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**EARLY T-PRECURSOR THYMOCYTES AS POTENTIAL TARGET CELLS
FOR HIV INFECTION (AIDS)**

MIDTERM REPORT

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Overview of research:

The research programme over this 1½ year period has followed very closely our original proposal, where we aimed to isolate very early T precursors from the human thymus, to determine if they expressed CD4 as in the murine thymus, and then to test if they could be targets for HIV infection. However, several of our findings were unexpected, although still very pertinent to the current views on the development of AIDS. This will lead to some shift of emphasis in the second half of the grant period.

We have prepared and conjugated all the monoclonal antibody reagents required for this previously murine T-cell laboratory to work on the human thymus. We have secured a reliable supply of infant thymus tissue. We have carried out a thorough flow cytometric analysis of human thymocyte populations, which has led to a paper describing several new observations. We have developed procedures to deplete human thymus cell suspensions of the more developed T-lineage cells, in order to obtain access to the very few early T precursors. The depleted populations contained many cells expressing CD4. We have analyzed these depleted preparations and isolated one subpopulations which had many of the characteristics we expected of an early T-lineage cell.

However on closer analysis this proposed early T precursor appears to be a form of thymic dendritic cell. It expresses very high levels of CD4, although it is CD3⁻8⁻2⁻; it also expresses high levels of class I and class II MHC products, a characteristic of dendritic cells which present antigen to T cells. In line with recent work on the transfer of HIV from peripheral dendritic cells to CD4⁺ T cells, this cell could well be a target for HIV infection, and a potent system for transfer of infection to developing T cells.

Our parallel work on the mouse thymus has led to the surprising finding that thymic T cells and dendritic cells have a common origin in an early thymic "lymphoid precursor", and that the development of these two interacting cell types is closely linked. This work has been submitted for publication. Thus, in retrospect, it is not surprising that in the human thymus an early thymic dendritic cell might resemble an early T lineage cell.

In the work planned for the second half of the grant period we will continue our search for the earliest T lineage precursor cell in the human thymus, to check if it bears CD4 and is a HIV target. However, we will also work with this new CD4 bearing thymic dendritic cell, and test directly if it can be infected with HIV, and whether that infection can be transferred to T cells. This will involve collaboration with Dr. Y. Rosenberg at the Jackson Foundation, USA and with Professor J. Mills and colleagues at the Macfarlane Burnet Centre, Melbourne, Australia.

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Detailed report

1. Preliminary studies on thymic populations in the infant human thymus

This laboratory has in the past specialized in the study of T-cell development in the mouse thymus. Thus this new programme involved a 6 month "lag" period while we prepared the reagents necessary for work on the human thymus, while we established a reliable supply line of human thymus tissue, and while we carried out basic preliminary analyses on human thymocyte suspensions to familiarize ourselves with our working material. Our collaboration with Dr. A. Boyd of this Institute, whose laboratory works with human lymphoid cells, greatly helped us during this preliminary phase.

Although we have purchased some of the monoclonal antibodies (mAb) required for our work, we made a strategic decision to obtain most of the hybridoma clones and to grow, purify, conjugate and calibrate most of the reagents ourselves. This represented a large financial saving, but took time. It has also given us considerable versatility: each mAb has been conjugated to biotin or directly to other fluorochromes, so we now have several choices of fluorescent colour for each mAb. This flexibility is essential for multiparameter flow-cytometry analysis, and in most cases the commercially available fluorescent mAb do not provide these options. Our present battery of mAb reagents is summarized in Table 1.

A good working rapport has been established with the cardiac surgeons at the Royal Children's Hospital, Parkville, Victoria. Samples of infant thymus (donors 1 month to 15 yr of age) are provided approximately twice a week, and are kept on ice until collection. The only practical problem is the arrival time of tissue in our laboratory, between 2 pm and 5 pm. To date we have preferred to work with tissue as fresh as possible, so most of the subpopulation isolation has been carried out during the night, ready for flow-cytometric analysis the next morning. Thanks to the enthusiasm and dedication of a student, Mr. Frank Sotzik, this approach has been working effectively.

Prior to attempting an isolation of very rare (<1%) early precursor populations, we carried out a thorough three-colour immunofluorescent analysis of the total human thymocyte population, using many of the standard markers. This was to "get our bearings", by comparison with our results using murine thymus. However, we realized that few laboratories had in fact carried out such an extensive study, and some new findings emerged. In particular we noted:

(1) The CD4⁻8⁻3⁻ thymocytes, the "conventional" early precursor population, did include some cells that lacked expression of the thymocyte T-lineage markers CD1, CD2 and even CD7. These cells were potentially very early T-lineage cells (prior to acquisition to these markers) or were non-T lineage cells (macrophages, dendritic cells, B cells or stromal cells).

(2) A population of CD4⁺8⁻3⁻ blast cells was observed, corresponding to one of the two types of "immature single positives" recently described in the murine thymus by our laboratory and other groups. This dividing cell population is a potential HIV target.

(3) Although mature peripheral T cells were, as expected, CD1⁻, the majority of mature medullary thymocytes (CD4⁺8⁻3⁺ and CD4⁻8⁺3⁺) expressed CD1. This result differs from the assumptions in most models of T-cell development, which suggest CD1 is lost at the stage of positive selection where a CD4⁺8⁺ cortical thymocyte down-regulates either CD4 or CD8.

These results on CD1 expression, although novel, are of no obvious direct relevance to HIV-infection and are not being pursued further. They have been written up (draft manuscript appended) and will be submitted for publication before 1 February of this year.

2. Depletion of the more differentiated thymocytes from human thymus cell suspensions

Whilst it is possible to sort rare cell populations directly from thymus cell suspensions following 3 or 4 colour immunofluorescent labelling, this becomes a very expensive and time-consuming exercise if the populations are <1% of the total cells. Accordingly we developed preliminary depletion procedures, similar to those we used successfully with murine thymocytes. There were three main problems in this approach:

(1) It was not entirely clear which mAb to choose for preliminary depletion of the human thymus. Since we wished to determine if CD4 was on the earliest T-lineage cells, anti-CD4 could not be used, leaving anti-CD3 and anti-CD8 as the main mAb for depletion of mature T cells and CD4⁺8⁺ cortical thymocytes. Since it is generally considered that the order of appearance of other thymocyte markers is CD7 before CD2 before CD1, we aimed to eliminate CD2 and CD1 bearing CD4⁻8⁻ thymocytes, leaving only the earliest CD7⁺ T precursors. However, Terstappen et al, (Blood 79, 666, 1992) have reported that CD2 is present even on the earliest thymocytes, and in view of this and our subsequent findings CD2 depletion may have been an inappropriate choice.

(2) There is some evidence that depletion procedures may strip certain surface molecules off human T cells without killing or eliminating them, causing some relatively mature cells to masquerade as early precursors (Hori & Spitz, J. Immunol. 146, 2116, 1991). To check on this possibility we avoided any depletion with anti-CD1, reserving this mAb for staining the final preparation to check for contamination with mature thymocytes, CD4⁺8⁺ cortical thymocytes or the more mature CD4⁻8⁻ cells. As described later, we may have to amend this strategy.

(3) Human thymocytes are difficult to kill with antibody and complement. However, depletion with immunomagnetic beads alone (the alternative high-efficiency procedure) is extremely expensive. We have overcome these problems by: (i) using higher levels of rabbit complement to ensure lysis of human thymocytes; (ii) following the complement kill with a final immunomagnetic bead depletion, when the number of cells to be treated is markedly reduced.

Establishment of the final reliable procedure has taken considerable time, but we now are able to efficiently deplete thymocytes with anti-CD3, anti-CD8 and anti-CD2, using one round of cytotoxic complement-mediated lysis, followed by two rounds of depletion with immunomagnetic beads (anti-mouse Ig coated dynabeads). When required, additional mAb

can be included at either or both steps, to remove non-T-lineage cells. Cells bearing significant levels of the depletion markers are completely eliminated, and the recovery of cells is usually around 0.5%. This served as our source of putative very early precursor T cells.

3. Analysis of depleted thymocyte populations

The depleted populations were confirmed to be CD3⁻ CD8⁻ CD2⁻. Of these approximately 50% were CD4⁻, 25% were CD4^{low} and 25% were CD4^{high}, encouraging the view that certain early T precursors express CD4. Most of the cells were CD7⁺, suggesting they were of the T lineage. None of the cells expressed CD34, which was disappointing; we hoped to see this marker, characteristic of multipotent bone marrow stem cells, on the earliest T-lineage thymocytes. However, many of the cells expressed CD44 and high levels of class I MHC, which from our work on mouse cells was expected on the earliest T precursors.

Three-colour immunofluorescent analysis was then used to analyze the depleted population more closely. Most preparations contained three groups or populations of cells:

(1) One variable group was class I MHC^{low}, CD44⁻, CD7⁺ and CD1⁺, and expressed levels of CD4 from zero to high. These CD1⁺ cells were almost certainly a spectrum of contaminating more mature thymocytes that the depletion procedures had failed to remove.

(2) A second group of cells was CD1⁻, class I MHC^{high}, CD44⁺, CD7⁻ negative to positive, and CD4⁻. We have not characterized this population further.

(3) A third group of cells was CD1⁻, class I MHC^{high}, CD7⁺ (but low), CD44⁺, and expressed high levels of CD4. This phenotype suggested it could be analogous to the "low CD4 precursor" we had described in the adult mouse thymus, except that the level of CD4 was markedly higher. This putative "early T precursor" was then examined more closely. One example of the analysis of the depleted thymocytes is presented in Fig. 1, showing the population expressing high levels of class I MHC and high levels of CD4.

4. Further investigation of the "high CD4" putative T precursor cell

Suspicious on the nature of "high CD4" population were aroused because of its light scatter characteristics - a high forward scatter indicated a very large size (larger than most T-cell blasts), and a high side scatter (much greater than T cells) suggested either a cell containing granules or having a highly irregular surface.

The cells were negative for markers of NK cells (CD16; CD56) or macrophages/monocytes (FMC17; FMC33). However, they stained strongly for class II MHC, a characteristic of dendritic cells.

The cells were sorted and cytopins examined for characteristic enzymes, or stained with Giemsa. The cells were negative for the macrophage/monocyte enzymes myeloperoxidase and "dual esterase". Morphologically the cells were large and pale staining, frequently with ovoid nuclei; the cytoplasm contained numerous small vacuoles, but there was no evidence of phagocytic activity. Occasionally a few granules were seen in the cytoplasm. Many of the cells appeared round, but some showed an irregular surface with fine extensions and dendrites.

When cultured in agar with factors known to induce myeloid cell expansion and development, no colonies were obtained.

However, when the cells were cultured from 4 to 12 hr in medium at 37° in Terasaki tray wells, over 80% showed a typical dendritic cell appearance, with veils and multiple cytoplasmic extensions. Presumably many of the cells had rounded up during the isolation procedure in the cold, explaining the more usual round appearance in cytospin preparations.

Although we have yet to do the functional studies, we tentatively conclude these cells are thymic dendritic cells.

5. Further studies on the developmental potential of the "low CD4" T-precursor cells in the mouse.

We have maintained our parallel studies on the murine early T-precursor cells, since it serves as a guidance for the work on the human thymus. We investigated whether these thymic precursor cells, which form both $\alpha\beta$ and $\gamma\delta$ T cells if transferred into an irradiated recipient thymus, and have retained a potential to form B lymphocytes if they artificially seed the bone marrow on intravenous transfer, are capable of forming other bone marrow derived thymus populations. Specifically we asked if they served as precursors of thymic B cells, macrophages or dendritic cells. This involved transfer of the purified precursors into the thymus of congenic irradiated recipients differing at the Ly 5 locus, then analysis of these minor thymic populations for donor-derived progeny cells. It was necessary to deplete the recipient thymus of the majority of T-lineage cells before these minor populations could be analyzed.

The thymic low CD4 precursor did not reconstitute thymic B cells, despite its B-cell precursor potential in bone marrow. Nor did it reconstitute thymic macrophages. However, it was an effective precursor of thymic dendritic cells, which were shown to have a turnover time very similar to the thymic T-lineage cells derived from the same precursor preparation. As yet we lack the clonal evidence that one and the same cell was capable of producing both T cells and dendritic cells, but this is now a distinct possibility. This related origin of thymic T cells and thymic dendritic cells may explain why dendritic cells share many T-cell surface molecules (e.g. CD8 α). It also makes it less surprising that in our human thymus preparations early dendritic cells somewhat resembled early T cells.

This work has been submitted for publication in Nature. Initial reviews were favourable, but with some more information requested before acceptance. A copy of the revised, resubmitted manuscript is appended.

Future Directions

To date we have no evidence for a very early T-lineage precursor cell in the human thymus expressing CD4. However, it seems likely we may have depleted such a cell if it expresses significant levels of CD2, as Terstappen suggests. Accordingly, we will repeat our depletions, using CD1 rather than CD2 to remove the relatively late T-precursor cells. Should we still fail to find an early T-lineage cell expressing CD4, we will document this clearly, since it would suggest that, in

contrast to the mouse, the postnatal human thymic T precursors are all of the CD4⁻8⁻ category. Should we find a CD4⁺CD2⁺ putative early precursor, we will revert to our original plan of (i) testing its ability to produce T cells in SCID-hu mice, in collaboration with Dr. Y. Rosenberg, and (ii) testing directly if it can be infected with HIV in culture.

However, the surprising finding of a putative thymic dendritic cell expressing high levels of CD4 is a promising new avenue we must explore. Such a cell could well serve as an HIV target, and as a very effective way of transmitting infection to the newly-developing T cells. In the peripheral lymphoid organs there is now evidence (from Dr. Y. Rosenberg and others) that dendritic cells are involved in the final pathogenesis of AIDS. Since dendritic cells in the thymus are believed to mediate negative selection, this would have the further effect of tolerizing and deleting any newly formed CD8⁺ T cells able to destroy the virus.

We will carry out functional studies to ensure the cells we isolate are able to stimulate T cells in an MLR-like reaction. We will also test if all human dendritic cells are of this type, expressing CD4, or if we have isolated an unusual subpopulation, perhaps of immature dendritic cells. We will test if our more conventional procedures for dendritic cell isolation give a better yield of this rare cell type.

Finally, when we have an optimal isolation procedure, we will collaborate, both with Dr. Rosenberg and colleagues at the Jackson Laboratories, and with Professor Mills and colleagues at the Macfarlane Burnet Centre, to see if these dendritic cells can be productively infected with HIV in culture, and if this infection can then be transmitted to T cells.

TABLE 1 Monoclonal Antibodies Prepared to ddate

mAb specificity	Hybridoma Clone	Forms available
CD1 (thymocytes, interdigitating cells)	OKT6	Ascites, purified, biotinylated, FITC-conjugated.
CD2 (thymocytes, NK cells, peripheral T cells)	LYM1	Ascites, purified, biotinylated, FITC-conjugated
CD3 (thymocytes, peripheral T cells)	OKT3	Purified, biotinylated, FITC-conjugated.
CD4 (thymocytes, T cell subset ["helper/inducer" T cells], monocytes)	OKT4	Supernatant, purified, biotinylated, FITC-conjugated. (Also CD4-PE PURCHASED FROM DAKO).
CD8 (thymocytes, T cell subset ["cytotoxic/suppressor" T cells], NK cells)	OKT8	Supernatant, purified, biotinylated, FITC-conjugated.
CD7 (thymocytes, major T cell subset, NK cells)	3a1	Purified, biotinylated, FITC-conjugated.
CD34 (haematopoietic precursor cells)	BIC35	Purified, biotinylated, FITC-conjugated.
CD44 (leukocytes, red cells, platelets)	Hermes-3	Ascites, purified, supernatant, biotinylated, FITC-conjugated.
Class I-MHC	W6-32	Purified, biotinylated, FITC-conjugated.
Class II-MHC	2.06	Ascites, supernatant
CD14 (monocytes)	FMC17 FMC33	Ascites Ascites
CD13 (granulocytes)	WEMG1	Ascites
CD19 (B cells)	FMC63	Ascites
Glycophorin (erythrocytes)	10F7MN	Supernatant

TABLE I (cont.)

mAb Specificity	Hybridoma Clone	Forms available
CD16 (Fc receptor)	HuNK-2	Purified (purchased from Silenus, Australia)
CD56 (NK cells)	NKH-1	Purified (purchased from Coulter)
Terminal Deoxynucleotidyl Transferase	HT-1 HT-3 HT-4	Purified (purchased from DAKO)

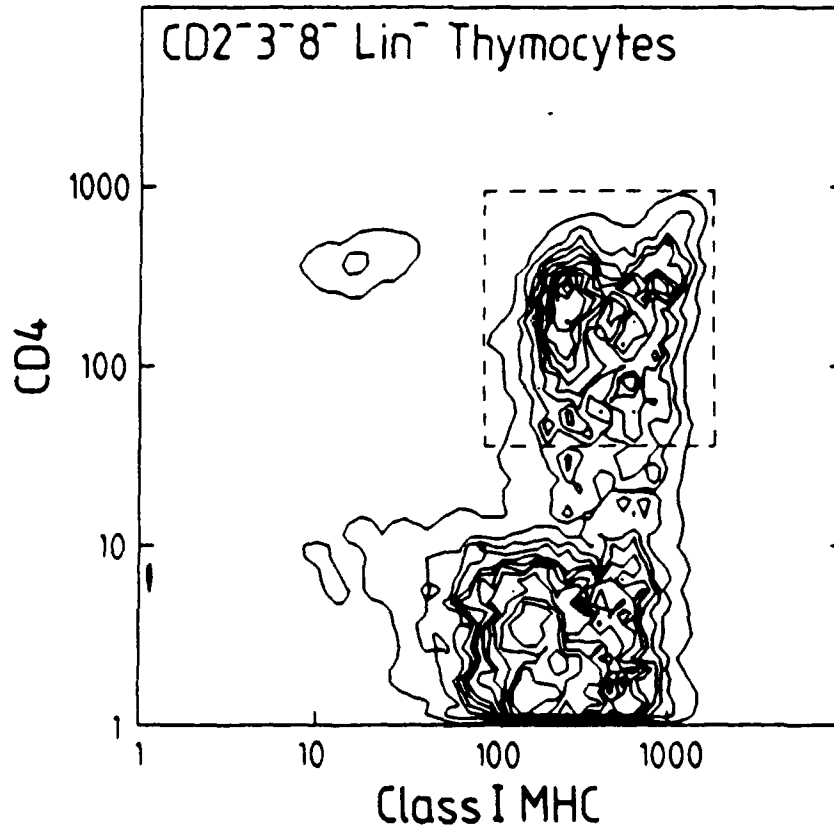


Figure 1 The distribution of class I MHC and CD4 on thymocytes depleted of cells expressing CD2, CD3 and CD8, together with depletion of macrophages, granulocytes, B cells and erythroid cells using lineage-specific markers. The broken line box indicates the population sorted and analyzed more closely. This "putative early T precursor" now seems to be a form of dendritic cell. In this experiment the level of class I MHC^{low} cells (some of which express CD4) is very low; these are more mature cell contaminants that have escaped the depletion process.