scatter profile by flow cytometry. Therefore, to validate the results from the phagocytosis quantification experiment in whole blood, granulocytes would have to be excluded from the analysis by CD15 staining or a prior density gradient separation step.

The work described in this chapter followed on from an earlier report by Wu et al presenting γδ T cells as professional phagocytes (223). The authors provided qualitative evidence of phagocytosis, some of which was reproduced in this thesis. They presented transmission electron microscopy images which showed the presence of both bacteria and γδ TCR MACS beads in the same cells. These cells were freshly isolated from peripheral blood and had a typical lymphocyte size and a round nucleus. Features of phagocytosis were observed including pseudopod-like dendritic processes, membrane ruffling and phagocytic vacuoles containing bacteria. Clustering of the γδ TCR MACS beads was also evident which likely corresponds to the γδ TCR punctate staining seen by confocal microscopy. However, the authors did not provide quantitative evidence that phagocytosis was a major feature of γδ T cells. In this chapter it was shown that small numbers of lymphocytes containing *E. coli* can be found not only in γδ T cell but also in NK cell and αβ T cell fractions in blindly-scored phagocytosis experiments. The proportion of lymphocytes undergoing phagocytosis was significantly lower than the proportion of monocytes undergoing phagocytosis, as would be expected comparing professional phagocytes to other cell types. In fact, in all the assays presented in this chapter, microscopy, microbiology or flow cytometry-based, monocytes phagocytosed much more frequently than any lymphocyte type. The data in this chapter therefore strongly suggests that γδ T cells, similarly to other lymphocytes, are not professional phagocytes in the sense that they are not proficient at taking up bacteria.

Although the question was investigated, whether a particular subtype of γδ T cells in the blood is proficient at phagocytosis was not determined. Further experiments would require first that such a cell be isolated to a satisfactory purity. One possible approach would be to scale-up the sample volume in the whole blood phagocytosis assay although that would substantially increase the cost in antibodies and FACS sorting charges. Perhaps more realistically, PBMCs could be used instead of whole blood which would increase the proportion of γδ T cells in the starting population with minimal sample processing. It

would also have the added advantage of removing granulocytes with high autofluorescence and therefore increase the accuracy of the sort. A larger number of starting γδ T cells could allow for a sufficient number of phagocytic γδ T cells to be isolated. It would also allow for a second FACS sort of the collected cells if the purity of the first sort was found to be low. Further experiments could then look at the other defining features of a professional phagocyte. As such, research could look for evidence of phagocytic cell-surface receptors, whether the uptake is actin-dependent, whether there is evidence of phagosome formation, microbicidal and degradative enzymes and a functional respiratory burst. In addition, the fate of the cell after phagocytosis could be investigated. Is the material phagocytosed by this γδ T cell loaded onto class II molecules such as in the transition from immature to mature dendritic cell? Or does the cell tend to be short-lived after phagocytosis, like a neutrophil?

It is difficult to think of the blood γδ T cell as a patrolling phagocyte mainly because other cells like monocytes and neutrophils fulfil that function already and circulate in abundance in the blood. A sub-population of γδ T cells in healthy blood expresses CD16 which binds IgG and therefore was the prime candidate for a phagocytic receptor for IgG opsonised bacteria. CD16A, the isoform of CD16 expressed by a proportion of T cells, NK cells, monocytes and macrophages, has been reported to mediate phagocytosis in non-phagocytic COS-1 cells when these are transduced with a CD16A construct (301;302). However, phagocytosis is an active process that involves both intracellular signalling and actin-mobilisation and therefore the presence of CD16 alone may not be enough to mediate phagocytosis. In addition, physical constraints of cell size and cytoplasmic volume are likely to play a part as well. A greater number of γδ T cells in healthy blood express CD16 than those that phagocytosed. It would have been interesting to have co-stained for CD16 and CD3/γδ TCR in the flow cytometry-based phagocytosis experiments to assess whether any correlation existed between CD16 expression and phagocytosis of IgG-coated *E. coli*. However, acquisition of antigen via CD16 may be relevant even if γδ T cells are not proficient at phagocytosis. Around the same time γδ T cells were reported to be capable of professional antigen presentation (233), NK cells were also reported to mature into an APC-like phenotype after the killing of target cells (258). CD16 expression correlates with cytotoxicity in both NK cells and γδ T cells, and antigen may be available for uptake in the

vicinity of the effector cells after cytolytic lysis of targets. Therefore, both γδ T cells and NK cells could possibly potentiate the adaptive immune response at peripheral inflammatory sites by performing class II presentation of antigens acquired from cytolytic targets in the local microenvironment. The following chapter describes experiments replicating previous published findings on uptake of IgG opsonised antigen and MHC class II presentation in γδ T cells. The last chapter will then evaluate whether engagement of CD16 on γδ T cells can lead to target cell lysis and antigen uptake followed by maturation of these cells into a γδ T-APC phenotype.

5 MHC class II antigen presentation by γδ T cells

5.1 Aims

- To replicate previously published work demonstrating class II presentation of influenza M1 antigen by *in vitro* stimulated γδ T cells
- To compare class II antigen presentation by activated γδ T cells with that of other similar lymphocytes

5.2 Introduction

Professional class II antigen presentation in γδ T cells was first described by Collins et al in cattle (232). Activated γδ T cells but not freshly isolated γδ T cells were found to quickly acquire the markers and morphological characteristics of professional antigen presenting cells. Activated γδ T cells displayed a developed cytoplasm and occasional membrane protrusions as is seen in dendritic cells (232). A few years later professional antigen presentation was first reported in human γδ T cells and was found to be comparable to that of dendritic cells (233;247). A subsequent study by a different group showed that the uptake of influenza M1 coated beads successfully resulted in antigen presentation in γδ T cells, using the same activation protocol as the earlier group but additionally implicating a receptor-mediated phagocytosis process in antigen uptake (223).

Further studies by Himoudi et al looked at the requirements for professional antigen presentation in γδ T cells. Their observations show a requirement for both TCR ligation, a particular cytokine milieu, and intriguingly interaction with an antibody coated target cell, again implicating a role for CD16 (246). The authors termed this conditioning "licencing" by drawing comparisons from the licensing of DCs by binding to CD40L (255-257). Interestingly, antigen presentation ability in γδ T cells was reversible as the removal of antibody coated target cells resulted in severe downregulation of the antigen presentation molecules MHC class II, CD80 and CD86 in *licensed* γδ T cells. This suggests that antigen presentation function in γδ T cells is likely to be a tightly regulated process that can occur at a specific time in the immune response, after antibody production by B cells, but that is quickly stopped when infected or tumour cells and antibody are no longer present. Therefore, antigen presentation by γδ T cells is probably both transient and locally induced

at the site of inflammation, thereby enhancing the immune function when needed but not exacerbating unnecessary inflammation when not.

It should be noted that evidence for class II antigen presentation has also been described for αβ T cells and NK cells. There is considerable evidence, dating as far back as 1978 (303), that activated αβ T cells can express MHC class II and present antigen to CD4 T cells *in vitro* (304-318). They have also been reported to express the co-stimulatory molecules CD80 and CD86 upon activation (319-323). However, the lack of a specific uptake receptor may severely restrict the ability of these cells to function as APC *in vivo*. A proportion of *ex vivo* γδ T cells from healthy donors express the Fc-receptor CD16 and were found to take up IgG-opsonised influenza M1 coated beads and then activate HLA-restricted M1-specific hybridomas by Wu et al (223). The hybridoma response, however, was halved in the presence of a CD16 blocking antibody, thereby strongly suggesting a role for the receptor in this process.

NK cells and T cells share a range of receptors (324) and are thought to arise from a common progenitor cell (325;326). As professional antigen presentation is not usually associated with either T cells or NK cells, it is unknown whether these share the intracellular machinery required for antigen processing and loading onto class II molecules, and specifically, what receptors mediate the uptake and activation of this pathway. Both αβ T cells and NK cells have been described to present antigen in certain instances (258;303;311-313;316;327;328). For αβ T cells, antigen presentation to other CD4 T cells is thought to promote the termination of the immune response because T cells activated by other T cells become unresponsive to subsequent stimulation by professional antigen cells (304). This is thought to be mediated by a more pronounced TCR and CD3 downregulation and a lack of sufficient co-stimulation (329). For NK cells, antigen presentation function has been described after the killing of tumour cell targets (258) which could potentially also play a role in anti-tumour T cell immunity.

The experiments described in this chapter set out to replicate previously published data on the ability of human blood γδ T cells to present antigen via the class II antigen presentation pathway (223). In addition, the same model was used to ask whether the ability of γδ T

cells to present class II antigen differs significantly from that of other lymphocytes (αβ T cells and NK cells).

5.3 γδ T cells can present influenza M1 antigen to specific CD4 T cell hybridomas in a chloroquine-sensitive, HLA-DR restricted manner

The experiments described in this section were replicated from those published by Wu et al. Wu et al showed that human blood γδ T cells could take up IgG opsonised influenza M1 protein coated beads and that these cells could then process and present this antigen on class II and activate antigen specific CD4 T cell lines (223). Before co-culture with antigen, the γδ T cells were cultured in the presence of the Vγ9Vδ2 TCR agonist IPP and IL-2 in the presence of irradiated B-LCL feeders, factors which had been previously shown to induce professional antigen presentation function in γδ T cells (233).

γδ T cells were isolated by MACS from PBMC, activated using the same protocol described, and incubated with influenza M1 coated beads, opsonised with anti-M1 rabbit polyclonal serum. Selected wells were pre-incubated for 1h with chloroquine, a potent inhibitor of antigen processing (330). Chloroquine interferes with antigen processing and receptor loading by increasing phagosomal and endosomal pH, thereby preventing antigen presentation without affecting antigen uptake (331;332). The same property can be exploited to enhance cross-presentation since dendritic cells incubated with chloroquine will tend to export exogenous virus antigen to the cytoplasm instead of via the class II route (333). After 1h co-culture with the antigen beads, a blocking anti-HLA-DR antibody or matched isotope control was added to triplicate wells and allowed to bind for 30 min at 37°C. Afterwards, an equal number of M1-specific CD4 T cell hybridoma cells were added to each well. The CD4 T cell hybridoma produces mouse IL-2 when specifically activated (261). Therefore, supernatants were collected at 24h and assayed for mouse IL-2 by ELISA.

The graph in Figure 5.1 depicts the results. This experiment was repeated in two donors with similar results. As expected, an antigen presentation response was detected in both cells treated with antigen beads only and isotype control wells. However, chloroquine pre-treatment for 1h or HLA-DR blocking fully suppressed activation of the hybridoma.

A weakness in this experiment and in the previously published work it replicates is that it does not include an additional control to test for the potential of chloroquine to directly induce suppression of T cell responses. The concentration used in this assay to inhibit antigen processing (50 μg/ml = 97μM) is moderate compared to published literature

(330;334-337). However, chloroquine at 100 μM has been shown to directly inhibit TCR-induced calcium signalling in T cell lines and primary T cells (338) and to inhibit proliferation and IL-2 production by T cell clones activated with anti-CD3 antibodies (339). An adequate control for the potential toxic effect of chloroquine on the T cell hybridoma would be to replace M1 protein with an M1 peptide that could be added to chloroquine wells but which had no need for processing for presentation. Alternatively, this potential toxic effect could be controlled for by adding chloroquine a few hours after incubation of the γδ T cells with antigen beads, to allow for antigen processing to take place.

The results described in Figure 5.1 reproduce the findings by Wu et al, showing that human γδ T cells can process and present via class II antigen delivered from IgG opsonised 1μm coated beads (223).

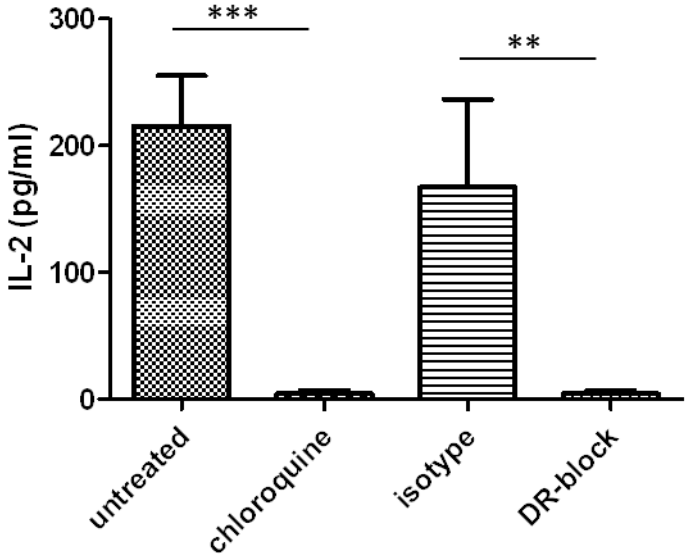

Figure 5.1 – Class II presentation of M1 influenza antigen by γδ T cells

Human γδ T cells activated with IPP, IL-2 and B-LCL feeders were tested for their ability to present M1 influenza antigen on class II. $2x10^4$ γδ T cells were tested in triplicate wells in four conditions: untreated; pre-incubated with 50 μg/ml of chloroquine; incubated with a blocking anti-HLA-DR antibody at 10 μg/ml; or incubated with a matched isotype control. Beads coated with influenza M1 protein and anti-M1 polyclonal IgG were added to all wells. After one hour, HLA-restricted M1-specific CD4 T cell hybridomas were added to all wells. Supernatants were collected at 24h and mouse IL-2 release by the hybridoma was measured by ELISA. One representative experiment of a total of two is shown.

5.4 γδ T cells induce influenza M1-specific CD4 T cell responses significantly more than other T cells or NK cells

To investigate whether γδ T cells are unique among lymphocytes in their ability to process IgG opsonised antigen and present it via MHC class II, I decided to test other lymphocytes in the same model, particularly αβ T cells and NK cells, as both of these share features with γδ T cells. Both γδ T cells and NK cells usually express CD16, unlike most freshly isolated αβ T cells (see Figure 6.2A). Therefore, if we postulate that CD16 mediates the phagocytosis of IgG-coated beads then NK cells should be likely candidates as they express ten times more CD16 receptors per cell than γδ T cells (Figure 6.2A, histogram).

To investigate whether NK cells or αβ T cells could take up, process and present the M1 antigen in a similar way to γδ T cells, all cell subsets were isolated from healthy donors by magnetic isolation. The cells were incubated with IgG coated M1 antigen beads and then co-cultured with M1-specific CD4 T cell hybridoma cells (A1C5 hybridoma). Presentation was measured by hybridoma mouse IL-2 release which was quantified by ELISA. As a negative control, all cell types were incubated with chloroquine, which was previously shown to block antigen presentation (Figure 5.1). As a positive control in this experiment, monocytes were isolated from the same donor in the same way. Only very small numbers of dendritic cells can be isolated directly from peripheral blood. Although monocytes are not professional antigen presenting cells, they have been shown to mediate antigen presentation to CD4 T cell hybridomas by the authors who made the hybridomas used in the experiments described in this thesis (340). The authors also noted that CD4 T cell hybridomas differ from normal antigen-specific CD4 T cells in that they are less costimulation dependent and therefore provide a readout that is mainly dependent on the levels of expression of peptide-MHC class II complexes only. Because the absolute values of IL-2 production vary significantly between donors (a range of 50 – 1500 pg/ml for positive control monocytes), the experiments were not pooled together and instead one representative experiment is shown in Figure 5.2.

In all donors, γδ T cells presented significantly more antigen than either αβ T cells or NK cells. The difference was larger between γδ T cells and αβ T cells ($p < 0.005$) than between γδ T cells and NK cells ($p < 0.05$) as measured by a One-way ANOVA with a Tukey's

multiple comparison test. Importantly, monocyte antigen presentation was consistently higher than γδ T cell antigen presentation, between 2 to 10-fold more in each donor.

In this experiment, γδ T cells were compared to other αβ T cells and NK cells in terms of their ability to present M1 influenza antigen via MHC class II. The results show that γδ T cells were significantly better at antigen presentation than the other lymphocytes tested. However, a small antigen presentation response could be seen in NK cell wells, which was investigated and is presented in detail in the following section.

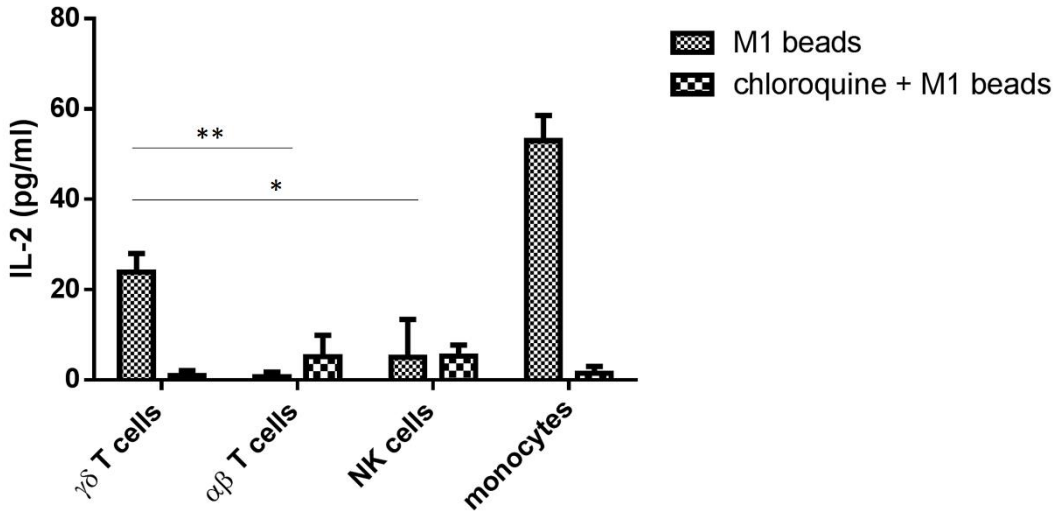

Figure 5.2 – γδ T cells present significantly more M1 influenza antigen than other lymphocytes

Human γδ T cells activated with IPP, IL-2 and B-LCL feeders were tested for their ability to present M1 influenza antigen on MHC class II in parallel with other lymphocytes and monocytes. $2x10^4$ γδ T cells, αβ T cells, NK cells or monocytes were isolated from the same donor PBMCs by MACS and seeded in two sets of triplicate wells. γδ T cells were activated overnight with IPP, IL-2 and irradiated non-HLA-DRB1*0101 B-LCLs. Half of the wells were then pre-incubated with 50 μg/ml of chloroquine for 1 h at 37°C. Opsonised M1 influenza coated beads were added to all wells. After one hour, HLA-restricted M1-specific CD4 T cell hybridomas were added to all wells. Supernatants were collected at 24h and mouse IL-2 production by the hybridoma was measured by ELISA. One representative experiment of a total of 10 is shown.

5.5 Antigen presentation signals seen in the NK fractions disappear when these are depleted of CD11c+ cells

In this section, the nature of the antigen presenting cells seen in NK cell wells in the antigen presentation assay described in Figure 5.2 was investigated by assessing the evidence for NK cell antigen presentation in the literature and then altering the NK cell isolation method accordingly.

One of the difficulties when studying NK cell behaviour is how to define NK cells. Historically, NK cells were defined on the basis of their ability to kill tumour cells and were noted to have the morphology of small lymphocytes (341). However, in the modern age of antibody based approaches, NK cells are usually identified by the CD3- CD56+ phenotype. The cells defined by this phenotype in healthy blood form a heterogeneous population. Burt et al showed that CD3- CD14- CD19- mononuclear cells from healthy human blood contain cells that co-express HLA-DR and CD56 (342), which are typical markers of antigen presenting cells and cytotoxic lymphocytes, respectively. These circulating CD56+ cells expressing high levels of HLA-DR were found to be phenotypically and functionally similar to conventional CD56- dendritic cells in the same report.

In agreement with these observations, some antigen presentation was observed in NK cell wells in the M1 model described in Section 5.4. For these experiments, NK cells were freshly isolated from healthy donor PBMCs using CD56 magnetic beads. This is a standard method for isolation of NK cells and leads to a high purity as measured by the expression of CD56 and the absence of CD3 in the purified cells. However, as Burt et al showed, this method of isolation of NK cells may lead to a variable number of CD56+ HLA-DR+ CD11c+ cells included in NK cell preparations (342). This is perhaps the reason why variable amounts of antigen presentation were initially seen in NK cell wells in the experiments described in Section 5.4.

In order to assess the ability of NK cells to present antigen in the M1 model, a further set of experiments was performed using a different method of isolation. NK cell fractions were purified by depleting PBMC fractions with magnetic beads conjugated to a cocktail of antibodies against T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells. A total of five separate experiments was performed using positive

selection CD56 MACS and another set of five separate experiments using the non-NK depletion MACS. The average results from triplicate NK cell wells in each experiment are shown in Figure 5.3. The results from monocyte wells in the same experiments are also included as a positive control. Strikingly, no M1 presentation was observed in NK cell wells using the non-NK depletion strategy whereas the positive selection method resulted in variable amounts of antigen presentation (Figure 5.3). The antibody cocktail in the non-NK depletion magnetic beads likely depletes the rare CD56+ HLA-DR+ CD11c+ cells that are found in mononuclear cells in healthy donors (342). Whether these cells are "NK cells" or "dendritic cells" is a matter of debate. A later study showed that the proportion of HLA-DR expressing NK cells in healthy blood is not insignificant (2-5.5%) and argues that these HLA-DR+ NK cells proliferate in response to IL-2, as opposed to an IL-2 driven HLA-DR expression in HLA-DR- NK cells as previously thought (343). This would argue against a dendritic cell nature as dendritic cells are thought to be terminally differentiated cells that arise from progenitor cells and therefore not capable of cell division (344). Regardless of cell type definitions, it proves an interesting example in how isolation procedures can affect the outcome and subsequent interpretation of an experiment.

The difficulty with defining NK cells experimentally is that we have yet to discover a single surface marker that can be used to discriminate them from other cell types. A significant number of "NK cell receptors" have been described (345) yet all of these can be acquired by activated αβ T cells and are often detected on freshly isolated γδ T cells, without any deliberate activation (324). However, it should be mentioned that antigen presentation function has been previously described for NK cells as long-term IL-2 activated NK cell clones acquire the capacity to process and present antigens to CD4 T cells (328). Also, NK cells were shown to acquire antigen presentation markers and the ability to present soluble immune complexes after a 72h co-culture with NK-lysis sensitive tumour cells (258). This indicates a role for target cell lysis or NK cell receptor engagement in the induction of an APC phenotype in NK cells.

It is possible that NK cells may share with γδ T cells the plasticity to acquire antigen presentation function but are not readily able to present antigen under steady-state conditions. Conceivably, flexible antigen presentation may be present in different cytotoxic cells types, taking advantage of the accessibility of antigen *in situ* after the cytotoxic lysis of

a virus of a tumour cell. If that is true, then perhaps that function could be shared by CD8 T cells as well as NK cells and γδ T cells. The following section compares freshly isolated γδ T cells and γδ T cells cultured in the presence of factors shown to induce an APC-like phenotype in these cells in their ability to present antigen via class II. Chapter 6 attempts to functionally link cell-mediated cytotoxicity and class II antigen presentation using different cell types.

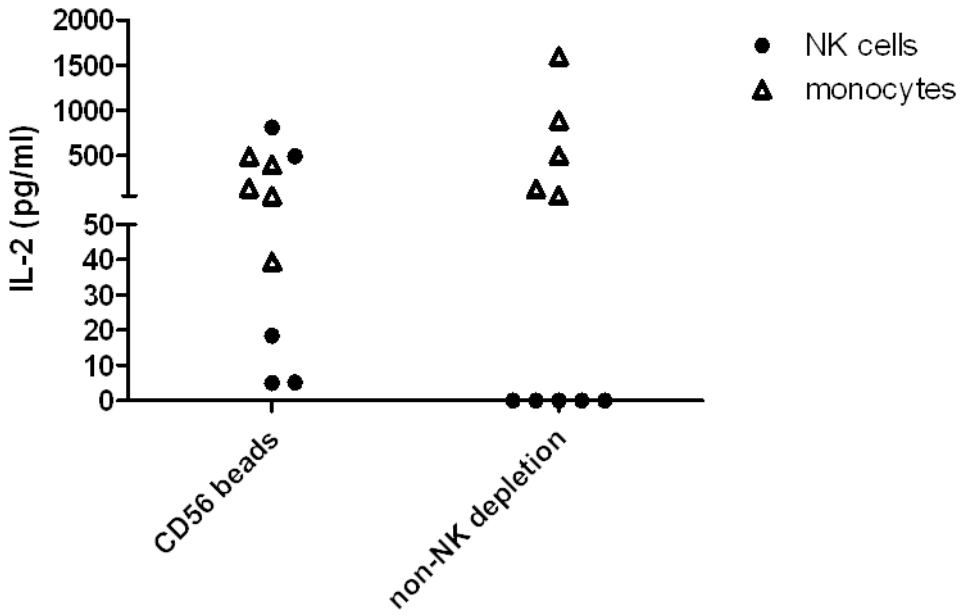

Figure 5.3 – Antigen presentation was seen in some wells of NK cells directly isolated with CD56 beads but not when NK cells were isolated by depletion of non-NK cell types

$2x10^4$ NK cells or monocytes from the same donor PBMCs were seeded in triplicate wells. Opsonised M1 influenza coated beads were added to all wells. After one hour, HLA-restricted M1-specific T cell hybridomas were added to all wells. Supernatants were collected at 24h and mouse IL-2 production by the hybridoma was measured by ELISA. The graph depicts average results from triplicate wells in ten separate experiments. The NK cells in these experiments were either isolated from PBMCs with CD56 magnetic beads (left) or by magnetic depletion of non-NK cell types (right). The plot highlights the NK cell results obtained using the two isolation procedures and includes monocytes from all experiments as a positive control.

5.6 Antigen presentation by freshly isolated versus activated γδ T cells

In the section, the ability of freshly isolated and cultured γδ T cells to present antigen via class II is compared using the influenza model described in this chapter. In the first report of professional antigen presentation by Brandes et al (233) and in the following paper from our own group, Wu et al (223), the requirements for antigen presentation by human blood γδ T cells were thought to be IPP, which is an agonist for the ubiquitous Vγ9Vδ2 TCR, IL-2 and B-LCL help. These stimuli were used to induce the maturation of freshly isolated γδ T cells into an APC phenotype.

A requirement for activation may be a way to control antigen presentation by γδ T cells avoiding unnecessary stimulation of CD4 T cell responses in the absence of foreign antigen, which could lead to autoimmunity. To elucidate the impact of activation in antigen presentation function in γδ T cells, freshly isolated γδ T cells were compared to *in vitro* activated γδ T cells from the same donor in an M1 presentation assay. γδ T cells were seeded in two sets of triplicate wells. Opsonised M1 influenza coated beads were added to half of the wells. After one hour, HLA-restricted M1-specific T cell hybridomas were added to the same wells and supernatants were collected at 24h. The other wells were incubated overnight with IPP, IL-2 and irradiated B-LCL feeder cells. Opsonised M1 influenza coated beads and HLA-restricted M1-specific T cell hybridomas were added the next day. Supernatants were collected at 24h and mouse IL-2 production by the hybridoma was measured by ELISA. Importantly, care was taken to ensure that the B-LCL feeder cells were originally from a non HLA-DRB1*0101 donor unlike in the original report by Brandes et al (233) and similar to the report by Wu et al. (223). This ensures that MHC class II molecules from the feeder cells did not activate the M1 specific hybridoma either by presentation by the feeders cells themselves or through transfer of MHC class II molecules from the feeder cells to neighbouring γδ T cells. Transfer of MHC class II molecules from APCs to neighbouring αβ T cells has been reported by different groups (346-349) and can equip them with the ability to present associated peptide antigens (349).

In the experiments described in Figure 5.4, activated γδ T cells presented more antigen than freshly isolated γδ T cells in each individual experiment. However, because of the high variability of absolute IL-2 values between individual experiments, the pooled data does

not shown a statistically significant difference between fresh and activated γδ T cells in their ability to present M1 antigen via class II.

This variability in M1 presentation readouts could be due to a number of factors specific to this antigen presentation assay, in addition to normal donor variability. Importantly, the small scale of the experiments (45 μl total volume per well) amplifies any small pipetting inaccuracies. This small scale is dictated by the rarity of γδ T cells in peripheral blood. In addition, the lack of appropriate quality control in the M1 bead preparation. M1 beads were freshly prepared before each experiment as beads that were batched and used in different experiments quickly lost their potency, possibly due to protein instability (data not shown). However, the conjugation of M1 protein to the beads does not allow for a quality control step to ascertain how much protein bound to the beads. Hence, any variability between bead preparations would lead to variability in the amount of antigen added per well between separate experiments.

It should be noted that class II antigen presentation could be seen with freshly isolated γδ T cells whereas all previous reports have used *in vitro* activated γδ T cells (223;233). This observation could be explained by taking into account evidence that was published on cross-presentation in γδ T cells after these experiments took place. The interaction between CD16 on the γδ T cell and an IgG-coated target has been described as an important stimulus for "licensing" of γδ T cells for professional antigen presentation. Himoudi et al showed that the presence of opsonised target cells was sufficient to induce a level of cross-presentation in activated γδ T cells similar to that of mature dendritic cells (246). Therefore, it is possible that the binding and uptake of M1 opsonised beads via CD16 on freshly isolated γδ T cells could play an important role in the partial maturation of these cells into an APC phenotype in the 24h time-frame that the assay takes to complete. NK cells also express CD16 and freshly isolated NK cell fractions displayed some M1 antigen presentation when isolated with CD56 beads but not when depleted of HLA-DR+ and CD11c+ cells (Figure 5.3). Therefore, I next investigated whether freshly isolated γδ T cells expressed HLA-DR and whether this marker was upregulated upon activation.

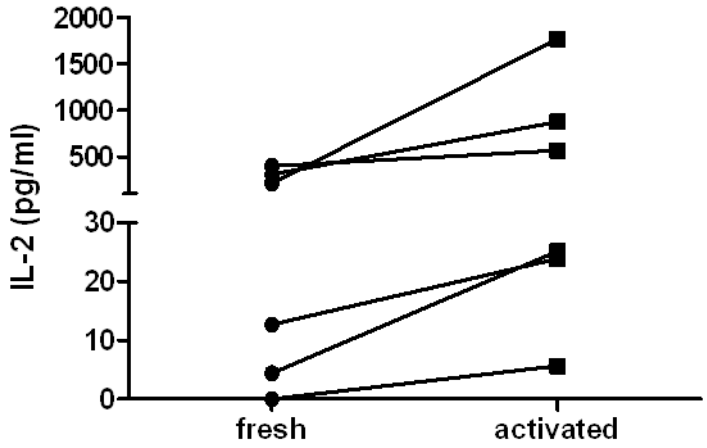

Figure 5.4 – Comparison of antigen presentation by freshly isolated γδ T cells and γδ T cells which have undergone γδ T APC maturation

Freshly isolated γδ T cells were seeded in two sets of triplicate wells. Opsonised M1 influenza coated beads were added to half of the wells. After one hour, HLA-restricted M1-specific T cell hybridomas were added to the same wells. Supernatants were collected at 24h. The other wells were incubated overnight with IPP, IL-2 and irradiated B-LCL feeder cells. Opsonised M1 influenza coated beads and HLA-restricted M1-specific T cell hybridomas were added the next day. Supernatants were collected at 24h. Mouse IL-2 production by the hybridoma was measured by ELISA. The average for each set of triplicate wells in five separate experiments is shown. Lines indicate pairing of cells isolated from the same donor PBMCs.

5.7 Antigen presentation markers and CD16 on antigen presenting γδ T cells

In this section, γδ T cells were probed for the expression of molecules required for antigen uptake and presentation. There are three essential requirements for class II presentation. The first is efficient antigen uptake (350). The second is synthesis of MHC class II molecules as exogenous antigen acquired by the cell is processed and loaded onto empty newly synthesised MHC class II molecules (351). The third is the presence on the APC of molecules capable of delivering co-stimulatory signals required for T cell activation (352). In humans, MHC class II molecules are encoded by three different loci, HLA-DR, -DQ and –DP, the best characterised of which is HLA-DR. Specific αβ T cell activation requires TCR stimulation by the cognate peptide-MHC complex and an accompanying second co-stimulatory signal through the T cell receptor CD28 as TCR triggering in the absence of co-stimulation can induce clonal anergy or T cell apoptosis (352). Physiologically, this could be a mechanism for contraction of the expanded pool of antigen-specific activated αβ T cells after clearance of the initiating antigen (304). Therefore, a professional antigen presenting cell must express both MHC class II and co-stimulatory molecules, such as CD80 and CD86.

To test for the presence of antigen presenting markers, γδ T cells were isolated by MACS from the same donor and separated into 3 samples. The first was stained straight after isolation. The others were activated for 18h with IPP, IL-2 and B-LCL feeder cells, which was previously reported to induce antigen presentation function in γδ T cells (233). One of these two remaining samples was then exposed to IgG-opsonised M1 antigen beads for 2h30 and then both samples were stained. All samples were stained for the antigen presentation markers HLA-DR, CD80 and CD86 and the possible antigen uptake receptor CD16. This experiment was performed on two donors at different times, both times yielding similar results. The results from one donor are shown in Figure 5.5.

As described before, freshly isolated γδ T cells from human peripheral blood expressed no CD80 or CD86 (233). However, I found that in both donors a proportion of freshly isolated γδ T cells expressed HLA-DR even before *in vitro* activation. Overnight culture with IPP, IL-2 and B-LCL feeder cells increased expression of HLA-DR, CD80 and CD86,

thereby inducing a mature APC phenotype in γδ T cells, similar to other reports (223;233;246).

CD16 was expressed on freshly isolated γδ T cells and was slightly increased after overnight activation. Interestingly, shortly after stimulation with IgG-opsonised beads, the number of CD16 receptors found on the surface of γδ T cells dramatically decreased. This suggests that the receptor may be involved in the internalisation of the opsonised antigen coated beads. However, a more conclusive experiment would need to test for CD16 in parallel after incubation with both opsonised and non-opsonised antigen coated beads, and with a larger number of donors.

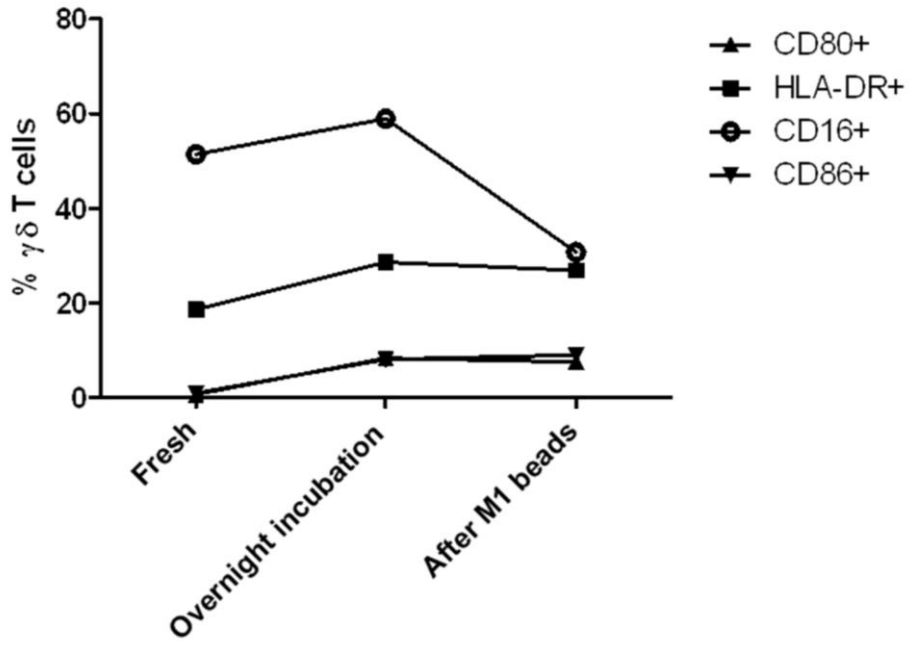

Figure 5.5 – CD16, class II antigen presentation markers and co-stimulatory molecules on γδ T cells

γδ T cells from one donor were stained with fluorochrome-conjugated antibodies against γδ TCR, CD3, HLA-DR, CD80, CD86 and CD16 at 3 different timepoints relevant to the M1 antigen presentation assay and analysed by flow cytometry. γδ T cells were stained: immediately after isolation from peripheral blood; after overnight incubation with IPP, IL-2 and B-LCL feeder cells; and after overnight incubation with IPP, IL-2 and B-LCL feeder cells and the further addition of IgG-opsonised M1 coated beads. The proportion of γδ T cells expressing the different markers at each timepoint is shown.

5.8 Summary

- γδ T cells presented influenza M1 antigen acquired from IgG-opsonised M1 protein coated beads on MHC class II
- Presentation of M1 antigen on MHC class II was significantly greater for γδ T cells than for αβ T cells and NK cells, and less than for monocytes
- HLA-DR is present on a proportion of freshly isolated γδ T cells but activation is required to induce expression of the co-stimulatory molecules CD80 and CD86

This chapter describes evidence of class II presentation by γδ T cells using opsonised influenza M1 antigen coated beads and an M1-specific HLA-DR restricted T cell hybridoma. The experiments described in this chapter were replicated from earlier work performed by a former member of the lab (223). In agreement with this previous work, γδ T cells isolated from human blood and cultured in the presence of IPP, IL-2 and B-LCL feeders were able to present M1 antigen and activate CD4 T cell antigen-specific responses. As in the previous work, this response was strongly inhibited by chloroquine, an inhibitor of antigen processing, and by HLA-DR blocking, implying a requirement for productive MHC-antigen/αβ TCR interactions to activate the CD4 T cell hybridoma.

A novel contribution of this chapter is the direct comparison of γδ T cells, αβ T cells and NK cells in the same class II antigen presentation assay. This is important because both αβ T cells and NK cells can display some antigen-presenting function *in vitro*. When compared side by side in the M1 model, γδ T cells were significantly better at class II M1 presentation than either αβ T cells or NK cells. NK cells did not significantly present M1 antigen when isolated by depletion of non-NK cell types. Some antigen presentation was seen in NK cells wells when these were isolated with CD56 beads which may be due to the presence of small numbers of CD56+ HLA-DR+ CD11c+ cells in healthy donor PBMCs.

It should be noted that other authors have shown more potent antigen presentation by human γδ T cells with different models. In the original report of Brandes et al (233), γδ T cells were found to induce the proliferation and differentiation of CD4 and CD8 T cells to a similar extent as mature DCs. Also, Himoudi et al showed a similar extent of PAX-5 tumour antigen presentation between γδ T cells and mature DCs (246). The differences found between the assay described in this chapter and the published findings should not

be due to insufficient activation of γδ T cells as these were stimulated according to the original protocol by Brandes et al (233) and express both HLA-DR and co-stimulatory molecules (Figure 5.5). Therefore, differences could be due to the type of antigen presented and therefore the route of antigen processing and presentation. For instance, different antigens have separate requirements in terms of the proteases required to cleave them and at what pH these enzymes are active (353).

Antigen presentation by human γδ T cells but not αβ T cells could be partially due to a greater responsiveness to activation induced MHC class II expression in γδ T cells. MHC class II expression in αβ T cells reportedly happens days after T cell activation (320) whereas for γδ T cells, the experiments in this chapter showed that a proportion of freshly isolated γδ T cells from healthy donors readily expressed MHC class II on their surface, which was increased following overnight activation with IPP, IL-2 and B-LCL feeders. γδ T cells stimulated with IPP were shown in the first report of professional antigen presentation by human γδ T cells to express levels of HLA-DR, CD80 and CD86 comparable to those of mature dendritic cells, whereas superantigen stimulated αβ T cells did not (233). As in this report, I also found that the antigen presentation markers HLA-DR and CD80/CD86 were upregulated in γδ T cells following activation with IPP, IL-2 and B-LCL feeders. In effect, although activation of γδ T cells enhanced their antigen presentation abilities, some presentation could be seen with freshly isolated γδ T cells but not freshly isolated αβ T cells or NK cells. The results described in this chapter lend support to the hypothesis that Vγ9Vδ2 T cells (the IPP responsive fraction of human blood γδ T cells) differ from αβ T cells and NK cells in their capacity to present antigen.

The next chapter attempts to contextualise antigen presentation by γδ T cells by investigating whether class II antigen presentation could follow cell-mediated cytotoxicity. γδ T cells are able to act as cytotoxic effectors against tumour or infected cells and are generally not infected by HIV (354). Therefore, I investigated whether cell-mediated cytotoxicity towards HIV infected cells can lead to antigen uptake and presentation in γδ T cells when it is triggered by CD16, a receptor implicated in both cytotoxicity and phagocytosis processes.

6 Cytotoxicity and antigen presentation by γδ T cells in an HIV-1 model

6.1 Aims

- To investigate whether the ADCC receptor CD16 is present on freshly isolated γδ T cells from healthy donors and if so to characterise these CD16+ γδ T cells in terms of expression of cell surface markers of T cell differentiation and whether they belong to a separate subtype of CD3/γδ TCR expressing cells distinguishable by flow cytometry
- To establish ADCC and antigen presentation models that are compatible with each other in order to look for a functional link between antigen acquired from ADCC targets and class II antigen presentation
- To investigate whether interactions between γδ T cell cytotoxic effectors and antibody-coated target cells can lead to target cell antigen acquisition by the effector γδ T cell
- To investigate whether antigen acquired by effector γδ T cells from cytotoxic targets can later be presented via MHC class II and activate antigen-specific αβ T cells

6.2 Introduction

In the experiments described in this thesis I investigated the possible roles of γδ T cells in phagocytosis and antigen presentation of exogenous antigen. In chapter 4 I examined the ability of γδ T cells to phagocytose *E. coli* as a model pathogen. In chapter 5 I examined the ability of γδ T cells to present antigens in an influenza model. In this chapter I build on this study of antigen presentation by γδ T cells by examining whether a functional link exists between the well-established ability of γδ T cells to carry out ADCC and the more recently reported ability of γδ T cells to present antigens. The major experimental approaches taken to address this question were the quantification of cell-mediated cytotoxicity against antibody-coated HIV-infected target cells and the detection of class II presentation of HIV-1 reverse transcriptase (RT) antigen using an antigen-specific hybridoma model. In this introduction I describe the theoretical background to these approaches.

The three major types of antigen-presenting cells are dendritic cells, macrophages and B cells. These cells are specialised to initiate or promote the development of antigen-specific T cell responses and are often called "professional antigen presenting cells" (355). Some of the main features that distinguish them from other cells capable of class II presentation are the abundance of receptors capable of binding and internalising exogenous antigen (350), and the ability to deliver an additional signal that accompanies direct antigen presentation and enhances and modulates the behaviour of the antigen-specific CD4+ αβ T cell (356).

Although αβ T cells are not generally thought of as professional APCs, reports of class II antigen presentation by αβ T cells date as far back as 1978 (303) and suggest that αβ T cells express MHC class II during the later stage of the immune response approximately 3 to 5 days after T cell activation (320). Most reports seem to suggest that there are at least two fundamental differences that distinguish αβ T cell antigen presenting cells from professional antigen presenting cells. The first is the lack of specific receptors for antigen uptake in αβ T cells (350). The second is a much lower level of expression of co-stimulatory molecules than professional APCs (357). These findings suggest that T cells may be restricted in the type of exogenous antigen they can present by the receptors expressed on their surface. For instance, class II antigen presentation in activated αβ T cells has been reported with antigens that bind to receptors expressed on their cell surface, such as HIV-gp120 that binds CD4 (305;308) but these cells were unable to present antigens taken up by fluid phase pinocytosis (313). It is conceivable that these limitations may also apply to class II antigen presentation in activated γδ T cells, which has not been the subject of extensive studies, and therefore that presentation may be limited to antigens that bind cell surface receptors.

Although antigen presentation by αβ T cells has been well documented (303-314), its role in the immune system remains elusive. It has been suggested that αβ T cell presentation may help dampen the immune response during the contraction phase, after the pathogen has been cleared. Evidence to support this is the delayed expression of MHC class II in these cells *in vivo*, and the low levels of co-stimulatory molecules, since MHC class II antigen presentation in the absence of a second signal can lead to anergy or apoptosis in the responding CD4+ αβ T cell (352).

Brandes and Moser examined expression of APC molecules by human blood γδ T cells (358). They reported that although infrequent in freshly isolated γδ T cells, 24h treatment with the Vγ9Vδ2 agonist IPP in the presence of IL-2 and B-LCL feeders induced levels of APC molecules similar to mature DCs, including MHC class II, and the co-stimulatory molecules CD80 and CD86. The rapid upregulation of MHC class II and high expression of co-stimulatory molecules suggest that the role of γδ T cell antigen presentation *in vivo* may be different to that of antigen presentation by αβ T cells.

One possible interpretation of these findings can be taken from studies with another cell type that shares many of its characteristics with γδ T cells, NK cells. γδ T cells and NK cells are both cytotoxic lymphocytes with a well-characterised role in the killing of cancer and virus or bacteria infected cells. Curiously, NK cells have been described to transiently acquire APC features *in vitro* in certain instances, which has been termed "presentation after killing" (259). The expression of MHC class II and CD86 can be induced on NK cells after 72h co-culture with cancer cell lines. Interestingly, upregulation of APC markers happens with cell lines susceptible to NK-cell lysis but not with cancer cell lines that evade NK-cell receptor mediated recognition (258). This suggests a role for target cell lysis or NK-cell receptor activation in the induction of a transient APC phenotype in NK cells. In addition, cross-linking of the receptors NKp30, NKp36 and CD16 but not NKG2D with antibodies also induces upregulation of MHC class II and CD86 on freshly isolated NK cells (258). This data suggests that in NK cells, class II antigen presentation in the overall immune response may be a temporally restricted phenomenon that takes place shortly after target cell lysis. A similar situation is conceivable with antigen presentation by γδ T cells. A number of target cell associated antigens will be available in the local microenvironment following cell lysis, and these antigens may be taken up by the effector cell and later presented via class II. The rapid upregulation and high levels of MHC class II and CD80/CD86 reported by Brandes and Moser (358) suggest that antigen presentation by activated γδ T cells may serve to amplify local antigen-specific CD4+ αβ T cell responses, as opposed to contracting the expanded αβ T cell pool in the later phase of the immune response. In addition, one of the critical steps for class II presentation is the uptake of exogenous antigen. In this thesis, the Fcγ receptor CD16 is hypothesised to be a possible antigen uptake receptor in γδ T cells.

There is evidence to support a role for the Fcγ receptor CD16 in professional antigen presentation by γδ T cells. In the original report by Wu et al, opsonisation of particulate antigen enhanced antigen presentation function by γδ T cells, whereas incubation with CD16 blocking antibodies before antigen pulsing decreased antigen presentation two-fold (223). Fcγ receptors are known to bind antibody coated antigen and this has been described to lead to internalisation of the antibody-antigen complex and processing for antigen presentation in macrophages and dendritic cells (359). Curiously, stable transduction with a CD16 construct confers the ability to phagocytose to non-phagocytic CHO cells (302), again supporting a role for this receptor in the internalisation of IgG coated antigen in non-professional phagocytes. CD16 is best characterised as an antibody-dependent cellular cytotoxicity (ADCC) receptor when expressed on NK cells, subsets of monocytes, γδ T cells and cytotoxic αβ T cells. In ADCC, an infected or tumour target cell is recognised via antibodies bound to its surface. Recognition of the tail region of the antibodies by CD16 receptors results in receptor clustering which activates a signalling cascade in the effector cell that culminates with the release of cytotoxic molecules which lyse the target. In this thesis it is hypothesized that CD16+ γδ T cells take up antibody coated exogenous antigen and present it via class II. This chapter investigates whether CD16+ γδ T cells can internalise and present antigen from antibody coated target cells following cell-mediated cytotoxicity.

Although limited research is available in the literature on CD16+ γδ T cells, in αβ T cells, CD16 is often described as an activation marker and tends to be found on late stage activated αβ T cells (289;360;361). Dieli et al (362) examined the T cell differentiation markers and functional activity of freshly isolated human blood γδ T cells and described a model for γδ T cell differentiation. This model is reminiscent of the central memory and effector memory model previously described by Lanzavecchia and colleagues for αβ T cells which proposed that different subsets of memory T cells exist and can be distinguished on the basis of their expression of the lymph-node-homing molecules CCR7 and CD62L (363). Naïve αβ T cells circulating in the blood use CD62L to bind high endothelial venules and CCR7 to migrate to lymph nodes. They also express the CD45RA isoform and the costimulatory receptor CD27. However, after primary antigen encounter surface expression of these two markers is switched off. CD62L is rapidly lost and CD27 is

downregulated at a slower rate. Successive differentiation stages can therefore be observed in αβ T cells, as naïve cells expressing CD62L, CCR7, CD45RA and CD27 lose expression of these markers upon activation.

The reports of Dieli et al (153;362) and Angelini et al (159) showed that expression of CD27 and CD45RA, migratory routes, and effector functions define four successive differentiation steps for peripheral blood γδ T cells. Based on the expression of CD45RA and CD27 on their cell surface, it is possible to distinguish naïve γδ T cells (CD27+ CD45RA+), central memory γδ T cells (CD27+ CD45RA-), effector memory γδ T cells (CD27- CD45RA-) and terminally differentiated effector memory γδ T cells (CD27- CD45RA+). Naïve γδ T cells (Tnaïve) are poorly represented in the blood of adult donors but frequent in cord blood and adult lymph nodes (362). In addition to CD27 and CD45RA, they express CD62L and CCR7 which allows them to home to secondary lymphoid organs. Central memory γδ T cells (TCM) are the most common subtype in the blood of healthy adults. They are generally non-effectors in terms of cytokine production and cytotoxic activity. However, they are the subtype with the strongest proliferative activity in response to antigenic stimulation (362). They can self-renew and give rise to both TEM and TEMRA through either antigenic driven or IL-15 driven stimulation in the absence of antigen, respectively (153).

Both types of effector memory γδ T cells are thought to home to inflamed tissue, as opposed to secondary lymphoid organs and are concordantly negative for CD62L and CCR7 but positive for receptors for inflammatory cytokines such as CXCR3, CCR5 and CCR6. They were the only subtypes recovered from the ascetic fluid and cerebrospinal fluid of patients with tuberculous peritonitis and tuberculous meningitis, respectively (362). They have very low proliferative activity in response to antigenic stimulation. However, they respond to infectious and cancerous insults in two complementary ways. Effector memory γδ T cells (TEM) produce large amounts of IFN-γ upon antigenic stimulation and show very limited cytotoxic ability (159). Terminally differentiated effector memory γδ T cells (TEMRA) are highly cytotoxic against cancer cell targets but have limited cytokine production potential in response to antigenic stimulation. They produce IFN-γ upon CD16 cross-linking. They express a variety of cytotoxic cell markers including perforin, CD16,

and receptors that are capable of detecting MHC class I expression on the surface of cells such as CD94, NKG2A, CD158 and NKAT2 (159).

Therefore, this model would predict that the CD16+ γδ T cells that are hypothesized to be capable of Fc-mediated phagocytosis and antigen presentation could be terminally differentiated effector memory T cells (TEMRA). TEMRA γδ T cells are more susceptible to apoptosis (153) and effector memory γδ T cell types have shorter telomeres than naïve or central memory γδ T cells (362), which is suggestive of a larger number of previous cell divisions. Therefore, this would suggest that these cells are short lived cells with limited proliferative capacity, which could limit antigen presentation function in γδ T cells to cells at the end of their life span and prevent autoimmunity. In this chapter, freshly isolated γδ T cells were stained for CD16 and T cell differentiation markers to investigate whether they belonged to the TEMRA subset or any of the other subsets described in this model.

In this chapter, I described investigation of CD16 as a potential route of antigen entry for MHC class II "presentation after killing" in γδ T cells by establishing an experimental model consisting of γδ T cells expressing CD16, HIV-1 infected target cells, an HIV-1 gp120 opsonising antibody and an antigen-specific CD4+ responder cell line. The monoclonal antibody mab2G12 was used, which binds gp120 expressed on the surface of HIV-1 infected cells and has been shown experimentally to induce ADCC (364).

Inducing ADCC against HIV-infected cells is one of the goals of current HIV vaccine research (365) and γδ T cells from HIV elite controllers have been reported to be efficient ADCC effectors (156). It should be noted however that in this thesis the HIV antigen presentation model is used as a tool to test for class II antigen presentation following ADCC and not to study the role of γδ T cells in HIV infection. The αβ T cell pool in humans has been estimated to respond to between 10^6 and 10^7 unique antigens (366). Therefore, the frequency of αβ T cells specific for a given antigen in autologous CD4 T cells will be very low. The number of γδ T cells that can be isolated from peripheral blood is also limited. In light of these two factors, an antigen-specific CD4+ T cell line was deemed more suitable to experimentally model γδ T cell class II antigen presentation. We had previously studied influenza M1 antigen presentation by γδ T cells using a hybridoma model (223). In addition, the antibody mab2G12 had been well characterised as an ADCC-

inducing antibody in HIV (364). Therefore, to study whether ADCC can lead to class II antigen presentation of target cell antigen by γδ T cells we obtained an HIV-1 hybridoma and HIV-1 peptide control from the same authors that produced the influenza M1 hybridoma (340).

In the ADCC and class II antigen presentation model described in this chapter, mab2G12 was used to opsonise a chronically HIV-1 infected lymphoma cell line (H9 cells) in order to trigger ADCC by γδ T cells. Monocytes from the same donors were used as a positive control for presentation after ADCC. Circulating monocytes, as DCs and macrophage precursors, exhibit several functions associated with antigen-presenting cells, such as phagocytosis and presence of endosomal/lysosomal degradative compartments particularly enriched in Lamp-1, MHC class II molecules, and other proteins related to antigen processing and MHC class II presentation (367) and also express Fcγ receptors, including CD64 and CD32, and a significant subset expresses CD16 (368). They are therefore ideally placed as positive controls to study Fcγ-dependent antigen acquisition and class II presentation.

To summarise, the experiments described in this chapter were designed to investigate a functional link between professional antigen presentation function by γδ T cells and CD16-mediated antigen uptake and ADCC. Following the hypothesis that the Fc receptor CD16 is involved in antigen uptake for presentation it was investigated whether a well-characterised functional consequence of recognition of target cells by this receptor – ADCC – correlates with antigen presentation by γδ T cells. In order to study this, an experimental model was constructed using HIV-1 infected target cells, an opsonising antibody previously shown to mediate ADCC, and an antigen-specific CD4 T cell hybridoma that responds to HIV-1 RT, presented on MHC class II.

6.3 Sub-populations of γδ T cells expressing different γδ TCR/CD3 levels and different γδ TCR/CD3 stoichiometry

It is not known whether the professional antigen presenting function described previously (233;246) for human blood γδ T cells is performed by all the γδ T cells present in the blood, or a specific subpopulation. The previous authors suggested that Vγ9Vδ2 T cells performed this function because the Vγ9Vδ2 TCR agonist IPP is used in the stimulation protocol to induce APC maturation in γδ T cells. However, B-LCL cells and IL-2 are also used in conjunction with IPP in this stimulation protocol and therefore other γδ T cell subsets other than Vγ9Vδ2 T cells may also be activated. In sum, little is known about the γδ TCR specificity of antigen presenting γδ T cells. Interestingly, additional differences other than TCR chain usage can be apparent in γδ TCR expression. When analysing γδ T cell populations by flow cytometry the presence of separate sub-populations with different levels of expression of γδ TCR and CD3 can be seen. Whether these sub-populations have disparate physiological functions is poorly understood but they have been mentioned previously in γδ T cell literature (159;369). This section presents data regarding the presence of distinct populations of γδ T cells in the blood which are apparent by flow cytometry based on differential expression of γδ TCR and CD3.

Angelini et al reported on the cell surface markers expressed and functional activity observed in Vδ2+ cells in freshly isolated PBMC (159). They found that in some donors two subsets of γδ T cells expressing different levels of TCR could be seen. They identified the γδ TCR^{low} subset as TEMRA and the γδ TCR^{high} as TEM. In accordance with their distinct levels of TCR expression, the γδ TCR^{high} subset responded more strongly than the γδ TCR^{low} subset to stimulation with the Vγ9Vδ2 TCR agonist BrHPP, as evidenced by a stronger induction of their ERK signaling pathway. Importantly, the γδ TCR^{low} subset strongly induced ERK signaling upon activation through Fc receptor ligation with an anti-CD16 antibody. The authors compared these two subsets to $CD56^{dim}$ and $CD56^{bright}$ NK cells, which are phenotypically classified as a more cytotoxic and a more cytokine producing subset of NK cells, respectively (370). Angelini et al therefore described γδ TCR^{high} cells as TCR-dependent helpers and γδ TCR^{low} cells as NK-like cytolytic effectors. γδ TCR^{low} NK-like effectors also displayed phenotypic markers that suggested they were at a later stage of T cell differentiation than the γδ TCR^{high} cytokine-producing effectors based on the

absence of CD27 and re-expression of CD45RA. A separate group had reported earlier observations of healthy human PBMC stained with antibodies against γδ TCR and CD3 and analysed by flow cytometry (369). The authors observed two separate populations double positive for these markers. The γδ TCR^{low} subset had a higher CD3 to TCR ratio than the γδ TCR^{high} subset. In addition, the γδ TCR^{low} subset was preferentially enriched in Vδ2+ cells. No functional activity was investigated by these authors.

To investigate whether populations expressing different levels of γδ TCR could be seen in this thesis, I constructed flow cytometry plots from different unrelated experiments, which involved either gating of γδ T cells by flow cytometry or assessing the purity of γδ T cell isolation processes (Fig 6.1). Different sub-populations of γδ T cells based on γδ TCR and CD3 staining could be distinguished when the anti-γδ TCR antibody clone B1.1 was used (Figure 6.1, A-C) but not when the magnetic isolation kit containing the anti-γδ TCR antibody clone 11F2 was used (Figure 6.1D). The differences observed could be due to the fluorochrome as clone B1.1 was conjugated to PE whereas clone 11F2 was conjugated to FITC. Since PE has a higher stain index than FITC it is better at resolving two distinct populations (371) which may explain the inability of the FITC conjugated antibody (clone 11F2) to detect distinct TCR levels in γδ T cell samples.

Whether the γδ TCR^{high} or the γδ TCR^{low} sub-population was more common varied between donors but was constant in the same donors bled at different times (data not shown). An enrichment of TEMRA CD8 T cells in the blood has been described in old age and is thought to arise from persistent viral infections, notably CMV (372) so a similar effect could be a contributing factor to a high frequency of γδ TCR^{low} cells in some individuals. However, the vast majority of donors were 25-35 years old and no correlation between age and the frequency of γδ TCR^{low} cells could be observed (data not shown). Intriguingly, in a few donors three separate sub-populations could be seen. The clearest dot plots display an extra sub-population that was γδ TCR^{medium} and expressed lower levels of CD3 than the γδ TCR^{high} and γδ TCR^{low} cells (Figure 6.1A second plot and Figure 6.1C fourth plot).

The γδ TCR^{low} subset had been previously described by Angelini et al to consist of CD16+ γδ T cells (159). In the next section I describe the frequency of CD16 expression in γδ T cells and sub-populations expressing different γδ TCR levels.

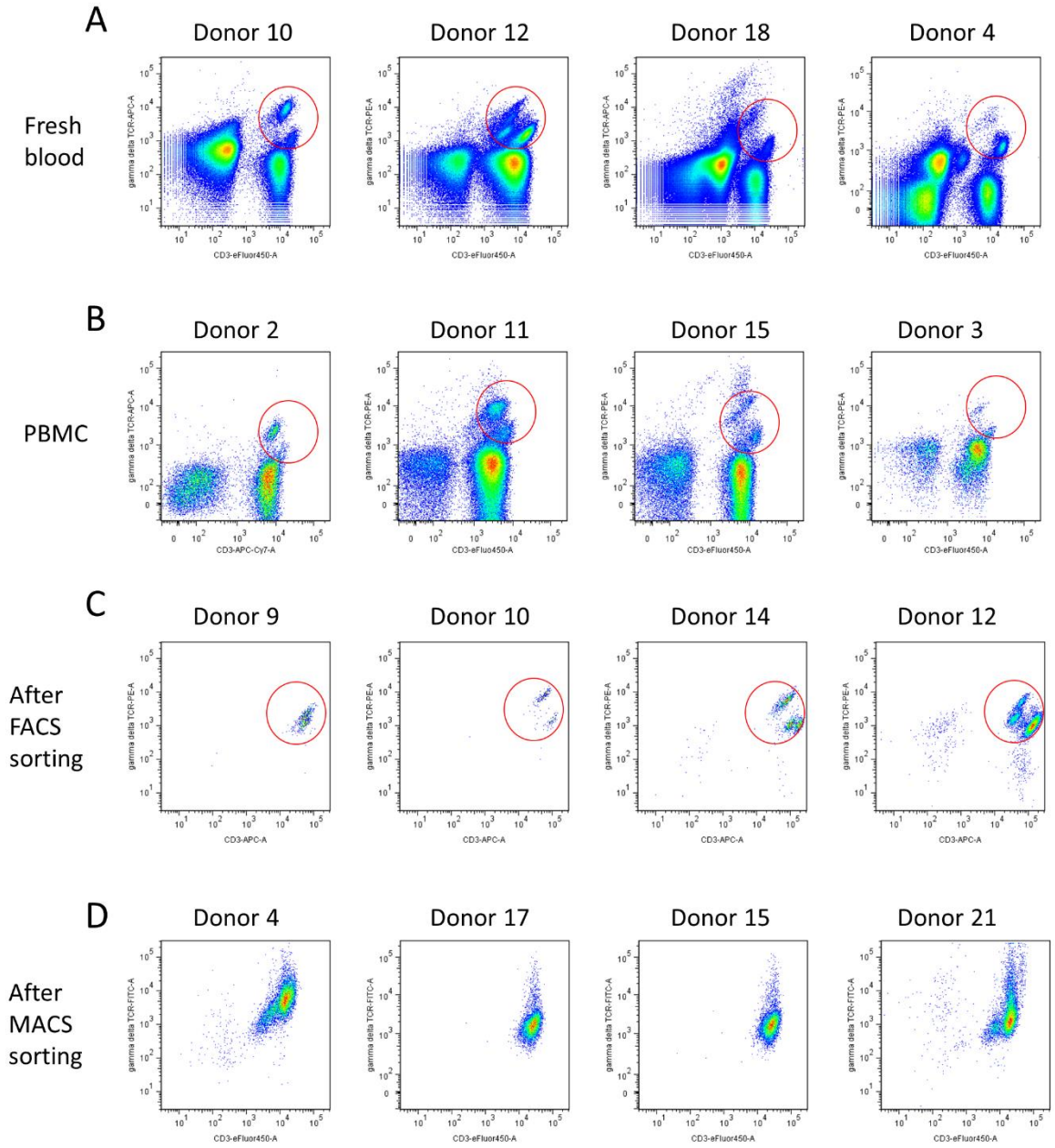

Figure 6.1 – Separate γδ T cell populations identified by flow cytometry based on γδ TCR versus CD3 staining

Examples of γδ TCR versus CD3 dot plots on healthy donor samples stained with γδ TCR and CD3 antibodies taken from different experiments in this thesis. The samples were (A) fresh blood, (B) PBMC, (C) γδ T cells FACS sorted from whole blood, or (D) γδ T cells MACS sorted from PBMC. A red circle highlights γδ T cell populations. Anti-γδ TCR antibody clone B1.1 for (A), (B) and (C). Anti-γδ TCR antibody clone 11F2 for (D).

6.4 Fcγ RIII (CD16) expression on γδ T cells

This thesis hypothesises that CD16 is an antigen uptake receptor for class II antigen presentation by γδ T cells. However, there is disagreement in the field regarding CD16 expression on γδ T cells. Different groups have reported that γδ T cells from healthy donor PBMCs express CD16 (159;160;223-225) while others have reported that they are negative for the marker and need to be activated *in vitro* to induce expression (158;226) or that they are associated with inflammatory conditions such as multiple sclerosis (224) and inflammatory bowel syndrome (173). However, most reports studying the function of CD16+ γδ T cells have used 2-4 weeks expanded γδ T cell cultures, either to expand γδ T cells to generate enough effectors to assay or because they are in the field of cancer immunotherapy where it would be valuable to expand effectors to infuse back into the patient and not because the initial population lacks CD16 (152;156). In this section, I investigated the presence of CD16 on human blood γδ T cells in freshly isolated PBMCs and additionally compared it with that of αβ T cells and NK cells in the same fractions.

PBMCs were isolated from 6 healthy donors and stained with antibodies against CD16, CD3, γδ TCR, αβ TCR and CD56. In this way 3 populations could be gated on and probed for CD16 expression: γδ T cells (CD3+ γδ TCR+), αβ T cells (CD3+ αβ TCR+) and NK cells (CD3- CD56+). It was found that donors were heterogeneous but that CD16 was consistently expressed in a larger percentage of γδ T cells than in αβ T cells from the same donor (Figure 6.2A, right plot). Another interesting observation is that the number of CD16 receptors expressed on the cell surface of γδ T cells was approximately an order of magnitude lower than that for NK cells, as measured by mean fluorescence intensity (MFI) (Figure 6.2A, histogram). Clemenceau et al found a similar difference in expression levels when they compared CD16 expression in αβ T cells and NK cells from the blood of healthy donors in a previous report (289). The physiological relevance of this phenomenon is not understood but it is possible it could mislead researchers into misrepresenting CD16 fluorescence in T cells as unspecific staining. I used a matched isotype control and also stained for NK cells in PBMCs from 6 healthy donors. On average 35.96% of γδ T cells (range 12.30 – 71.57%) compared to 3.73% of αβ T cells (range 0.53 – 7.54%) from the same donors expressed CD16 on their cell surface. CD16 is therefore present on a

proportion of freshly isolated γδ T cells and so would be expected that were used throughout the experiments described in this thesis.

As described in the previous section, γδ T cells from human PBMCs often contain more than one subset of γδ TCR/CD3 double positive cells. CD16 has been previously ascribed to the γδ TCR^{low} subpopulation (159). I plotted CD16 against γδ TCR expression to investigate whether CD16+ γδ T cells belonged to this subset. In all donors I observed CD16+ γδ T cells segregated into one or two populations. The γδ TCR^{high} population contained CD16+ cells in all donors (Figure 6.2B, 2 examples shown). However, most donors also had a second separate population of $CD16^{bright}$ γδ T cells within the γδ TCR^{low} population (Figure 6.2B, Donor 1). This cell population expressed levels of CD16 closer to those expressed by NK cells and therefore likely corresponds to the NK-like cytolytic effectors described by Angelini et al (159).

In conclusion, the CD3+ γδ TCR^{high} sub-population in healthy blood contains CD16+ cells but the $CD3^{high}$ γδ TCR^{low} sub-population can contain a separate distinct $CD16^{bright}$ population. In the next section freshly MACS isolated γδ T cells were stained with differentiation markers in addition to CD16 in order to investigate whether CD16+ γδ T cells belong to the TEMRA subset.

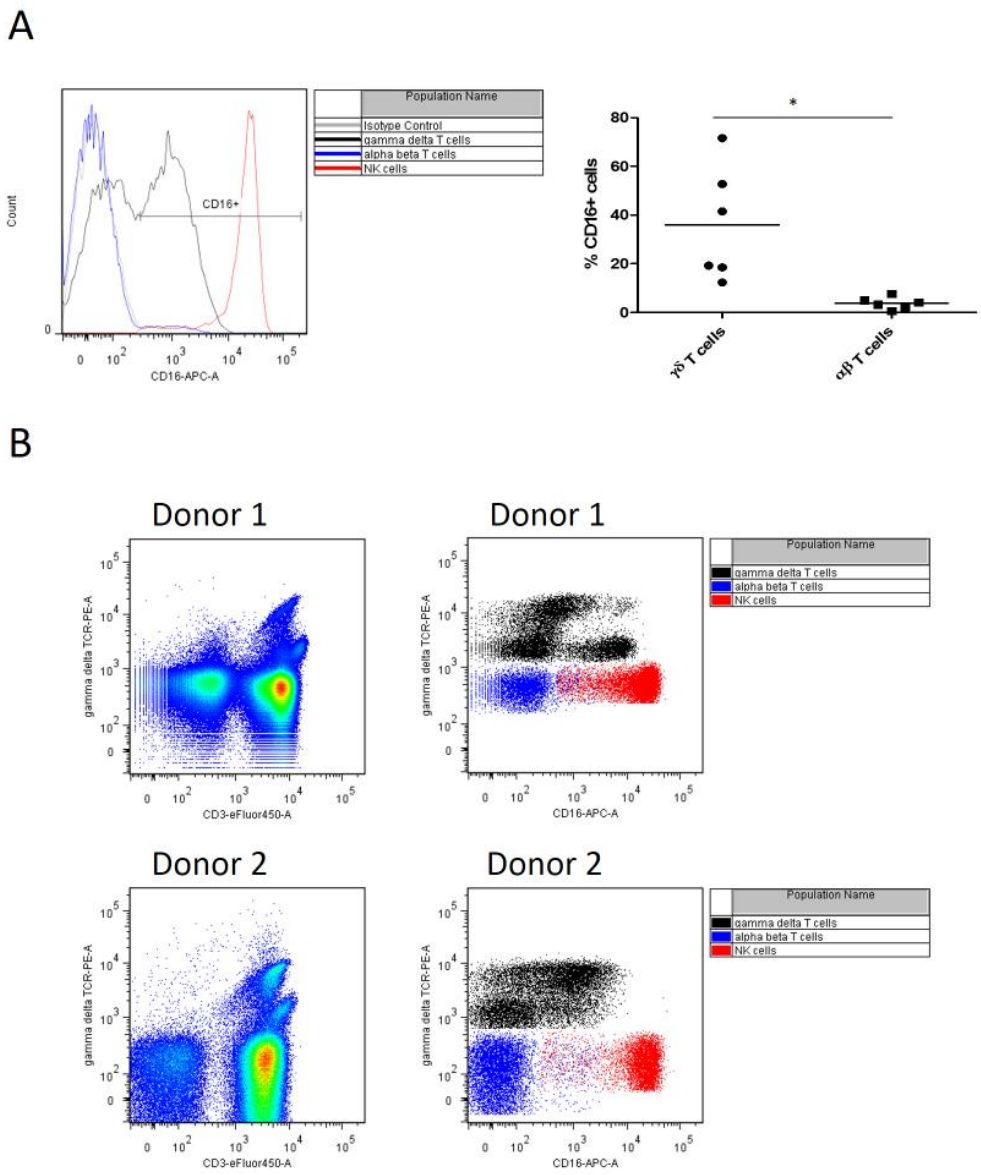

Figure 6.2 – CD16 is expressed on γδ T cells in human blood.

Freshly isolated PBMCs were stained with antibodies against CD3, γδ TCR, αβ TCR and CD16 or appropriate isotype controls. The samples were analysed for CD16 expression by flow cytometry in γδ T cells, αβ T cells and NK cells to clarify whether CD16 is present on *ex vivo* γδ T cells from healthy donors without *in vitro* activation. (A) Left: Histogram depicting fluorescence in the CD16 channel for gated lymphocytes: αβ T cells in blue, γδ T cells in black and NK cells in red. A CD16+ gate is drawn based on the isotype control. Right: Pooled data from the 6 donors showing variability of CD16 expression in *ex vivo* γδ T cells and αβ T cells. (B) Multiple distinct sub-populations of γδ T cells can be discriminated in a single donor based on CD16 and the level of γδ TCR expression. Example from 2 donors. Left: PBMCs stained with CD3 and γδ TCR to show the presence of γδ T cell sub-populations expressing different levels of γδ TCR within a single donor. Right: gated γδ T cells (black), gated αβ T cells (blue) and gated NK cells (red) plotted in a CD16 x γδ TCR scatter plot to illustrate the distribution of CD16 expression within different sub-populations of γδ T cells in the same donor.

6.5 Differentiation status of CD16+ γδ T cells

CD16+ αβ T cells in peripheral blood have been previously described as TEMRA (289). TEMRA cells are thought to be late-stage activated T cells, with high effector function but low proliferative potential. As was shown in the previous section, CD16 is expressed on a significantly greater proportion of healthy peripheral blood γδ T cells than αβ T cells. I decided to investigate whether CD16+ γδ T cells present in healthy blood had a similar phenotype.

To investigate the phenotype of CD16+ γδ T cells, γδ T cells were isolated by MACS from 5 healthy donors PBMCs and stained for CD27, CD45RA and CD16. The majority (56.70%) of freshly isolated γδ T cells were central memory T cells (Figure 6.3A) which is not surprising as the majority of γδ T cells in the blood are thought to encounter their cognate antigen very early in life (123). However, the frequency and distribution of effector memory γδ T cell subsets was variable.

Dieli et al first reported that TEMRA γδ T cells were absent from healthy peripheral blood and lymph nodes but abundant at sites of inflammation (153). However, the authors did find TEM γδ T cells in the blood with frequencies of 26.5±17.3% of γδ T cells in the 15 healthy donors tested. A later publication from Caccamo et al in the same group reported on FACS sorted Tnaïve, TCM, TEM and TEMRA γδ T cells from MACS purified γδ T cells isolated from healthy PBMCs. The frequencies reported for the four subsets in the ten healthy donors tested were 15±4% Tnaïve, 46±10% TCM, 32±8% TEM and 7±2% TEMRA (153). Angelini et al also studied TEM and TEMRA γδ T cells in healthy PBMCs from 26 healthy donors (159). They reported a frequency of 0.001-0.04% TEM γδ T cells and 0.003-0.3% TEMRA γδ T cells in PBMC so assuming an average frequency of 1% γδ T cells in PBMCs that would equate to 0.01-4% TEM and 0.03-30% TEMRA in γδ T cells.

In this thesis, the proportions of the four subsets of γδ T cells found in the 5 donors tested were 9±6% Tnaïve, 57±18% TCM, 19±13% TEM and 16±16% TEMRA. A high variability in the distribution of effector memory γδ T cell subsets was also found as shown in Figure 6.3A. One donor had predominantly TEMRA effectors (donor 1), two donors had predominantly TEM effectors (donors 2 and 5), one donor had low frequencies of both subsets (donor 3) and one donor had high frequencies of both subsets (donor 4).

Angelini et al suggested that elevated numbers of TEMRA γδ T cells found in some donors could be a consequence of subclinical infections or exposure to environmental pathogens (159) and these cells have also been shown to be rapidly and specifically mobilised during acute psychological stress (373). However, this thesis found that two out of five healthy donors recruited for this study exhibited a high proportion of TEMRA γδ T cells (27.85% and 37.44% of total γδ T cells) so high variability in the basal frequencies of effector memory γδ T cell subsets in the blood may be normal. Another possibility is that the incidence of acute psychological stress among post-doctoral researchers is greater than in the general population.

In this work, and in contrast to Angelini et al, it was found that CD16+ γδ T cells were as likely to belong to the TEMRA subtype as to the TEM subtype (mean 24.48% for TEM versus 27.35% for TEMRA) with wide donor variability for both (range 3.24-45.64% for TEM and 7.18-72.23% for TEMRA) (Figure 6.3). CD16+ γδ T cells were also represented in the TCM pool (mean 44.54%, range 8.19-83.45%). Both the data presented in Figure 6.3 and the reports by Dieli et al and Angelini et al relate to freshly isolated γδ T cells from human blood. However, most groups studying γδ T cells use cells expanded *in vitro* with phosphoantigens. In expanded cultures, cytotoxic CD16+ cells have been reported to be either TEM or a mix of TCM and TEM cells (152). In light of this variability, a greater number of donors would need to be tested to discern accurate patterns for CD16 expression in γδ T cell subtypes.

The most striking observation for this experiment was that a discrete population of $CD27^{high}$ cells in the Tnaïve cell gate completely disappeared when gating on CD16+ γδ T cells, in all donors. This population can be seen in Figure 6.3A in the upper right quadrants but not in the CD16+ gated γδ T cells in Figure 6.3B. CD27 expression in Vγ9Vδ2 T cells is associated with enhanced survival and proliferation upon activation (141), again supporting the notion that CD16+ γδ T cells are more susceptible to apoptosis and have little proliferative ability (153).

Overall, 44.53% of CD16+ γδ T cells belonged to the TCM pool and 51.85% to the effector memory subsets, whether TEM or TEMRA (Figure 6.3). This suggests that at least half of CD16+ γδ T cells are readily available for immediate effector function after isolation

from peripheral blood. Freshly isolated γδ T cells are therefore able to exert immediate CD16-mediated cytotoxicity and conceivably uptake of IgG coated antigen.

In the next section I describe experiments to investigate a connection between ADCC and antigen uptake by γδ T cells.

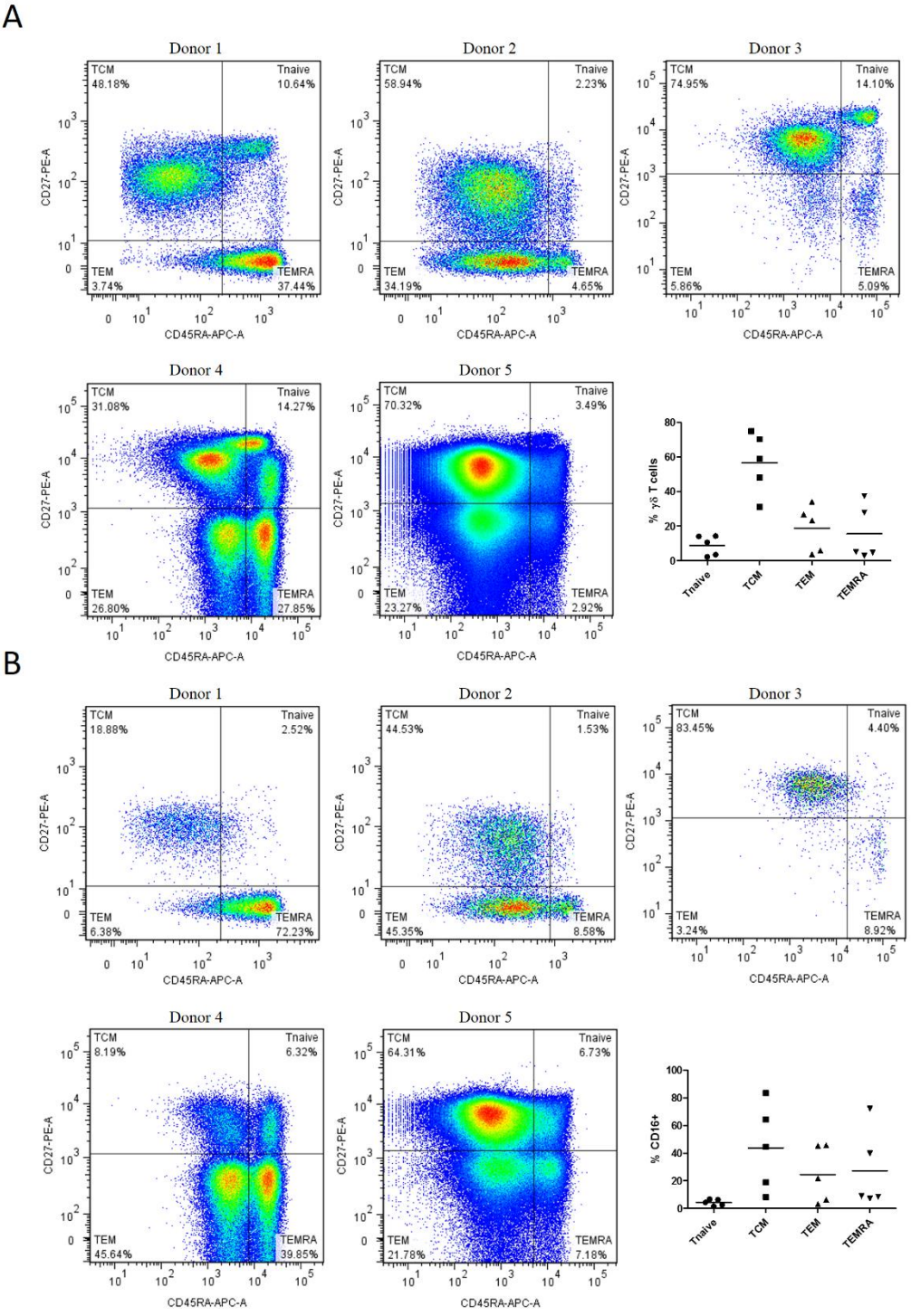

Figure 6.3 – CD16+ γδ T cells present in the blood of healthy donors preferentially display an effector memory T cell phenotype.

γδ T cells were magnetically isolated from PBMCs from 5 healthy donors and stained with antibodies against CD45RA, CD27 and CD16 to investigate their differentiation phenotype by flow cytometry. (A) CD45RA and CD27 expression by flow cytometry on γδ T cells from 5 healthy donors. A graph with pooled data is shown. (B) CD45RA and CD27 expression on gated CD16+ γδ T cells from the same donors. A graph with pooled data is shown.

6.6 Acquisition of target cell antigen through ADCC interactions

Engagement of target cells through the immunologic synapses of γδ T cells (159;374), NK cells (375) and CD8 αβ T cells (376) has been described to lead to the synaptic transfer of target cell antigens to the effector cell. This could therefore be a mechanism by which antigen can be acquired for class II presentation in NK cells and γδ T cells. In γδ T cells, synaptic transfer of target cell antigens was more frequent in TEMRA cells than in any of the other Tnaïve, TCM or TEM subtypes which was explained by a greater ability of these cells to engage targets and establish synapses as compared to the other subtypes (159). Therefore, this suggests that CD16+ γδ T cells may be able to acquire target cell antigen through interactions with opsonised target cells.

Acquisition of antigen from antibody coated targets was tested using an experimental model in which Rituximab coated Daudi cells were used as ADCC targets. The reason for using Daudi and not HIV-1 infected target cells as in section 6.4 onwards is that the glass slides needed for microscopy are not suitable for use in a Category III laboratory due to the sharps hazard they present. There are a number of reports in the literature that Vγ9Vδ2 T cells engage and lyse Daudi cells (156;377-385) and Rituximab mediates ADCC by γδ T cells towards these cells (152;156). Antigen acquired from lysed targets via CD16 could then conceivably be presented by the effector γδ T cell via MHC class II.

To evaluate whether antigen can be acquired through engagement of target cells via CD16, γδ T cells and Daudi B-cell lymphoma cells were fluorescently labelled with CFSE and DiI, respectively. Daudi B-cell lymphoma cells were then coated with the CD20-specific antibody Rituximab which is known to induce ADCC towards Daudi (152). γδ T cells and Daudi cells were co-cultured in the presence of the Vγ9Vδ2 agonist IPP which has been shown to promote synaptic transfer by γδ T cells (374). After 1h, the co-cultures were fixed with paraformaldehyde and inspected by confocal microscopy. γδ T cells were probed for the presence of Daudi cell fragments to observe whether target cell antigen can be acquired during ADCC.

Figure 6.4 shows representative results of 3 healthy donors tested in parallel. Orange Daudi fragments were visible on green-fluorescent γδ T cells. Two examples are shown on Figure 6.4A and 6.4B. One notable γδ T cell – Daudi cell interaction seemed to be mediated by a

membrane nanotube (Figure 6.4B). Membrane nanotubes have been found connecting NK cells and EBV-transformed B cells where they could traffic cell surface proteins over many tens of microns (386). To the best of my knowledge this is the first time they have been described in γδ T cells but this observation would need to be repeated as it was only found once in all the images scanned.

On average, Daudi fragments were visible on 12.95% of γδ T cells (Figure 6.4C). Dimethyl amiloride, a macropinocytosis blocker, was not able to significantly reduce Daudi cell antigen acquisition by the γδ T cells. However, incubation with cytochalasin D, a phagocytosis blocker, significantly reduced uptake of Daudi fragments by γδ T cells. Interpretation of these results should take into consideration that cytochalasin D is also known to reduce cell motility (387) and hence may reduce the number of γδ T cell – Daudi contacts. Although in our experiment the frequency of γδ T cell – Daudi cell conjugates in media and in media containing cytochalasin D was not statistically different (Figure 6.4C), a previous publication had shown that the number of γδ T cells found in conjugates with Daudi was significantly reduced from a mean of 77% to 20% by the same concentration of cytochalasin D that I used in this experiment (374).

In conclusion, cell fragments, conceivably containing various target cell antigens, may transfer between an antibody-coated target and an activated γδ T cell. It should be noted however that although the data is consistent with this interpretation, it alone does not provide sufficient evidence to draw strong conclusions. For instance, the dye used to label the target cells is a general membrane lipophilic dye and could therefore transfer between the membranes of the interacting cells without an associated transfer of target cell antigen. A similar membrane dye was used in the original report of antigen transfer in γδ T cell – Daudi cell conjugates (374). Uptake of target cell antigen by the effector cell may be detected if this antigen is then processed through the MHC class II route for presentation. In addition, signals in the local microenvironment may promote maturation of the effector cell into a functioning APC by promoting the expression of MHC class II and co-stimulatory molecules. This hypothesis would equate actively cytolytic γδ T cells to actively phagocytic immature DCs, which following antigen internalisation respond to cues in the local microenvironment and mature into a professional APC phenotype and initiate antigen-specific CD4 T cell responses.

To evaluate whether antigen acquired from cytotoxic targets could be presented by the effector γδ T cell, a model was established with opsonised HIV-1 infected cells as targets and an HIV-1 RT specific CD4 T cell hybridoma as responder cells. The development of this model and the resulting data on cytotoxicity and class II presentation by γδ T cells are explained in detail in the remainder of this chapter.

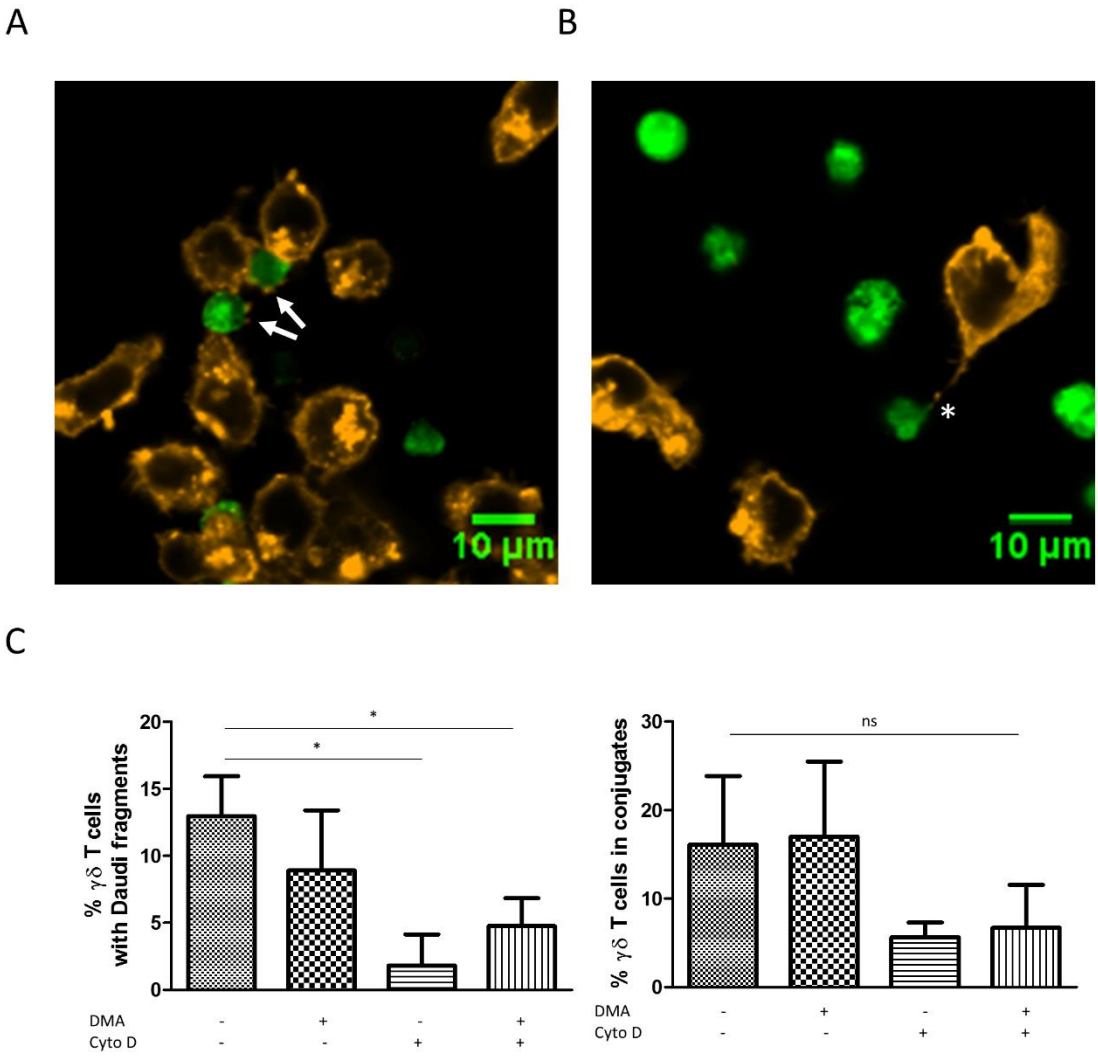

Figure 6.4 – Target cell antigen may be acquired by γδ T cells through ADCC interactions

To investigate whether ADCC can result in target cell antigen acquisition by γδ T cells, effector: target co-cultures were observed by confocal microscopy. Daudi cells were labelled with a yellow-fluorescent dye and coated in Rituximab, an antibody known to promote γδ T cell ADCC towards Daudi. Daudi cells and CFSE-labelled γδ T cells were seeded together in polylysine-coated slides in media containing IPP. When indicated, the macropinocytosis blocker dimethylamiloride (DMA) was added at 250 µM or the phagocytosis inhibitor cytochalasin D (Cyto D) at 5 µM to investigate the possible mechanism of antigen acquisition. The cells were co-cultured for 1h at 37°C, fixed in PFA and sealed with a hard-set mounting media. (A),(B) Interaction of γδ T cells (green) and Rituximab-opsonised Daudi cells (orange). White arrows denote Daudi fragments visible on the γδ T cells. An asterisk denotes a structure resembling a membrane nanotube between a Daudi cell and a γδ T cell. (C) Quantification of apparent Daudi fragments on γδ T cells after co-culture. Results are the average of 3 healthy donors assayed in the same experiment.

6.7 Opsonisation of HIV-1 infected cells with mab2G12

γδ T cells expressing the cell-surface receptor CD16 can be found in human healthy blood as shown by Figure 6.2A in this thesis and in reports from a number of groups (159;160;223-225). The presence of this receptor suggests that freshly isolated γδ T cells are capable of recognising antibody coated cells and exerting ADCC. There are a number of reports in the literature documenting ADCC by γδ T cells towards various cancer cell lines including breast cancer (152), lymphoma (152), head and neck cancer (156) and neuroblastoma (388), as well as primary follicular lymphoma (155). In addition, the report by Wu et al showing class II antigen presentation in γδ T cells also supported a role for CD16 in antigen presentation, as CD16 blocking prior to incubation with opsonised antigen reduced antigen presentation two-fold (223). This section describes establishment of an experimental model to investigate whether antigen acquired during ADCC can be later presented on MHC class II.

In order to model ADCC and then probe for class II antigen presentation by the same cells we acquired a responder hybridoma cell line that has been shown to respond specifically to presentation of HIV-1 RT antigen on HLA-DR1*0101 (340). To complete this model we acquired an HIV-1 infected cell line and a monoclonal antibody that has been shown to mediate ADCC against HIV-1 infected cells. The human monoclonal antibody 2G12 (mab2G12) was isolated from an asymptomatic HIV-1 infected patient in 1990. Buchacher et al immortalised B lymphocytes from the peripheral blood of this HIV-1 infected patient using EBV transformation, making this antibody available for the wider HIV research community (389). It was characterised as an anti-gp120 antibody, of the IgG1 isotype, with broadly neutralizing activity against HIV-1 strains and an ability to mediate ADCC (364).

The ADCC-mediating mab2G12 was used to opsonise HIV-1 chronically infected H9 cells. These cells then served as targets to investigate whether antigen was acquired and processed onto MHC class II as a result of ADCC by γδ T cells. HIV-1 infection was first confirmed by testing supernatants from the infected and uninfected cells with an HIV-1 p24 ELISA (Figure 6.5A). Binding of mab2G12 to the infected cell line was then tested by flow cytometry. The antibody binds gp120 expressed on the surface of the HIV-infected cells. The antibody specifically bound HIV-infected H9 cells but did not bind uninfected H9 cells (Figure 6.5B).

HIV-1 infected H9 cells opsonised with mab2G12 were then used as ADCC targets in the experiments described in the rest of this chapter.

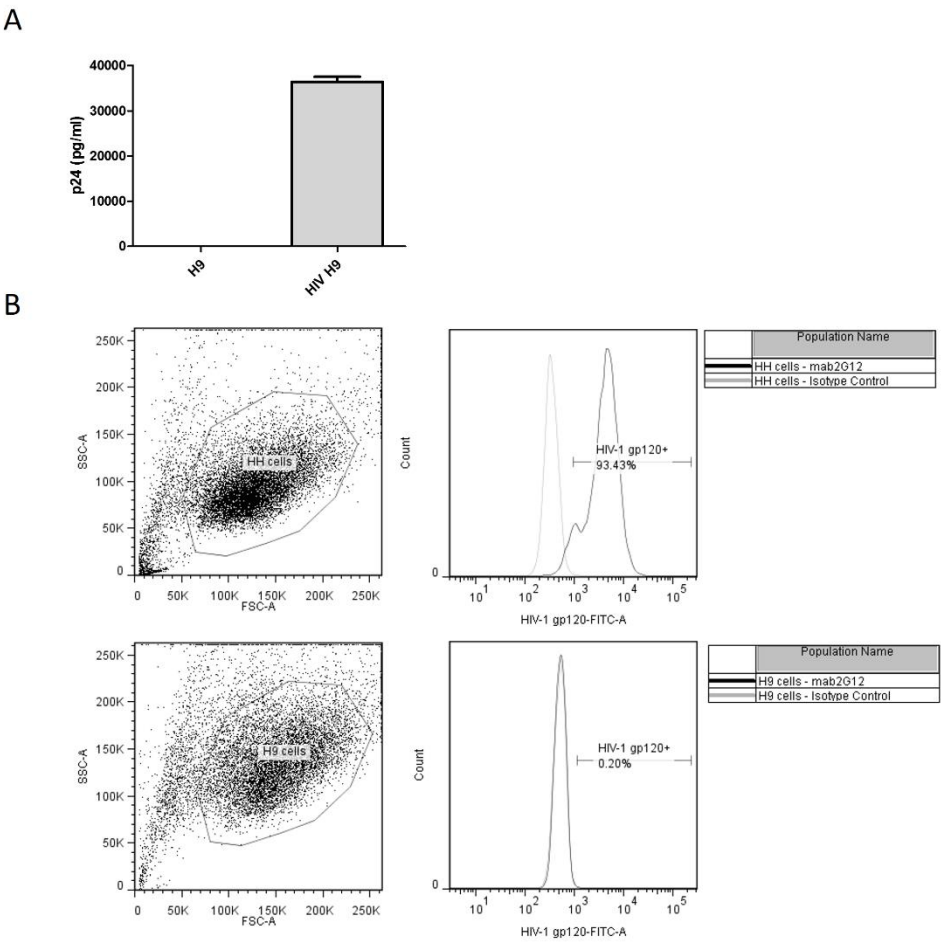

Figure 6.5 – mab2G12 binds HIV-1 gp120 on the surface of HIV-infected targets.

To build an ADCC model, HIV-1 chronically infected H9 cells were chosen as targets and the ADCC-mediating antibody mab2G12 as an opsonising reagent. (A) Supernatant from chronically HIV-1 infected H9 cells and uninfected H9 cells was tested with an HIV-1 p24 ELISA to confirm infection. (B) HIV-1 infected H9 cells and uninfected H9 cells were incubated with 10μg/ml of mab2G12 antibody to test binding of the antibody to the target cells. The cells were then counterstained with a FITC conjugated secondary antibody and HIV-1 gp120 expression was assessed by flow cytometry.

6.8 Development and validation of a non-radioactive flow cytometry based assay to test cell-mediated cytotoxicity against HIV-1 infected cells

In order to assess the ability of γδ T cells to engage with opsonised HIV-infected H9 cells and exert ADCC, a non-radioactive assay to measure direct cytotoxicity and ADCC was developed based on the previously published FATAL assay (390) and RFADCC assay developed for mab2G12 (390;391). This flow cytometry based assay was used as an alternative to the standard chromium release assay (392-397) due to the safety regulations preventing the transfer of HIV-1 infected cells from Category III containment to a laboratory rated for experiments involving radioisotopes. The assay uses targets labelled with the lipophilic PKH26 fluorescent dye which after incubation with effectors are stained with a fixable viability dye (Live/Dead Fixable Far Red, Life Technologies) to discriminate between live and dead cells (398). The pre-labelling of targets allows the operator to specifically gate and assess lysis of these cells. The use of a fixable viability dye allows for testing of biological hazard samples since paraformaldehyde fixation is required for the safe flow cytometry of HIV-infected cells. Figure 6.6 describes the development of this flow cytometry based assay.

To distinguish between live and dead target cells by flow cytometry, gates were set up using live and dead controls which were included in each experiment. The dead control consisted of a sample of PKH26-labelled target cells snap frozen in the absence of a cryoprotectant to induce cell death and permeabilise the membrane thereby allowing the viability dye to stain intracellular amine groups. Cell death was confirmed by trypan blue staining. This control was used to draw a dead gate as pictured in Figure 6.6C. An unmanipulated sample of PKH26-labelled target cells was also used as a live cell control. In Figure 6.6A and C we see an overlap of the dead cell control in black and the live cell control in grey. It is clear that the light scatter properties of the target cells change with cell death as the lysed cells show a reduced forward scatter (FSC) (a measure of cell size) and increased side scatter (SSC) (a measure of refractivity or granularity). Both dead and live target cells maintain their membrane labelling and can be gated on using PKH26 fluorescence in the PE channel (Figure 6.6B). After gating on PKH26+ events, live and dead cells can be discriminated based on the brightness of the viability dye and the scatter properties of the cell (Figure 6.6C). Unlike non-fixable viability dyes such as PI and 7-AAD which bind to DNA and

only permeate dead cells, fixable viability dyes bind to targets expressed on both the cell surface and the intracellular milieu, and therefore stain both live and dead cells. The distinction between live and dead is therefore based on the increased brightness of staining that results from the dye permeating dead cells and not the presence or absence of staining as in non-fixable viability dyes. The dead target cell gate was drawn based on live and dead cell controls as shown in Figure 6.6C and was used to quantify target cell lysis.

Figures 6.6D, E and F show an example of an experiment well containing both effectors and targets. Targets were gated on based on PKH26 fluorescence (Figure 6.6E) and then probed for cell lysis by quantifying the % of cells in the dead cell gate (Figure 6.6F).

In order to validate the flow cytometry assay against the standard chromium release assay, an experiment was performed in a Category II laboratory in which uninfected K562 cells were used as targets and PBMCs were used as effectors. PBMCs were co-cultured in parallel with both PKH26-labelled and chromium-labelled target cells. The effectors and targets were cultured at varying effector to target cell ratios (E:T) for 4h in parallel. Lysis of targets was then measured by quantifying chromium release or calculating the proportion of cells within the dead cell gate as previously described. Specific lysis in the chromium release assay was calculated according to the following formula, where spontaneous lysis refers to wells with target cells in the absence of effectors and maximum lysis refers to target cells in the absence of effectors in the presence of 1% Triton:

$$\%\,Specific\ Lysis = \frac{(Sample\ cpm - Spontaneous\ Lysis\ Control\ cpm)}{(Maximum\ Lysis\ Control\ cpm - Spontaneous\ Lysis\ Control\ cpm)} * 100\%\%$$

Specific lysis in the flow cytometry cytotoxicity assay was calculated by subtracting average spontaneous lysis from sample wells. In this validation experiment, the chromium release assay and the flow cytometry cytotoxicity assay both showed dose dependent specific killing of target cells but the chromium assay appeared to be more sensitive as it detected on average 60% higher lysis values than the flow cytometry alternative for the same effector to target cell ratios (Figure 6.6G).

This result is not surprising considering that the chromium release assay measures a marker released continuously during the 4h lysis period that accumulates in the supernatant without significant decay whereas the flow cytometry assay only measures target cells still

present at the end of the assay. Lysed target cells may degrade into cell fragments so would not be present for counting at the end of the assay. Also, staining with fixable viability dyes requires extensive washing steps which are likely to result in the loss of some target cells unlike staining with non-fixable viability dyes such as propidium iodide (PI) and 7-amino-actinomycin (7-AAD) which are added to the cell suspension before flow cytometry.

In conclusion, a cytotoxicity assay was developed that allows for the safe detection of target cell lysis under Category III containment and this assay detected specific target cell lysis in a dose-dependent manner. This assay was used in the following section to investigate γδ T cell mediated cytotoxicity and ADCC against HIV-1 infected H9 cells.

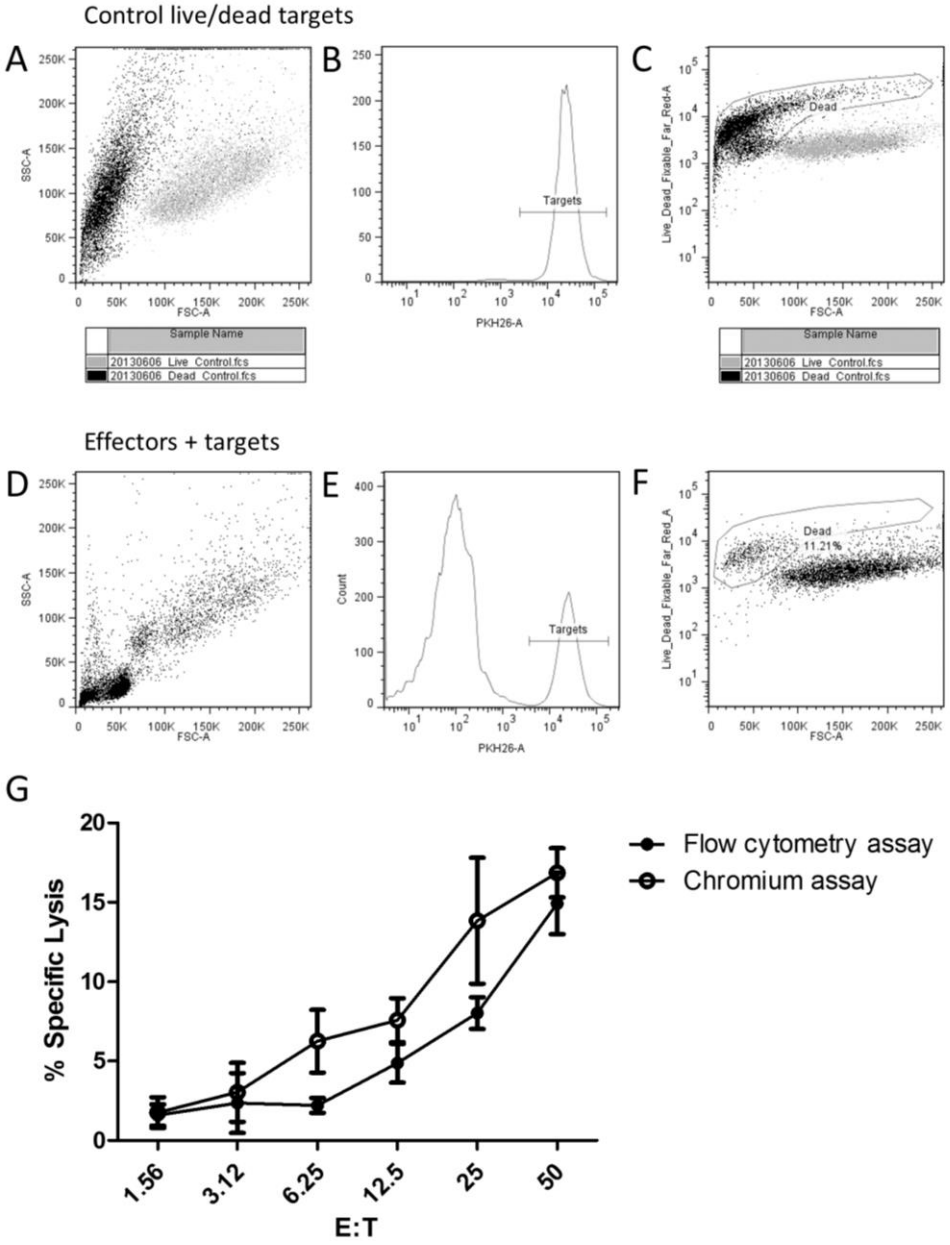

Figure 6.6 – Development of a non-radioactive flow cytometry based cytotoxicity assay.

K562 targets were labelled with PKH26 and co-cultured with PBMC at different effector to target cell ratios before staining with a fixable viability dye. Unmanipulated targets and targets snap-frozen in the absence of a cryoprotectant were used as control live and dead targets to set up gates. (A) Light scatter overlay of control live (grey dots) and dead (black dots) targets. (B) Gating of PKH26-labelled control targets. (C) Control live and dead targets were used to draw a dead gate based on FSC and brighter staining with the fixable viability dye. This gate was then used to probe for target cell lysis in assay wells. (D) Light scatter dot plot of PBMC effectors and K562 targets. (E) Targets are gated on PKH26+. (F) The proportion of lysed targets is quantified in the dead gate. (G) Comparison between this flow cytometry assay and a standard chromium release assay performed in parallel with the same donor PBMCs and K562 targets.

6.9 Antibody-mediated cellular cytotoxicity against mab2G12 opsonised HIV-1 infected cells

In order to test whether target cell lysis via ADCC can lead to antigen acquisition and presentation via MHC II, the flow cytometry-based cytotoxicity assay described in section 6.8 was used to investigate ADCC against the mab2G12 opsonised HIV-1 H9 target cells described in section 6.7. The targets were coated with the mab2G12 antibody or isotype control and co-cultured with or without effector cells for 4h. Target cell lysis was measured using the flow cytometry-based cytotoxicity assay. Figure 6.7A shows one of two ADCC experiments using freshly MACS purified γδ T cells or NK cells as effectors. Addition of either γδ T cell or NK effector cells did not significantly increase lysis of HIV-1 infected H9 cells (HH cells), either via direct cytotoxicity or ADCC. Essentially, no effector cell function was detected.

ADCC is usually measured *in vitro* with freshly isolated PBMCs without any further isolation steps and this ADCC activity is usually attributed to NK cells (173;399;400) since these cells are the most frequent cells expressing Fcγ receptors in PBMCs. PBMCs have been shown to have lower ADCC activity after cryopreservation. However, if thawed cryopreserved PBMCs are rested overnight this appears to restore their ADCC activity (401). It is conceivable that if cells undergo both PBMC isolation by density gradient centrifugation and positive MACS isolation this may negatively impact their ADCC function. Therefore, in all the following experiments, MACS isolated cells were rested overnight. Low dose IL-2 (100 IU/ml) was added to complete culture media to support survival of NK cells and T cells.

Figure 6.7C shows representative plots of one of three independent experiments investigating ADCC against the mab2G12 opsonised HIV-infected cell line using MACS isolated effector cells that had been rested overnight. γδ T cells and NK cells from the same donor were used. Both overnight cultured γδ T cells and NK cells were also tested for expression of the Fc receptor CD16 by flow cytometry, and found to express the marker on a significant proportion of cells (Figure 6.7B). There was statistically significant direct cytotoxicity against the HIV-infected cell line for both γδ T cells and NK cells but the addition of opsonising antibody had no significant effect on target cell killing.

Binding of mab2G12 to the surface of the cell line had been previously confirmed using the same antibody concentration that was used in this assay so insufficient opsonisation is unlikely to be the reason for the observed lack of ADCC (Figure 6.5). A possible explanation for this result is that high direct cytotoxicity against the leukaemia cell line could be masking ADCC. To test this hypothesis, effectors were co-cultured with uninfected H9 cells for 4h resulting in the lysis of 22.13% of H9 targets by γδ T cells and 43.58% of H9 targets by NK cells (Figure 6.8). For HIV-infected targets, co-culture with γδ T cells resulted in 9.14% lysis in the absence and 9.46% in the presence of opsonising antibody. In co-culture with NK cells, there was 57.39% lysis of HIV-infected targets without antibody and 58.66% in the presence of opsonising antibody. In this model, direct cytotoxicity against the H9 cell line was very high and cell-mediated cytotoxicity in the presence or absence of antibody could not be distinguished.

In previous publications measuring ADCC responses with mab2G12 and other antibodies other investigators have used CEM.NKR as target cells (364;401;402). CEM.NKR is a naturally isolated cell clone from the human T lymphosarcoma cell line CEM that is resistant to natural killer (NK) cell-mediated lysis (403). NK cell-mediated lysis refers to cytotoxicity induced by a variety of receptors first described in NK cells but also present in CD8 T cells and γδ T cells (345). These receptors bind ligands expressed on the surface of the tumour cells and include for instance NKG2D (61;404;405) and NKG2C (406). Human γδ T cells kill a variety of tumour cell lines *in vitro*, including leukaemia and lymphoma, melanoma, neuroblastoma, and multiple types of carcinoma (407-410). Therefore, the target cell used to measure ADCC must be carefully selected.

I next tested the mab2G12 - HIV-infected H9 cell model with the CD4 T cell hybridoma that detects MHC class II presentation of HIV-1 RT, as investigating MHC class II antigen presentation of target cell antigen by γδ T cells was the main goal of the work described in this chapter. These experiments are described in the following concluding section.

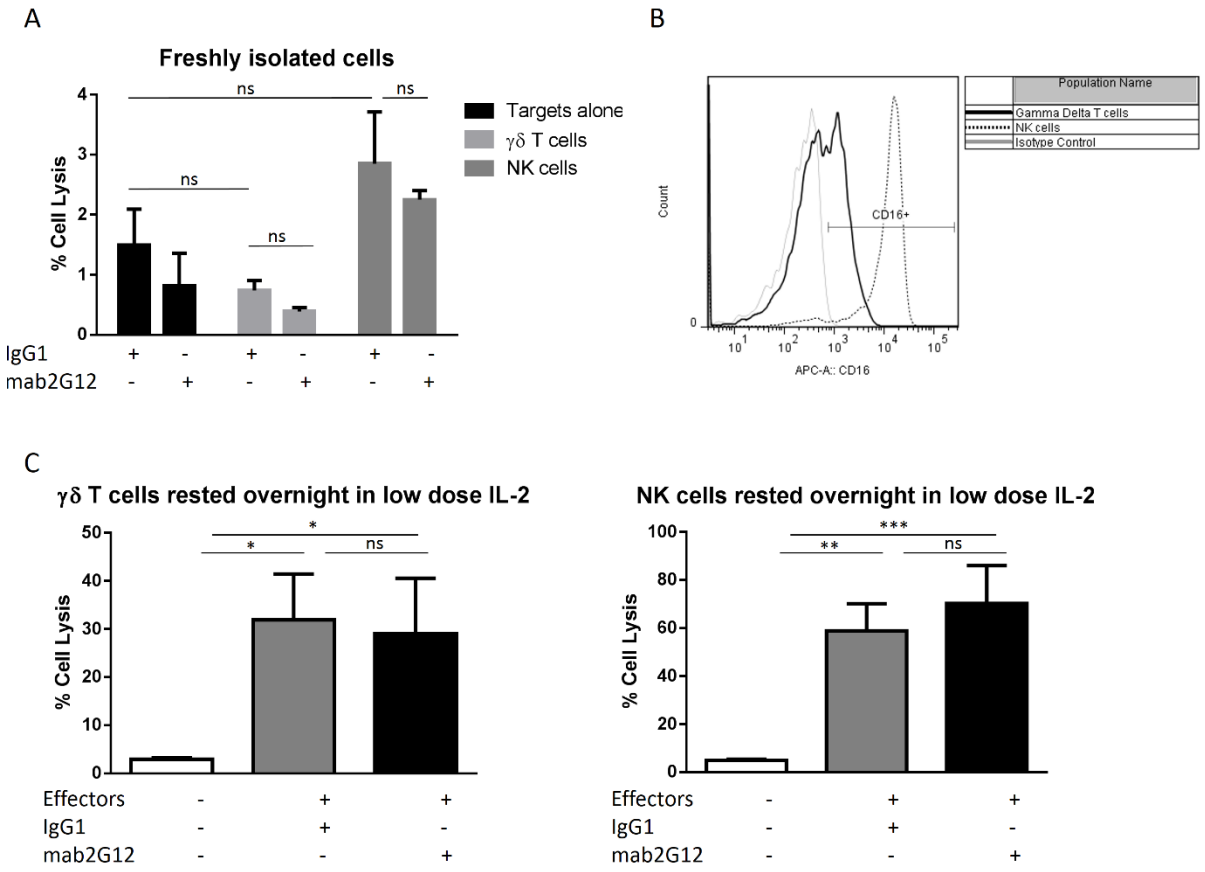

Figure 6.7 – Antibody-dependent cellular cytotoxicity was not detected with mab2G12 opsonised HIV-infected H9 targets.

γδ T cells and NK cells were isolated from PBMCs by MACS and used as effectors in a cell-mediated cytotoxicity assay. HIV-infected H9 target cells were opsonised with mab2G12 at 10μg/ml to promote ADCC. Human IgG1 was used as an isotype control. 10^5 effectors and 10^4 targets were co-cultured in triplicate wells for 4h. Target cell lysis was quantified using the previously described flow cytometry method. (A) Target cell lysis after co-culture with freshly MACS isolated γδ T cells and NK cells. One representative experiment out of a total of 2 is shown. (B) Expression of CD16 by γδ T cells and NK cells rested overnight in complete RPMI with 100 IU/ml IL-2. (C) Target cell lysis by γδ T cells (left panel) and NK cells (right panel), rested overnight in complete RPMI with 100 IU/ml IL-2. γδ T cells and NK cells were assayed in separate plates but are from the same donor. One representative experiment out of a total of 3 is shown.

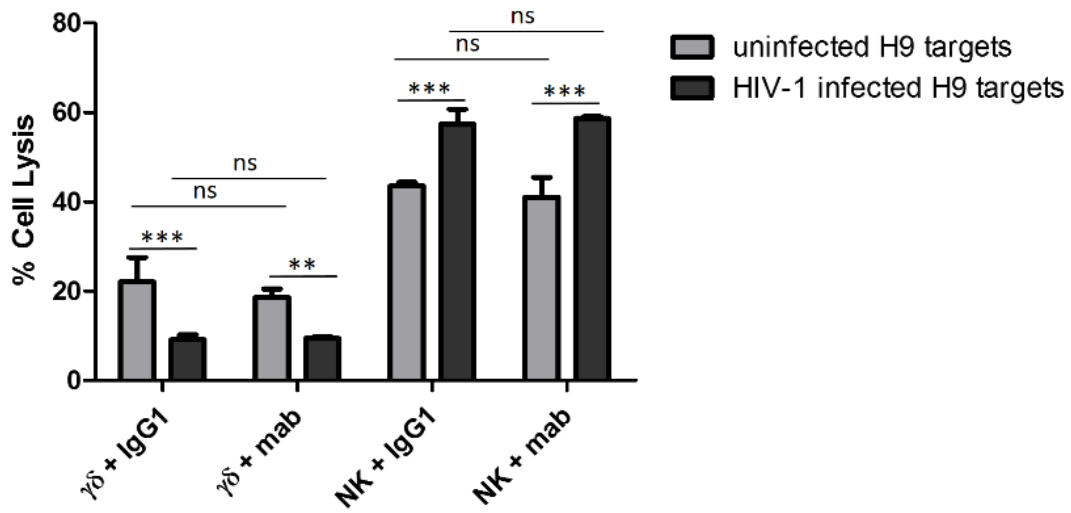

Figure 6.8 – γδ T cells and NK cells show high cell-mediated cytotoxicity against the H9 lymphoma cell line

γδ T cells and NK cells were isolated from PBMC by MACS, seeded at 10^5 cells/well in triplicate and rested overnight in complete RPMI with 100 IU/ml IL-2. 10^4 uninfected or HIV-1 infected H9 cells were added per well after pre-incubation with 10 μg/ml mab2G1 or human IgG1. Target cell lysis was quantified using the previously described flow cytometry method. One experiment of a total of 2 is shown. Data was analysed with a two-way ANOVA and a Tukey's multiple comparisons test.

6.10 MHC II presentation of antigen acquired from mab2G12 opsonised HIV-1 infected cells

Wu et al showed that blocking of CD16 on γδ T cells before incubation with opsonised influenza antigen decreased subsequent MHC class II presentation of influenza M1 antigen by γδ T cells two-fold (223). Therefore, the authors hypothesized that Fcγ receptor interactions could lead to antigen acquisition and class II presentation in γδ T cells.

To test for class II antigen presentation in the mab2G12-HIV-1 infected cells model, monocytes were isolated from the blood of healthy HLA-DRB1*0101+ volunteers and co-cultured with HIV-infected H9 cells or uninfected H9 cells in the presence of opsonising mab2G12 antibody, isotype control or media alone. As a control for uptake and presentation of particulate antigen, neat supernatant from the HIV-infected cell line containing intact viral particles with HIV-1 RT was added to a number of monocytes wells instead of target cells. To positive control wells, an HIV-1 RT peptide was added that specifically activates the CD4+ 1ACD5 hybridoma cell line when bound to the outside of empty MHC class II pockets in antigen presenting cells (340). Activation of the hybridoma was measured by the release of mouse IL-2 and not by proliferation as is used for primary human CD4 T cells because the cell line divides very rapidly in culture even in the absence of any stimulation.

Three independent experiments were performed, of which a representative example is shown in Figure 6.9. Opsonised HIV-infected H9 cells were the most potent source of antigen for presentation in monocytes. There was a statistically significant increase in MHC class II antigen presentation of HIV-1 RT when target cells were coated with antibody compared to in the presence of isotype control or media alone. No significant presentation was observed when supernatant from infected cell cultures was added. These results strongly suggest that IgG – Fcγ receptor interactions can indeed increase productive HIV-1 RT antigen internalisation or routing for MHC class II presentation.

Next, the same experiment was performed using γδ T cells instead of monocytes (Figure 6.10A). Freshly isolated γδ T cells were stimulated with IPP, IL-2 and B-LCL feeders to mature into an APC phenotype as described previously (223;233). MHC class II and CD16 expression was confirmed by flow cytometry (Figure 6.10B). Very low MHC class II

antigen presentation of HIV-1 RT was consistently observed in four independent experiments with γδ T cells, two of which were performed in parallel with monocytes from the same donor. All of the supernatants in the γδ T cell wells were tested neat in a mouse IL-2 ELISA. However, the levels of IL-2 detected were low and the variation between replicates means that no statistically significant conclusions could be drawn. Similar conclusions were drawn using freshly isolated γδ T cells tested in parallel with monocytes (Figure 6.11).

Interestingly, the HIV-1 RT peptide positive control failed to activate the hybridoma cell line in the γδ T cell experiments (Figure 6.10A) even though it strongly activated the hybridoma in the monocyte experiments (Figure 6.9). Since the γδ T cell APCs could be shown to express MHC class II by flow cytometry (Figure 6.10B), and the peptide control relies on binding to empty MHC class II molecules on the outside of the cell, one possible explanation is that the MHC class II molecules evident by flow cytometry on activated γδ T cells may not possess empty pockets. Empty MHC class II molecules are not present on the surface of all MHC class II+ haematopoietic cells. In the mouse at least, empty MHC class II molecules are found on the surface of dendritic cells but are absent from the surface of B cells and macrophages (411). In humans empty MHC class II molecules expressed on the cell membrane are progressively lost during monocyte to dendritic cell differentiation (411;412). Empty MHC class II molecules bind peptides on the outside of the cell without the need for processing and activate antigen-specific T cell lines and hybridomas (413). Therefore, if γδ T cells activated for professional antigen presentation more closely resemble mature than immature dendritic cells it then follows that the MHC class II molecules expressed on their surface may not be empty.

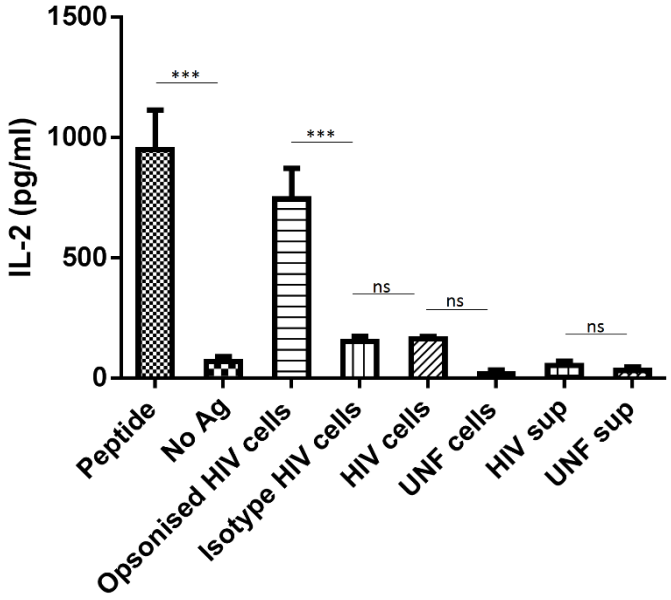

Figure 6.9 – HIV-1 RT is presented by MHC class II in monocytes after co-culture with opsonised HIV-1 infected targets.

It was hypothesized that antigen may be acquired by the effector cell during ADCC. To test whether this antigen can be presented by MHC class II on the effector cell an antigen presentation assay was developed using a HIV-1 RT specific, HLA-DRB1*0101-restricted CD4 hybridoma cell line. Monocytes have been reported to act as APCs with this hybridoma cell line. To validate this assay, I magnetically isolated monocytes from HLA-DRB1*0101 donors. 10^5 monocytes were co-cultured with 10^4 mab2G12 opsonised HIV-1 infected H9 cells in triplicate wells. Alternatively, wells received either non-opsonised HIV infected cells, uninfected targets, supernatant from HIV-1 infected and uninfected cultures, or an HIV-1 RT peptide. After 4h, 10^5 HLA-DRB1*0101-restricted HIV-1-specific CD4 hybridoma cells were added to all wells. Supernatants were collected at 24h and analysed for mouse IL-2 release by ELISA. One representative experiment out of a total of 3 is shown. UNF = uninfected

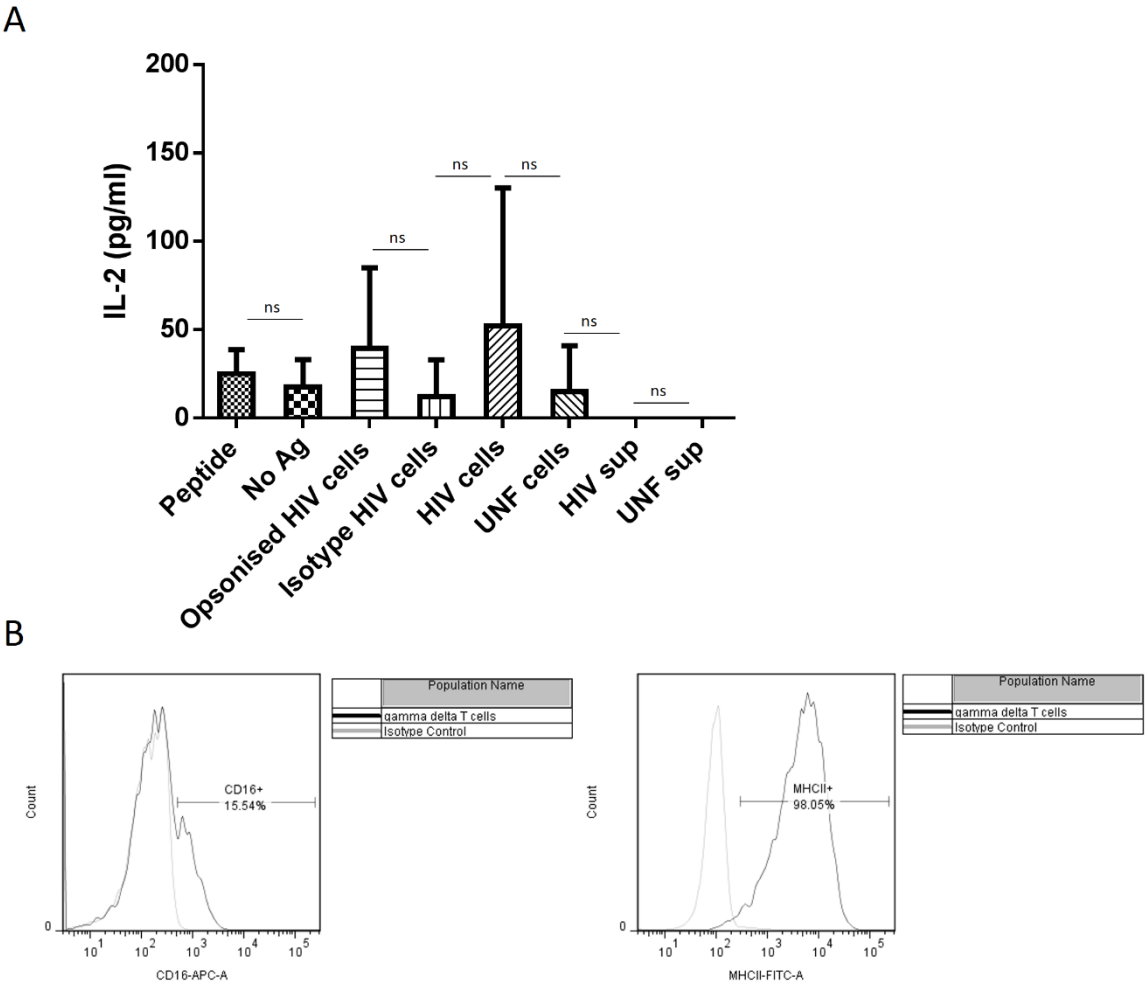

Figure 6.10 – Activated MHC class II+ γδ T cells did not present HIV-1 RT after co-culture with opsonised HIV-1 infected targets

To test whether γδ T cells can present antigen acquired through ADCC interactions, γδ T cells were co-cultured with HIV-infected targets and class II antigen presentation was assessed with an HIV-specific CD4 T cell hybridoma. (A) γδ T cells were isolated from healthy donors by MACS and matured into an APC phenotype in the presence of IPP, IL-2 and non HLA-DRB1*0101 B-LCL cells. 10^5 γδ T-APCs were then co-cultured in triplicate wells with 10^5 mab2G12 opsonised HIV-1 infected H9 cells. Alternatively, wells received either non-opsonised HIV infected cells, uninfected cells, supernatant from HIV-1 infected and uninfected cultures, or an HIV-1 RT peptide. After 4h, 10^5 HLA-DRB1*0101-restricted HIV-1-specific CD4 hybridoma cells were added to all wells. Supernatants were collected at 24h and analysed for mouse IL-2 release by ELISA. One representative experiment out of a total of 3 is shown. (B) Mature γδ T cells were stained with CD16 and MHC class II antibodies and tested by flow cytometry. UNF = uninfected

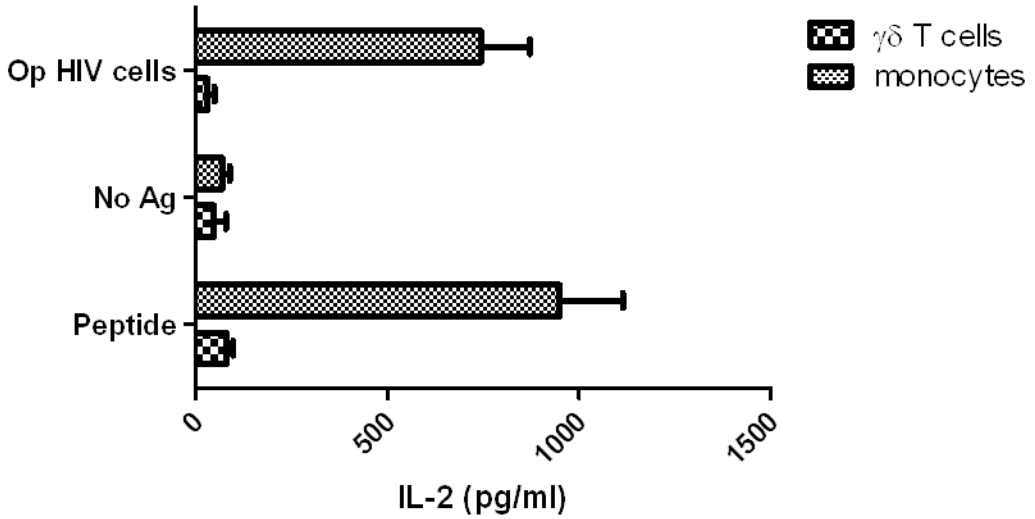

Figure 6.11 - Comparison of MHC class II presentation of HIV-1 RT by freshly isolated γδ T cells and monocytes from the same donor.

γδ T cells and monocytes were isolated by MACS from PBMCs from HLA-DRB1*0101 donors. 10^5 freshly isolated cells were seeded per well and incubated with either 10^5 opsonised HIV-1 infected H9 cells, peptide positive control or no antigen. After 4h, 10^5 HLA-DRB1*0101-restricted HIV-1-specific CD4 hybridoma cells were added to all wells. Supernatants were collected at 24h and analysed for mouse IL-2 release by ELISA. One representative experiment out of a total of 2 is shown.

6.11 Summary

- Effector memory and central memory CD16+ γδ T cells are present in freshly isolated γδ T cells from healthy donors
- Target cell antigen may be acquired by cytotoxic γδ T cells through ADCC interactions
- A flow cytometry-based alternative to the chromium release assay was established to measure ADCC against HIV-1 infected cells
- Monocytes successfully presented HIV-1 target cell associated antigen on MHC class II and presentation was significantly enhanced by opsonisation of the target cells with an ADCC-mediating antibody
- No γδ T cell ADCC against HIV-1 infected cells was detected
- No γδ T cell MHC class II presentation of HIV-1 target cell associated antigen was detected

The role of γδ T cells as cytotoxic effectors is well-documented *in vitro* (126;147;148;151;152;155-157;379;414-417) and supported by *in vivo* evidence in a murine cutaneous tumour model (145) and in humans in cancer clinical trials in advanced renal cell carcinoma and hormone-refractory prostate cancer (418;419). The experiments described in this chapter investigated whether antigen from cytotoxic target cells could be acquired via NK-cell receptors such as CD16 expressed on γδ T cells and later presented on MHC class II to CD4 T cells. CD16 was hypothesised to be a candidate receptor for antigen uptake in γδ T cells as it mediates phagocytosis in human macrophages and a subset of monocytes (420-422) and it can mediate phagocytosis when transduced into the non-phagocytic CHO cell line (302).

Previous reports in the literature disagree on whether the Fcγ receptor CD16 is (159;160;223-225) or is not (158;226) present on peripheral blood γδ T cells in healthy donors. In this chapter, freshly isolated γδ T cells were tested for expression of CD16. In agreement with most of the reports in the literature, an average of 35.96% of γδ T cells (range 12.30 – 71.57%) compared to 3.73% of αβ T cells (range 0.53 – 7.54%) from the six healthy donors tested expressed CD16 on their cell surface (Figure 6.2A).

CD16+ γδ T cells were also examined for levels of TCR expression and the presence of T cell differentiation markers. A previous report by Angelini et al (159) that examined CD16 expression in γδ T cells from freshly isolated PBMCs in twenty-six healthy donors, suggested CD16+ γδ T cells could express low levels of TCR and exhibit TEMRA T cell differentiation markers. In the experiments described in this chapter, CD16+ γδ T cells were found in both the γδ TCR^{high} and γδ TCR^{low} subsets but the latter contained brighter CD16+ cells in most donors, in agreement with Angelini et al.

Studies documenting TEMRA αβ T cells suggest that CD8+ TEMRA accumulate with old age (423). CD8+ TEMRA respond rapidly to cytokines and express high levels of the IL-15 receptor but have a lower ability to proliferate, a lower resistance to cell death, and express lower levels of the anti-apoptotic Bcl-2 molecule, as compared to naïve, central memory and effector memory CD8+ T cells (424). They are thought to be generated from a TCM subset upon homeostatic proliferation in the absence of antigen and migrate preferentially to inflammatory sites (424). Similarly to the previous studies on CD8+ αβ T cells, Caccamo et al showed lack of proliferation in TEMRA γδ T cells upon TCR stimulation with phosphoantigen and IL-2 (153). These cells did divide in response to IL-15 alone but did not accumulate in culture due a high rate of cell death and correspondingly low expression of the anti-apoptotic Bcl-2 protein. These results suggest that TEMRA γδ T cells are more susceptible to apoptosis than the other γδ T cell subsets. Therefore, if CD16+ γδ T cells do belong exclusively to the TEMRA phenotype then they would be expected to be short-lived cells, relatively rare, and with a low proliferative capacity and therefore difficult to expand *in vitro* for larger studies on CD16-mediated phagocytosis and antigen presentation by γδ T cells.

In this work, it was found that CD16+ γδ T cells were as likely to belong to the TEMRA subtype as to the TEM subtype (mean 24.48% for TEM versus 27.35% for TEMRA) with high donor variability for both (range 3.24-45.64% for TEM and 7.18-72.23% for TEMRA) (Figure 6.3). In addition, CD16+ γδ T cells were also represented in the TCM pool (mean 44.54%, range 8.19-83.45%). These findings were therefore not in agreement with Angelini et al's previous report suggesting that CD16 can be used experimentally to discriminate TEMRA γδ T cells from the other Tnaïve, TCM and TEM γδ T cell subsets (159). The presence of different differentiation phenotypes on CD16+ γδ T cells from different

donors may affect the outcome of experiments testing for functions such as phagocytosis, ADCC and antigen presentation of IgG-coated targets. For instance, TEM γδ T cells have been described to have a preference for cytokine responses over cytolytic responses whereas the opposite was found to be true for TEMRA γδ T cells (153;159). Functional responses following CD16 ligation could also differ between the two effector subtypes. For example, *in vivo* CMV-expanded γδ T cells from transplantation recipients with CMV infections have been found to express CD16 but interaction with IgG-opsonised CMV infected targets resulted in IFN-γ production rather than ADCC (160), suggesting that the CMV-expanded γδ T cells could have belonged to the TEM subset.

One difference between the experiments described in this chapter and the reports of Angelini et al (159) and Caccamo et al (153) is that I first gated using a pan-γδ TCR antibody whereas these authors first gated on Vγ2+ cells. Vγ2+ cells reportedly make up the vast majority of γδ T cells in healthy adult peripheral blood (83;84) and so it would be difficult to conclude that CD16 expression in TEM and TCM γδ T cells is largely accounted for by non-Vγ2+ subsets. Other subsets of γδ T cells include Vδ1+ cells or the less well studied Vδ3+ cells which have both been implicated in protective responses against herpes viruses such as CMV (73;77-82). The predominance of Vγ2+ cells in adult life is thought to be shaped by the encounter of Vγ9Vδ2 antigens in early life (86). These antigens are found in many pathogenic and commensal bacteria (87). However, an environment in which other non-Vγ2 γδ TCR antigens were prevalent could shape the γδ TCR repertoire in a different way, and a predominance of non-Vγ2 subsets has been described in a study of healthy adults in Ghana (85).

Overall, the phenotyping studies described in this chapter demonstrate that effector memory and central memory CD16+ γδ T cells are present in freshly isolated γδ T cells from healthy donors. Therefore, the freshly isolated γδ T cells used throughout this thesis contain cells readily available for immediate effector function including CD16-mediated cytotoxicity and conceivably uptake of IgG coated antigen.

In order to investigate the acquisition of antigen from IgG opsonised target cells by γδ T cells, microscopy experiments were set up using Rituximab-coated Daudi cells as targets instead of mab2G12-coated HIV-1 infected H9 cells due to the sharps hazard presented

by glass slides in the presence of infectious HIV-1. I was interested in whether previously published results documenting Daudi cell antigen uptake by IPP-activated γδ T cells could be replicated (374). γδ T cells recognise and kill Daudi cells via recognition of heat shock proteins (hsp60) on the surface of Daudi cells via the Vγ9Vδ2 TCR (377;378) and have also been shown to recognise and kill Daudi cells coated with Rituximab, an anti-CD20 monoclonal antibody, via their CD16 receptor (152). Daudi cells and γδ T cells were fluorescently labelled and co-cultured in microscopy slides. Fluorescently labelled Daudi cell fragments were found in a proportion of γδ T cells (Figure 6.4). This was partially blocked by cytochalasin D, a drug that blocks actin polymerisation consequently preventing active cell movement. This suggests acquisition of Daudi cell fragments was not a passive phenomenon and likely involved either cell-cell contact or an active membrane remodelling process such as in receptor-mediated internalisation of antigen. These results agree with previously published data (374).

The fate of the acquired antigen in the cytotoxic effector γδ T cell is not known. The use of a lipophilic dye in both this and the previously published study (374) means there is a possibility of passive dye transfer between membrane lipids during cell-cell contact. However, another possibility is that antigen could indeed be acquired by the effector γδ T cell and enter the exogenous antigen presentation pathway. There is some evidence to suggest that NK-receptor mediated lysis of influenza-infected cells can result in the acquisition of hemagglutinin by NK cells and subsequent induction of specific T cell proliferation through NK cell antigen presentation (258).

Therefore, a model was developed in which antigen acquired through interactions between IgG opsonised targets and CD16 on γδ T cells could be detected on MHC class II (Figures 6.9-6.11). This model was developed using HIV-1 infected H9 target cells, the mab2G12 monoclonal antibody, and the 1ACD5 HIV-1 RT-specific T cell hybridoma, to allow for testing of cell-mediated cytotoxicity and antigen presentation using cytotoxic effector cells as APCs. In addition, a flow cytometry alternative to the chromium release assay was adapted from previously published assays (390;391) to measure cell-mediated cytotoxicity of HIV-1 infected cells in Category III containment. The experiments were able to detect cell-mediated cytotoxicity by γδ T cells and NK cells but not ADCC (Figures 6.7-6.8). This result may have been caused by these particular target cells being highly susceptible to

recognition and killing triggered via cytotoxicity receptors other than CD16. It is possible that target cells were recognised via ADCC interactions but that ADCC was in direct competition with other NK-receptor recognition mechanisms. CEM.NKR cells have been infected by HIV-1 and used to detect ADCC with the mab2G12 antibody because they have a low background susceptibility to cytotoxicity via NK receptors (364). Therefore, an improvement to the model described in this chapter would be to acquire CEM.NKR cells and use the supernatant from HIV-infected H9 cells to infect them to then use HIV-infected CEM.NKR cells as ADCC targets and a source of antigen for subsequent antigen presentation assays.

The HIV-1 RT model was used to test for class II presentation of the target cell associated antigen following a 4h co-culture of effectors and targets in the presence or absence of target cell opsonising antibody. MHC class II presentation of HIV-1 RT antigen was detected in monocytes and was greatly enhanced when the target cells were coated with antibody (Figure 6.9) suggesting that antibody coating successfully promotes productive HIV-1 RT antigen internalisation or routing for MHC class II presentation. However, no class II presentation of HIV-1 RT by γδ T cells could be detected (Figure 6.10-6.11). This was the case for both γδ T cells that were pre-cultured overnight with IPP, IL-2 and B-LCL feeder cells in order to induce maturation of a γδ T-APC phenotype (223;233;244;246;247) and for freshly isolated γδ T cells. Nearly all γδ T-APCs expressed MHC class II as detected by flow cytometry (Figure 6.10).

Although presentation of HIV-1 RT by γδ T cells was not detected in this model, there have been reports of professional antigen presentation by this cell type. Brandes and Moser first reported MHC class II presentation of tetanus toxoid (TT) and Mycobacterium tuberculosis purified protein derivative (PPD) (233) and our own group then described MHC class II presentation of M1 influenza protein (223). There have also been reports of cross-presentation of influenza (247) and the PAX-5 cancer antigen (246). HIV-1 RT may have a particular biochemical property which prevents it from being significantly presented by γδ T cells, for instance a pH-dependent enzyme might be required to process it for presentation and the necessary level of acidification of the phagosome may not be available in antigen presenting γδ T cells. In agreement with this, Meuter et al found that γδ T-APCs display delayed endosomal acidification and antigen proteolysis after FITC-BSA

endocytosis compared with monocyte-derived DCs and suggested that these conditions could favour cross-presentation as opposed to MHC class II presentation of exogenous antigen (247).

Overall, the results from this chapter do not support the hypothesis that ADCC can lead to antigen uptake and class II presentation in γδ T cells. Class II antigen presentation by monocytes was significantly increased in the presence of opsonising antibody but no significant class II antigen presentation of HIV-1 RT antigen could be detected for γδ T cells under the same conditions. Although γδ T cells express receptors such as NKG2D and CD16 that allow them to interact with tumour and infected cells and IgG-coated particles, they might be restricted in the type of antigen that can be presented due to differences in their ability to process particular antigens as compared to professional antigen presenting cells.

7 Discussion

The discovery of T and B cells dates back to the seminal work of JFAP Miller who first demonstrated in 1961 the essential role of the thymus in immune function by showing that mice thymectomized immediately after birth had poorly developed lymphoid tissues, impaired immune responses and a high susceptibility to infection (425). He ultimately proposed the existence of two major subsets of lymphocytes in mammals, bone marrow-derived antibody-producing cells and thymus-derived helper cells (426;427). At this time there was a great interest in transplantation and in the immunological basis for graft rejection. Later advances demonstrated MHC restriction in T cell effector function (428;429) and revealed the basis of TCR diversity through the cloning and analysis of the genetic organization of the TCR β chain (430-432). It was unknown how T cells depended on the appropriate MHC being expressed by the target cell until a landmark paper by Bjorkman et al in 1987 which presented the first crystal structure of an MHC protein (433) leading the authors to postulate that TCR engages the peptide-binding groove of MHC along with bound peptide (434). These advances paved the way to a flurry of research on T cell function, including the molecular basis for T cell activation (352), the identification of different T cell subsets based on different effector functions (435;436), the stages of T cell development in the thymus (437;438), the development of tolerance towards self (439) and the induction and maintenance of T cell memory (440).

However, all these studies on T cell function relate to αβ T cell function. The cloning of the γ chain of the TCR in 1984 (53) led to the discovery of γδ T cells but did not generate as much interest as αβ T cells as these cells were rare, their immunological function was poorly understood, and they were not restricted by MHC. Therefore, despite 30 years of study, many aspects of γδ T cell biology remain elusive. It appears that γδ T cells stand somewhere at the interface between αβ T cells and NK cells in terms of their function. Like NK cells, γδ T cells rapidly produce cytokines upon recognition of conserved microbial and endogenous danger signals, and they are potent killers of infected and tumour cells. Like αβ T cells, they carry variable antigen receptors that are generated from rearranged gene segments coding for variable (V), diversity (D), joining (J) and constant (C) genes. γδ T cell receptors (TCRs) are highly limited in their diversity when compared to αβ TCRs and many recognise conserved molecules similarly to pattern recognition

receptors. Similarly to αβ T cells, γδ T cells exhibit immunological memory, as antigen-experienced γδ T cells remain after a primary infection and respond more rapidly to a second challenge with the same pathogen (130;441-443). γδ T cells are therefore unusual in that they combine expression of adaptive immune receptors with rapid innate-like responses. Interestingly, the tripartite division of the adaptive immune compartment into αβ T cells, γδ T cells and B cells seems to be highly conserved among jawed vertebrates and a similar tripartite division is present in primitive jawless vertebrates such as the lamprey (58). This high degree of conservation suggests that a tripartite organisation may be optimal for adaptive immune function. Therefore, even though at first glance most of the functions performed by γδ T cells overlap with αβ T cells and NK cells, they may have unique roles in immunity that elude us at present.

The work described in this thesis follows on from two interesting publications describing novel dendritic cell-like properties of human blood γδ T cells. In 2005, Brandes et al (233) were the first to report professional antigen presentation function by *in vitro* activated human blood γδ T cells. Wu et al (223) then described professional phagocytosis and antigen presentation in these cells. Although no contradicting reports have subsequently been published, the hypothesis that γδ T cells are professional phagocytes and professional antigen presenting cells remains controversial in the field. One of the difficulties is that it is not always obvious how to translate *in vitro* findings to an understanding of the immune system as a whole. Additionally, the word "professional" is largely qualitative in this context so it is difficult to objectively determine whether or not γδ T cells are professional phagocytes or professional APCs.

The word "professional" in professional phagocytes or professional antigen presenting cells is used to convey information about the role that we believe these cells have in the overall immune response. The main role that professional phagocytes perform *in vivo* is to capture and destroy bacteria and dead cell debris. Typically, this "professional phagocytes" category would include neutrophils and the cells of the mononuclear phagocyte system such as monocytes, macrophages and immature dendritic cells. Upon phagocytosis, immature dendritic cells undergo maturation and migrate to draining lymph nodes, where they present the exogenous antigen they acquired to antigen-specific αβ T cells, thereby linking innate immune function – phagocytosis – with adaptive immune function – antigen-

specific responses. B cells also capture and present antigen at lymph nodes to antigen-specific αβ T cells, and macrophages phagocytose and present antigen at other tissue sites where they reside. Dendritic cells are the initiators of adaptive immunity as they are thought to be the only APC capable of activating naïve T cells. B cells and macrophages activate effector and memory T cells. Together, dendritic cells, B cells and macrophages orchestrate the antigen-specific immune response and are called "professional antigen presenting cells".

In order to advance our understanding of phagocytosis and exogenous antigen presentation via class II in γδ T cells beyond the previous reports that described these phenomenon largely qualitatively, the experiments described in this thesis were designed to quantify these activities in γδ T cells and to compare them with other cells of the immune system.

In the work described in this thesis, phagocytosis by γδ T cells was quantified by a number of methods using microscopy, flow cytometry and microbiology techniques. Although phagocytosis by γδ T cells could be detected using these methods, the number of phagocytosing cells was small and not significantly different from αβ T cells and NK cells. The numbers of γδ T cells found to phagocytose varied from approximately 1 in 67 cells to 1 in 40 cells but since γδ T cell isolation typically results in purities of 96-98%, 1 in 25 cells to 1 in 50 cells could have been non-γδ T cell contaminating cells. The combination of apparently low phagocytic activity by γδ T cells and the presence of contaminating cell types means that the true number of phagocytic γδ T cells is difficult to discern. An assay using whole blood did result in statistically significant numbers of γδ T cells found to phagocytose, at an average frequency of 12% of γδ T cells. These phagocytic γδ T cells additionally produced reactive oxygen species upon bacterial engulfment. However, these phagocytic γδ T cells could only be isolated to a low purity (6.25%) with strong granulocyte contamination (54.69%) found in the phagocytic CD3+ γδ TCR+ FACS sorted fraction. Hence, these results would need to be confirmed by repeating the same experiments but additionally including an exclusion marker for granulocytes such as CD15 or by using PBMC fractions instead of whole blood, since these are typically depleted of granulocytes. Light microscopy inspection of the FACS sorted fractions following staining with haematology stains revealed an unusual morphology, with the largest group of FACS sorted

phagocytic CD3+ γδ TCR+ cells composed of cells with multi-lobed nuclei, again suggesting the contamination of this fraction with granulocytes. Interestingly, previous reports in the literature had suggested the presence of a small proportion of γδ TCR+ cells in the granulocyte fraction of healthy white blood cells. I investigated whether a γδ TCR could be found in granulocytes by flow cytometry and Western blot but did not find any evidence of such. These results highlight the fact that the research of rare cell phenotypes is made difficult by the technical limitations of the experimental techniques available. In particular, cell separation procedures do not yield 100% purities and fluorescence-based methods can be confounded by cell types with strong autofluorescence. Importantly, the lower the frequency of the cell population of interest the higher the statistical impact that these factors could have on the resulting data.

In my opinion the levels of phagocytosis found experimentally in γδ T cells indicate that they do not warrant the "professional" phagocyte label as professional phagocytes tested in parallel such as monocytes were consistently much more efficient at phagocytosis than γδ T cells. For instance, monocytes phagocytosed bacteria 24-fold more frequently than γδ T cells in a blindly-scored microscopy assay, and required 100-fold less bacteria than γδ T cells for phagocytosis to be detectable in a gentamicin protection assay. Although there have been no further reports of phagocytosis by γδ T cells in the literature since the beginning of the work described in this thesis, there have been novel reports of professional antigen presentation performed by *in vitro* activated γδ T cells (244;246;247). Therefore, since antigen uptake is a pre-requisite for antigen presentation it could be tested in *in vitro* activated γδ T-APCs as a future avenue of research.

In further work described in this thesis I investigated class II antigen presentation by human blood γδ T cells. The results described in this thesis support the earlier findings of Wu et al (223) by replicating MHC class II antigen presentation of influenza M1 by γδ T cells. In addition, γδ T cells were compared with other immune cells in their ability to present via class II. γδ T cells presented significantly more antigen than αβ T cells, NK cells and less than monocytes tested in parallel. In addition, they upregulated the APC markers MHC class II, CD80 and CD86 after overnight activation with the Vγ9Vδ2 TCR agonist IPP and IL-2, in the presence of B-LCL feeders. Therefore, this thesis supports the notion that γδ T cells are capable of MHC class II antigen presentation to αβ T cells.

The requirements for antigen presentation by γδ T cells are not fully understood. A transcriptome analysis reported by Pont et al (251) found that APC markers were not expressed by human blood γδ T cells after IPP and IL-2 activation, which suggests a critical role for B-LCL help in the maturation of γδ T cells into an APC phenotype. In culture, γδ T cells form tight clusters with B-LCL cells (444). It is therefore likely that these cells provide critical cues that induce the maturation of γδ T cells, which may be triggered by cell surface receptor interactions or soluble mediators. Whether a different feeder cell type would be able to support a γδ T-APC functional switch has not been explored but it would be important to research the signals that govern γδ T-APC maturation in order to understand the timing and location of class II antigen presentation by γδ T cells in the overall immune response. The rapid kinetics of γδ T cell activation suggest that antigen presentation function by this cell type may take place shortly after activation during an immune response, contrary to αβ T cells, which upregulate APC markers more slowly (320). Therefore, γδ T cell antigen presentation could serve to potentiate the immune response as opposed to dampening it during the later contraction phase.

During my experiments I detected class II antigen presentation by freshly isolated γδ T cells whereas previous authors had only tested class II antigen presentation in γδ T cells activated *in vitro* with IPP, IL-2 and B-LCL feeders. It is possible that the cross-linking of Fcγ receptors expressed on the freshly isolated γδ T cells with IgG-opsonised antigen-coated beads could activate the γδ T cells to upregulate MHC class II and therefore potentiate antigen presentation by these cells. There is evidence that Fcγ receptor cross-linking on γδ T cells can lead to IFN-γ (155;159;160) production by these cells, and IFN-γ potently induces the expression of MHC class II genes in most cell types (445). Similarly, activation via NK cell receptors has been previously reported to upregulate APC markers in NK cells, endowing them with a transient ability to present antigen via class II (259).

It is conceivable that the local microenvironment at an infection site or tumour site could stimulate the induction of an APC phenotype in γδ T cells (and perhaps NK cells) through the interaction of these cells with antibody-coated targets and NK receptor ligands. This APC function may be a short-lived phenomenon but may nonetheless contribute to the clearing of infection by promoting local antigen-specific CD4+ αβ T cell responses. Accordingly, Himoudi et al showed that the removal of the antibody coated target cell

reverted the induction of the co-stimulatory molecules CD80 and CD86 in antigen presenting γδ T cells (246). In addition, *ex vivo* data tracking the phenotype of bovine γδ T cells over the course of a foot-and-mouth virus infection described high cytotoxicity and acquired MHC class II antigen presentation function during the acute phase of infection (260). γδ T cell cytotoxicity and MHC class II expression peaked at day 2 post-infection, and MHC class II antigen presentation was highest at day 3. However, by day 5 post-infection, γδ T cells were found to have lost their antigen presenting ability together with MHC class II expression. It is possible that γδ T-APCs had migrated to draining lymph nodes and were therefore not found in the blood samples collected at that point. However, a different interpretation, which is also in agreement with Himoudi et al's data, is that it is the continuous stimulation at the local infection or tumour tissue that stimulates an APC phenotype in cytotoxic effector cells. Therefore, class II antigen presentation by these cells may take place locally as migration to draining lymph nodes would result in the loss of continuous contact with target cells which may be required for the maintenance of an APC phenotype.

In this thesis it was hypothesised that the ADCC receptor CD16 might be able to mediate antigen uptake and promote APC maturation in γδ T cells. Therefore, experiments were designed in order to investigate whether a correlation between ADCC and MHC class II antigen presentation by γδ T cells could be detected. ADCC was measured by co-culturing γδ T cells with opsonised and non-opsonised HIV-infected targets and quantifying the proportion of lysed targets. MHC class II antigen presentation by γδ T cells was detected by adding an antigen-specific CD4 T cell hybridoma after 4h co-culture, which responds specifically to presentation of HIV-1 RT on HLA-DRB1*0101. NK cells and monocytes were used as positive controls for ADCC and antigen presentation, respectively. The uninfected and HIV-infected lymphoma cells used as targets showed a high susceptibility to lysis by NK cells and γδ T cells in the absence of opsonising antibody and no ADCC was detected with either effector cell type. MHC class II antigen presentation of target cell antigen by monocytes was significantly potentiated by the opsonisation of target cells, suggesting that IgG – Fcγ receptor interactions can increase productive HIV-1 RT antigen internalisation or routing for MHC class II presentation. However, no significant MHC class II antigen presentation was detected in γδ T cells.

One possible explanation for the lack of MHC class II antigen presentation observed in the HIV model is that γδ T cells may be restricted in the type of antigen they can process and present by the intracellular machinery they express. For instance, acidification of the phagosome has been shown to vary in intensity and timing between neutrophils, macrophages and dendritic cells (23). Different antigens may require different enzymes for processing which are optimal at different pH. Meuter et al followed endosomal acidification in γδ T cells and dendritic cells pulsed with FITC-BSA and concluded that γδ T cells display a delayed endosomal acidification when compared to dendritic cells (247). Therefore, the types of antigen that can be presented by γδ T cells may be restricted by the intracellular processing machinery they express.

A major technical challenge during research with human γδ T cells is their rarity. From a collection of 50 ml of blood from a healthy donor it is usual to recover approximately $3x10^5$ to $3x10^6$ γδ T cells. The rarity of these cells imposes limits on the experimental techniques that can be used to study them. Microscopy techniques are particularly attractive since these require small numbers of cells. However, microscopy is labour-intensive and presents a risk of operator subjectivity during image analysis. To minimise this subjectivity experiments can be designed so that image analysis is performed blindly as in the quantitative confocal phagocytosis assay described in this thesis. Another way to study rare cell types is to minimise the number of cell isolation processing steps to avoid cell loss. An example is the whole blood phagocytosis assay described in this thesis, where freshly taken whole blood samples were tested in a phagocytosis assay by adding fluorescent bacteria to them and later stained for cell surface markers. Wash steps were kept to a minimum and a one-step red blood cell lysis and fixative solution was used at the end. However, when I FACS sorted phagocytic CD3+ γδ TCR+ cells directly from whole blood for further characterisation only 300-500 cells from this fraction were recovered. This made it difficult to test for purity and be able to clearly interpret the results.

γδ T cell isolation from the small number of HLA-DRB1*0101+ donors available did not generate the required numbers of γδ T cells needed for the M1 antigen presentation assay as described by Wu et al (223). Therefore, when testing for antigen presentation in this model I reduced the number of γδ T cells to $2x10^4$ per well, one fifth of what was originally described by Wu et al. I also reduced the number of responding CD4+ T cells to $2x10^4$ per

well and adjusted the volume accordingly, to maintain the same cell concentration and APC to CD4 T cell ratio as in the original assay. The same number of cells was also used for monocytes, αβ T cells and NK cells tested in parallel for antigen presentation ability in the same assay. This difference may have affected the experiments in some way as my M1 antigen presentation results were reproducibly lower than those described by Wu et al. The frequency of the DRB1*0101 allele is relatively high at 8.39% of Caucasians, 4.6% of Hispanics, 3.0% of African-Americans and 3.0% of Asian-Pacific Americans as calculated using the bioinformatics tool available in the United States of America National Marrow Donor Program "Be the Match" (https://bioinformatics.bethematchclinical.org). However, this frequency means that a limited number of suitable donors could be recruited and that using non-HLA typed leukocyte cones from the National Blood Service would not be practical. It is possible that the previous authors' study used HLA-DRB1*0101+ donors with a higher circulating number of γδ T cells than the ones I used as the frequency of γδ T cells in the blood can vary substantially between donors.

To test for cell-mediated cytotoxicity and ADCC I initially used γδ T cell cultures expanded with IPP, IL-2 and B-LCL feeders for two weeks. The expanded cultures had variable levels of CD16 expression and, although cytotoxic, could not be seen to present antigen via class II. For this reason, I decided to use freshly isolated γδ T cells or overnight activated γδ T cells for the experiments described in this thesis. Expression of CD16 on a proportion of γδ T cells was confirmed by flow cytometry prior to cytotoxicity experiments.

An interesting line of work in the future could be to develop a γδ T cell expansion protocol able to promote the accumulation of CD16+ γδ T cell effector cells in culture. Cytotoxic CD16+ effector γδ T cells are described to be short-lived terminally differentiated cells with a low potential for proliferation and hence would likely need to be generated from a precursor pool, such as expanded central memory T cells. Central memory Vγ9Vδ2 T cells have been shown to give rise to terminally differentiated effector memory cells in the presence of IL-15 and in the absence of antigen (153). Therefore, one possible approach could be to induce clonal expansion of central memory Vγ9Vδ2 T cells with IPP and IL-2 and then induce differentiation of these cells with IL-15.

With respect to the broader hypothesis that human blood γδ T cells perform professional phagocytosis and antigen presentation *in vivo*, both phagocytosis of opsonised bacteria and MHC class II antigen presentation could be reliably detected in γδ T cells *in vitro* during the work described in this thesis. However, the phagocytic events were rare relative to monocytes and although presentation of exogenous antigen was significantly greater for γδ T cells than for αβ T cells or NK cells it was also lower than for monocytes. Therefore, the data described in this thesis do not support previous literature reporting that the potency of antigen presentation performed by *in vitro* activated γδ T-APCs is comparable to that of mature dendritic cells (233;244;246;358). It is possible that these myeloid functions may be restricted to a particular type of γδ TCR-expressing cells as discussed in a review by Kabelitz and He (446). Further studies of subsets of γδ T cells may identify functional differences that may help resolve the question of the non-redundant role of γδ T cells and why the tripartite division of adaptive immunity into αβ T cells, γδ T cells and B cells is so well conserved in evolutionary terms.

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