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# Immune memory in $CD4^+$ $CD45RA^+$ T cells

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## SUMMARY

This study addresses the question of whether human peripheral $CD4^+$ $CD45RA^+$ T cells possess antigen-specific immune memory. $CD4^+$ $CD45RA^+$ T cells were isolated by a combination of positive and negative selection. Putative $CD4^+$ $CD45RA^+$ cells expressed CD45RA (98·9%) and contained $<0{\cdot}1\%$ $CD4^+$ $CD45RO^+$ and $<0{\cdot}5\%$ $CD4^+$ $CD45RA^+$ $CD45RO^+$ cells. Putative $CD45RO^+$ cells expressed CD45RO (90%) and contained 9% $CD45RA^+$ $CD45RO^+$ and $<0{\cdot}1\%$ $CD4^+$ $CD45RA^+$ cells. The responder frequency of *Dermatophagoides pteronyssinus*-stimulated $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells was determined in two atopic donors and found to be 1 : 11 314 and 1 : 8031 for $CD4^+$ $CD45RA^+$ and 1 : 1463 and 1 : 1408 for $CD4^+$ $CD45RO^+$ T cells. The responder frequencies of $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells from two non-atopic, but exposed, donors were 1 : 78 031 and 1 : 176 903 for $CD4^+$ $CD45RA^+$ and 1 : 9136 and 1 : 13 136 for $CD4^+$ $CD45RO^+$ T cells. T cells specific for *D. pteronyssinus* were cloned at limiting dilution following 10 days of bulk culture with *D. pteronyssinus* antigen. Sixty-eight clones were obtained from $CD4^+$ $CD45RO^+$ and 24 from $CD4^+$ $CD45RA^+$ T cells. All clones were $CD3^+$ $CD4^+$ $CD45RO^+$ and proliferated in response to *D. pteronyssinus* antigens. Of 40 clones tested, none responded to *Tubercule bacillus* purified protein derivative (PPD). No difference was seen in the pattern of interleukin-4 (IL-4) or interferon-$\gamma$ (IFN-$\gamma$) producing clones derived from $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ precursors, although freshly isolated and polyclonally activated $CD4^+$ $CD45RA^+$ T cells produced 20–30-fold lower levels of IL-4 and IFN-$\gamma$ than their $CD4^+$ $CD45RO^+$ counterparts. Sixty per cent of the clones used the same pool of V$\beta$ genes. These data support the hypothesis that immune memory resides in $CD4^+$ $CD45RA^+$ as well as $CD4^+$ $CD45RO^+$ T cells during the chronic immune response to inhaled antigen.

## INTRODUCTION

Immunological memory may be defined as the capacity of the immune system to respond more rapidly and more vigorously to an antigen when it is subsequently encountered. B cells undergo immunoglobulin variable gene re-arrangement following contact with antigen, and naive B cells can be distinguished from memory B cells by the presence of membrane IgD and IgM and by their inability to secrete specific antibody when stimulated *in vitro*.[1] It is more difficult to identify memory T cells. Re-arrangement of T-cell receptor (TCR) genes occurs in the thymus. During an immune response, the TCR genes of mature T cells do not undergo re-arrangement but those whose receptors possess the appropriate affinity for peptide–major histocompatibility complex (MHC) are selected and preferentially expanded. Thus T-cell immune memory is signalled by an increase in the number of T cells specific for a given antigen. Immune memory cells were originally believed to be long-lived, but recent evidence suggests that both B- and T-cell memory is maintained by short-lived cells that are driven by antigen that persists for months and probably years.[1,2] In some cases, immune memory can last a very long time. The immune response to bee venom proteins, for example, can persist for 20 years or longer without an intervening sting.[3] It is possible that such long-lived memory responses are maintained in part by antigen cross-reactivity,[4] such as has been observed between the grass pollen antigen Rye I and bee venom phospholipase $A_2$.[5]

The T-cell marker that is believed to most closely related to immune memory is the common leucocyte antigen CD45.[6] The CD45 molecule is expressed on the surface of T cells in different molecular weight isoforms generated by the alternative splicing of three exons (A, B, C) encoded by the leucocyte

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Abbreviations: PPD, purified protein derivative of tubercule bacillus; RAST, radioallergosorbent test; RT-PCR, reverse transcription-polymerase chain reaction.

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common antigen gene. The surface expression of these different isoforms indicates the state of activation of the T cell. Cells expressing the high molecular weight (MW) 220 000 isoform express the exons CD45RA, CD45RB and CD45RC, while the 205 000 MW isoform expresses the exons for CD45RA (190 000 MW) and CD45RB (180 000 MW). Two T-cell populations can be identified, one that expresses CD45RABC and CD45RAB (commonly referred to as CD45RA cells) and one that expresses CD45RB and CD45RO (commonly referred to as CD4RO cells), believed to represent naive T cells. After *in vitro* or *in vivo* activation, $CD45RA^+$ cells rapidly lose CD45RA and become positive for CD45RO.[7,8] *In vivo*, expression of CD45RO ($RA^-$ human, $RB^-$ mouse or $RC^-$ rat) is associated with immune experience. Such cells are present in low numbers at birth but increase with age.[9] Recent thymic emigrants in the rat are of the $RO^+$ ($CD45RC^-$) phenotype[10] but rapidly express CD45RA. The same has been reported for newborn infants.[11] T-helper type 1 (Th1) and Th2 patterns of cytokine production are reportedly imprinted in memory T cells.[12,13] Antigen-specific $CD8^+$ T cells that fail to express CD45RO have been described.[14] CD45RO cells express lower levels of bcl-2 and are more prone to apoptosis.[15,16] This observation has been difficult to reconcile with the view that RO cells are responsible for long-term memory.

It was originally believed that $CD45RO^+$ T cells could not revert to $CD45RA^+$ T cells. However, Rothstein *et al.*[17] have shown that following stimulation of human $CD45RA^+$ cell lines, CD45RA was fully re-expressed by days 14–16, while CD45RO was expressed throughout the stimulatory cycle. CD45RC is expressed at high levels on naive rat T cells and at low levels following activation. However, $CD45RC^{low}$ T cells transferred to congenic nude rats[18] re-expressed CD45RC at a high level, indicating that these cells had reverted to the naive phenotype. These cells were found to be capable of inducing graft-versus-host (GVH) responses, indicating that reversion bought with it functional as well as phenotypic changes.[19] Evidence that $CD45RA^+$ cells possess memory was also demonstrated in a T–B cell co-stimulation assay.[20,21] In humans, differing survival curves for the two subsets in radiation-treated patients indicated that there may be reversion *in vivo* from the CD45RO to the CD45RA phenotype.[22] Thus conversion from $CD45RA^+$ to $CD45RA^-$ does not appear to be either unidirectional or irreversible.

In this study we have tested the hypothesis that immunological memory resides in $CD4^+$ $CD45RA^+$ as well as $CD4^+$ $CD45RO^+$ T cells by comparison of the clonal response of $CD4^+$ $CD45RO^+$ and $CD4^+$ $CD45RA^+$ T cells from atopic and non-atopic donors to *Dermatophagoides pteronyssinus*. As expected, we observed a greater *D. pteronyssinus*-specific responder frequency of $CD45RO^+$ compared with $CD45RA^+$ atopic donor T cells. However, the responder frequency of atopic donor $CD45RA^+$ T cells was comparable with that of non-atopic, but exposed, donor $CD45RO^+$ T cells. The clones generated were comparable in terms of V$\beta$ gene usage and cytokine profile. We conclude that immune memory resides in $CD4^+$ $CD45RA^+$ as well as $CD4^+$ $CD45RO^+$ T cells and that these molecules reflect the state of activation of T cells rather than their immunological memory.

## MATERIALS AND METHODS

*Reagents*

Lymphoprep was purchased from Nycomed (Birmingham, UK); Detach-a-beads from Dynal (Wirral, UK); anti-CD45RO (UCHL-1) and anti-CD45RA (B-C15) from Serotec (Kidlington, UK); and Hanks' phosphate-buffered saline (HBSS), fetal calf serum (FCS), RPMI-1640 and 50 mM 2-mercaptoethanol from Gibco BRL (Abingdon, UK). Fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-labelled anti-CD4, CD8, CD19 and CD3 were from Becton Dickinson (Oxford, UK). Human AB serum, sodium pyruvate, cell freezing medium and *p*-nitrophenyl phosphate were from Sigma Ltd (Poole, UK). *Dermatophagoides pteronyssinus* extract was a kind gift from Smith-Kline Beecham (Great Burgh, UK). Purified protein derivative (PPD) was obtained from Public Health Laboratories (Porton Down, UK). Interleukin-4 (IL-4) and interferon-$\gamma$ (IFN-$\gamma$) enzyme-linked immunosorbent assay (ELISA) kits were from Cambridge Biosciences (Cambridge, UK). V$\beta$ primers were purchased from Clontech (Palo Alto, CA).

*Preparation of peripheral blood mononuclear cells*

Two *D. pteronyssinus*-sensitive volunteers were selected for cloning. Both had a strongly positive *D. pteronyssinus* skin prick test (patient 1, 18 mm; patient 2, 24 mm wheal diameter), a class 3 or greater radioallergosorbent test (RAST) and a clinical history of asthma (patient 1) and rhinitis (patient 2) associated with exposure to *D. pteronyssinus*. Peripheral blood (160 ml) was collected by venepuncture into sodium citrate, diluted 1 : 1 with HBSS, layered onto Lymphoprep and centrifuged at 800 ***g*** for 20 min at 18°. The interface containing mononuclear cells (MNC) was then collected and washed three times with HBSS at 200 ***g*** for 10 min at 4°. The cells were then resuspended at $10 \times 10^6$/ml in RPMI-1640/2% FCS. PBMC, $10 \times 10^6$, were kept for feeders and the remainder used for $CD4^+$ purification.

*Purification of $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells*

$CD4^+$ cells were isolated by positive selection using anti-CD4 Dynabeads (Dynal), which were added at a 3 : 1 bead : target cell ratio and mixed by rotation for 40 min at 4°. A magnet was applied for 2 min and unbound cells aspirated. The remaining magnetic beads were gently resuspended in 1 ml of RPMI-1640/2% FCS and reapplied to the magnet for 30 seconds and the supernatant aspirated. This washing procedure was repeated four times in total and the beads resuspended in 100 $\mu$l of RPMI-1640/2% FCS. Bound $CD4^+$ cells were released by addition of the Detach-a-bead antibody with rotation for 60 min at 18°. The magnet was applied for a further 2 min and the released $CD4^+$ cells collected. The bound beads were then washed with 1 ml RPMI/2% FCS, the magnet reapplied for 30 seconds, and the released $CD4^+$ cells collected as before. This washing procedure was repeated twice and all the released $CD4^+$ cells were pooled and washed five times by centrifugation at 200 ***g*** for 1 min, to remove any residual Dynabeads. An aliquot of $CD4^+$ cells was taken for fluorescence-activated cell sorter (FACS) analysis to check purity. The remainder were counted and resuspended at $10 \times 10^6$/ml and divided into two equal aliquots.

$CD4^+$ cells were purified into $CD45RA^+$ and $CD45RO^+$ cells by negative selection with 40 $\mu$l of anti-CD45RA (B-C15)

or 300 $\mu$l of anti-CD45RO (UCHL-1) of a 1 $\mu$g/ml solution of antibody per $1 \times 10^6$ cells and incubated for 20 min at 4°. The CD4$^+$ cells were washed twice with RPMI-1640/2% FCS, resuspended in 200 $\mu$l and mixed by rotation at an anti-mouse immunoglobulin Dynabead : cell ratio of 40 : 1 for 20 min at 4°. The cells were resuspended to 1 ml and applied to the magnet and unbound cells removed and kept. The beads were then resuspended in 1 ml of medium and reapplied to the magnet; the supernatant was then removed and added to the kept sample. This was repeated a further four times, resulting in a total of 6 ml of purified cells. The purity of the cells was tested by FACS analysis using anti-CD4, anti-CD8, anti-CD45RA, anti-CD45RO and anti-CD19 antibodies. In six separate experiments (two for cloning and four for limiting dilution analysis) a high degree of purity was achieved (Fig. 1 and Table 1). Purity of CD4$^+$ cells was >99% with <0·1% CD8 and <0·1% CD19 contamination. CD45RA$^+$ cells were 98·9% ($\pm$0·2) pure. The main contaminants were CD45RA/CD45RO double-positive cells (0·5%$\pm$0·1) with only 0·1%$\pm$0·1 CD45RO$^+$ cells. CD45RO$^+$ cell purities were lower (90·1%$\pm$0·3), with the main contaminants again being CD45RA/CD45RO double-positive cells (8·95%$\pm$4·9) and only 0·58%$\pm$0·3 CD45RA$^+$ cells.

*Limiting dilution analysis*

Aliquots of CD45RA- and CD45RO-purified cells were prepared at concentrations from $1 \times 10^5$/ml to $1 \times 10^1$/ml in complete medium [RPMI-1640, 10% normal human serum (NHS), 1% sodium pyruvate, 1% non-essential amino acids (NAA), 50 mM 2-mercaptoethanol] in Terasaki plates at 10 $\mu$l/well together with 10 $\mu$l of X-irradiated (4000 Gy) autologous feeders ($1 \times 10^6$/ml) and *D. pteronyssinus* extract (final concentration 10 $\mu$g/ml) for 10 days at 37°, 5% $CO_2$. On day 5 10 $\mu$l of culture supernatant was aspirated and 10 $\mu$l of complete medium containing 20 U/ml of IL-2 was added. On day 10 the plates were scored for cell growth blind and the cloning frequency calculated. Wells were scored positive when proliferation was clearly visible.

Dermatophagoides pteronyssinus-*induced proliferation of CD45RA$^+$ and CD45RO$^+$ CD4$^+$ T cells in the primary bulk cultures*

Purified CD45RA and CD45RO cells were resuspended at $1 \times 10^6$/ml in complete medium, added to 96-well microtitre plates at 100 $\mu$l/well and stimulated with *D. pteronyssinus* extract (final concentration 10 $\mu$g/ml) together with 100 $\mu$l of X-irradiated (4000 Gy) autologous feeders ($1 \times 10^6$/ml) at 37°, 5% $CO_2$. On day 5 IL-2 was added to the cultures (final concentration 20 U/ml). On day 8 triplicate wells were pulsed with [$^3$H]thymidine ($\mu$Ci/well) and harvested on day 9 and counted on a $\beta$-scintillation counter.

*Generation of* D. pteronyssinus-*specific CD4$^+$ T-cell clones*

Purified CD45RA and CD45RO cells at $1 \times 10^6$/ml in complete medium were added to 96-well microtitre plates at 100 $\mu$l/well and stimulated with *D. pteronyssinus* extract (10 $\mu$g/ml) and 100 $\mu$l of X-irradiated (4000 Gy) autologous feeders ($1 \times 10^6$/ml) at 37°, 5% $CO_2$. On day 5, IL-2 (20 U/ml) was added to the cultures. On day 10, the cells were washed and diluted to 0·3 cells/well and transferred to new 96-well

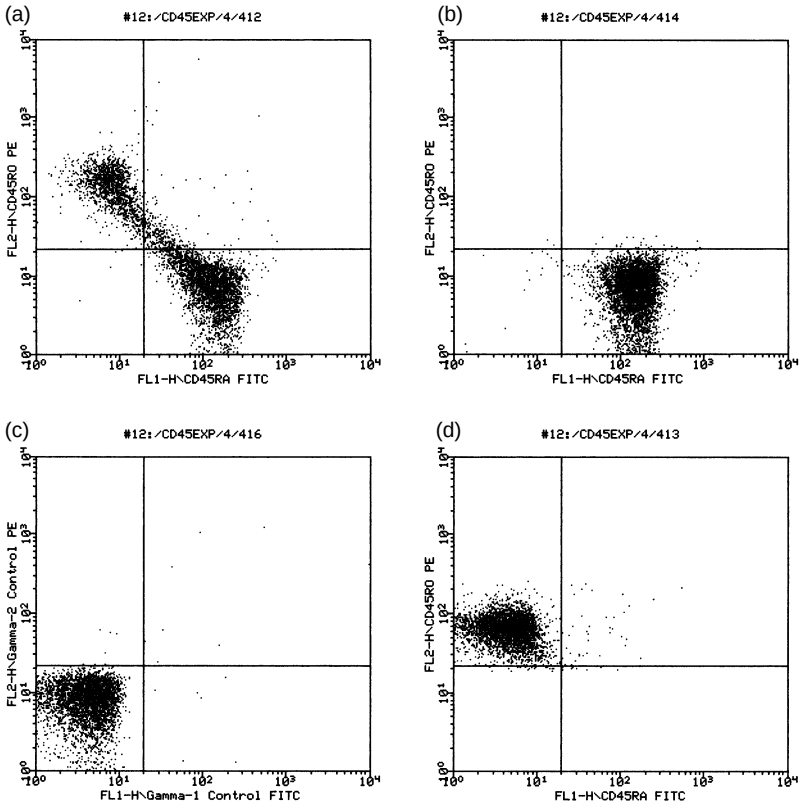


**Figure 1.** FACS profile of purified peripheral blood CD4$^+$ T cells stained for CD45RA$^+$ and CD45RO$^+$. (a) purified CD4$^+$ T cells, (b) purified CD4$^+$ CD45RA$^+$ T cells, (c) FITC and PE negative control, (d) purified CD4$^+$ CD45RO$^+$ T cells.

**Table 1.** Purity of isolated $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells

| | % $CD45RA^+$ $CD4^+$ T cells | % $CD45RO^+$ $CD4^+$ T cells |
|---|---|---|
| $CD45RA^+$ | $98{\cdot}9 \pm 0{\cdot}2$ | $0{\cdot}58 \pm 0{\cdot}3$ |
| $CD45RO^+$ | $0{\cdot}1 \pm 0{\cdot}1$ | $90{\cdot}1 \pm 4{\cdot}9$ |
| $CD45RA^+$ $CD45RO^+$ | $0{\cdot}5 \pm 0{\cdot}1$ | $8{\cdot}95 \pm 4.9$ |

$CD4^+$ T cells were purified by positive selection using immunomagnetic Dynabeads and Detach-a-bead™. The CD4 cells were then further purified into $CD45RA^+$ and $CD45RO^+$ CD4 T cells using negative selection with immunomagnetic beads coated with either B-C15 (anti-CD45RA) or UCHL-1 (anti CD45RO). The table shows the purity of $CD45RA^+$ and $CD45RO^+$ purified populations as measured by FACS. All data presented were derived from these four experiments.

microtitre plates and restimulated with *D. pteronyssinus* (10 $\mu$g/ml) and 100 $\mu$l of X-irradiated (4000 Gy) autologous feeders ($1 \times 10^6$/ml) at 37°, 5% $CO_2$. On days 5 and 10 the cells were fed with complete medium supplemented with IL-2 (20 U/ml). On day 14 positive wells were scored and restimulated with phytohaemagglutinin (PHA; 5 $\mu$g/ml final concentration) and X-irradiated (4000 Gy) heterologous feeders ($1 \times 10^5$/well) in complete medium containing IL-2 (50 U/ml). On days 5 and 10 putative clones were fed with complete medium plus IL-2 (20 U/ml) without PHA. Every 14 days clones were restimulated with PHA and irradiated feeder cells as before. This cycle of stimulation and feeding was used to expand the clones with aliquots frozen in cell-freezing medium at regular intervals.

*Testing specificity of clones*

On day 14 of the cycle, clones ($2 \times 10^4$/well) were incubated with autologous X-irradiated (4000 Gy) feeder cells ($1 \times 10^5$/well) with or without *D. pteronyssinus* (10 $\mu$g/ml) or purified *Der p* 1 (10 $\mu$g/ml) in 96-well microtitre plates in complete medium without IL-2. Cells were incubated for 48 h at 37°, 5% $CO_2$. [$^3$H]thymidine was added for the last 18 h of culture. The cells were then harvested and radioactivity counted in a Matrix $\beta$-counter (Canberra Packard, Pangbourne, UK). Results are expressed as triplicate mean $\pm$ SD counts per minute (c.p.m.).

*Cytokine analysis*

On day 14 of the cycle, clones ($2 \times 10^4$/well) were incubated in 1 ml of complete medium without IL-2 in 24-well plates together with ionomycin (400 ng/ml) and phorbol myristate acetate (PMA; 10 ng/ml) for 24 h. Supernatants were harvested and analysed for IFN-$\gamma$ and IL-4 production using commercially available ELISA reagents (Cambridge Biosciences). Briefly, 96-well microtitre plates were coated with either anti-IL-4 or anti-IFN-$\gamma$ (1 $\mu$g/ml) in $NaHCO_3$ buffer, pH 9·6, 0·1 M, overnight at 4°. All volumes used were 50 $\mu$l. Plates were washed three times with phosphate-buffered saline (PBS), pH 7·5, 0·05 M, containing 0·5% Tween-20, and samples together with standards and controls diluted in complete medium added to the plate and incubated for 1 h at room temperature. Plates were washed as above and incubated with biotinylated anti-IL-4 or anti-IFN-$\gamma$ (1 $\mu$g/ml in complete medium) for 1 h at room temperature. Plates were washed as above and incubated with streptavidin–alkaline–phosphatase (Sigma Ltd) diluted 1/1000 in assay diluent for 1 h at room temperature. The plates were washed a final time as above and incubated for 1 h with *p*-nitrophenyl phosphate (1 mg/ml in $\beta$-ethanolamine buffer, pH 9·8, 0·1 M). Colour development was measured at 405 nm and cytokine concentrations determined by reference to a standards purchased from Quantakine ELISA kits (R&D Systems, Abingdon, UK). Sensitivity of the assays was 30 pg/ml IL-4 and 30 pg/ml IFN-$\gamma$. Results are expressed as pg/ml/$2 \times 10^4$ cells.

*V$\beta$ gene expression*

*Dermatophagoides pteronyssinus*-specific T-cell clone V$\beta$ gene expression was studied using a panel of primers recognizing V$\beta$1–24 (Clontech), according to the manufacturers' protocol. Briefly, total RNA was prepared from $1 \times 10^7$ cloned cells by phenol–guanidine thiocyanate extraction and the yield determined by optical density at 280 nm. Two micrograms of total RNA were reverse transcribed using AMV reverse transcriptase (RT). cDNA was then aliquotted into 25 polymerase chain reaction (PCR) tubes together with C$\beta$ 3′ primer and one of the V$\beta$ 5′ primers. The positive control for the PCR was cDNA together with C$\alpha$ 3′ and 5′ primers. Between 24 and 27 amplification cycles were then performed and the products run on a 3% agarose ethidium bromide gel. Products were detected using an Eagle Eye III transluminator (Stratagene, Cambridge, UK). A single band in one V$\beta$ channel together with a single band in the control C$\alpha$ channel was taken to indicate clonality.

*Data analysis*

Cloning frequencies were calculated using weighted mean statistics as described by Taswell.[23] T-cell proliferation is expressed as the geometric mean of three replicates.

## RESULTS

### The responder frequency of *D. pteronyssinus*-specific $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells

The responder frequency of *D. pteronyssinus*-specific $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells in the peripheral blood of two *D. pteronyssinus*-allergic donors was determined by limiting dilution analysis. $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells were purified as described in the Materials and Methods (Fig. 1 and Table 1). There was $<0{\cdot}1$ cross-contamination of $CD4^+$ $CD45RA^+$ with

$CD4^+$ $CD45RO^+$ T cells. Pure $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells were cultured at different cell densities with *D. pteronyssinus* and the responder frequency determined on day 10. The responder frequency of *D. pteronyssinus*-responsive $CD4^+$ T cells (Fig. 2) was lower for $CD4^+$ $CD45RA^+$ (patient 1, $1:8031 \pm 2100$; patient 2, $1:11\,314 \pm 2500$) than $CD4^+$ $CD45RO^+$ T cells (patient 1, $1:1408 \pm 325$; patient 2, $1:1463 \pm 220$). The responder frequency for two non-atopic, but *D. pteronyssinus*-exposed, subjects (Fig. 2) was lower for $CD4^+$ $CD45RA^+$ (non-atopic 1, $1:78\,031 \pm 2800$, non-atopic 2, $1:176\,903 \pm 28\,600$) than $CD4^+$ $CD45RO^+$ T cells (non-atopic 1, $1/9136 \pm 521$; non-atopic 2, $1:13\,136 \pm 1020$).

### The polyclonal response of CD45RA$^+$ and CD45RO$^+$ CD4$^+$ T cells to *D. pteronyssinus*

The polyclonal response of $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells to *D. pteronyssinus* was determined in bulk culture. Purified $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells ($1 \times 10^5$) were cultured for 9 days with *D. pteronyssinus* and $1 \times 10^5$ autologous irradiated feeder cells. With both atopic donors a significant but three-fold lower proliferative response was seen with $CD4^+$ $CD45RA^+$ T cells compared with $CD4^+$ $CD45RO^+$ T cells (Fig. 3). In both cases there was little

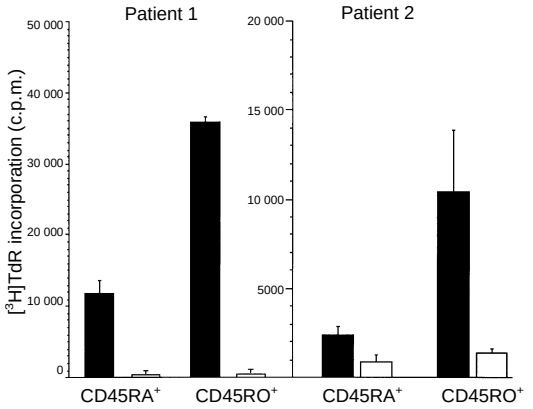


**Figure 3.** The polyclonal response of purified $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells to *D. pteronyssinus* antigen. Cells were seeded at $1 \times 10^5$/well together with $1 \times 10^5$/well autologous irradiated feeder cells with (shaded bars) or without (open bars) 10 $\mu$g/ml *D. pteronyssinus* antigen. On day 5 IL-2 was added at a final concentration of 20 U/ml. On day 9 the cultures were pulsed with [$^3$H]thymidine [$^3$H]TdR, harvested 18 h later and counted in a $\beta$-scintillation counter. Results are expressed as net [$^3$H]TdR incorporation (c.p.m.).

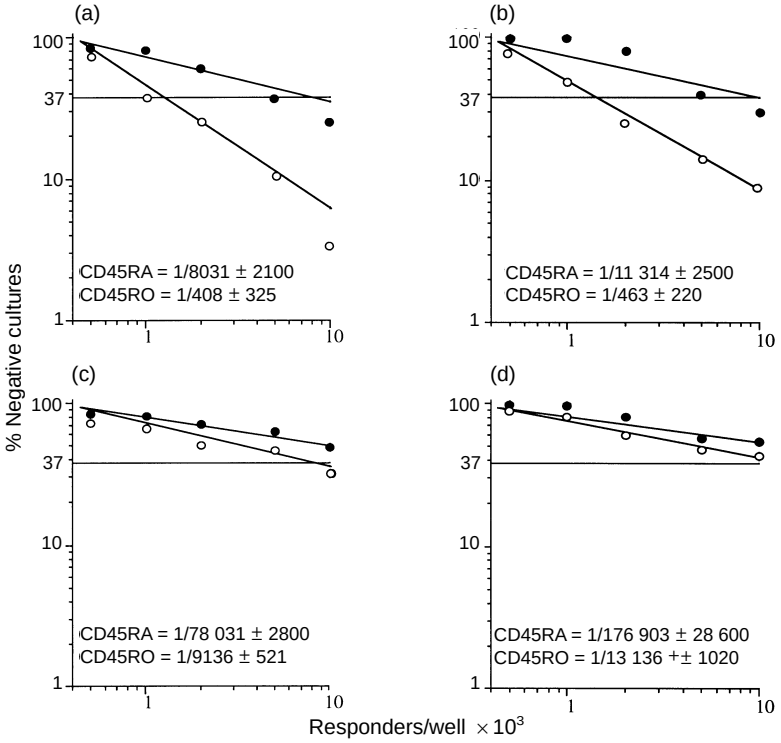


**Figure 2.** Limiting dilution analysis of *D. pteronyssinus* antigen-stimulated $CD45RA^+$ (closed circles) and $CD45RO^+$ (open circles) $CD4^+$ T cells. $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells were seeded into Terasaki plates from 1000 to 0·1 cells/well, together with irradiated autologous feeders, at $1 \times 10^4$ cells/well. *Dermatophagoides pteronyssinus* antigen was added at 10 $\mu$g/ml final concentration. On days 5 and 10 the cultures were fed with 20 U/ml IL-2 in complete medium. On day 10 the plates were scored for the presence of cell growth. Responder frequencies were calculated using weighted mean statistics as described by Taswell.[23] (a) Patient 1 showed a responder frequency of $1/8031 \pm 2100$ in the $CD45RA^+$ and $1/1408 \pm 325$ in the $CD45RO^+$ population. (b) Patient 2 showed a responder frequency of $1/11\,314 \pm 2500$ in the $CD45RA^+$ and $1/1463 \pm 220$ in the $CD45RO^+$ population. (c) The responder frequency of non-atopic control 1 was $1/78\,031 \pm 2800$ ($CD45RA^+$) and $1/9136 \pm 521$ ($CD45RO^+$). (d) Non-atopic control 2 responder frequency was $1/176\,903 \pm 28\,600$ ($CD45RA^+$) and $1/13\,136 \pm 1020$ ($CD45RO^+$).

(patient 2) or no (patient 1) proliferative response in cultures where *D. pteronyssinus* was absent.

### Generation of *D. pteronyssinus*-specific CD4$^+$ T-cell clones from CD45RA$^+$ and CD45RO$^+$ precursors

*Dermatophagoides pteronyssinus*-specific clones were generated at limiting dilution from 10-day bulk cultures of CD4$^+$ CD45RA$^+$ and CD4$^+$ CD45RO$^+$ T cells, as described in the Materials and Methods. A total of 51 clones was derived from the CD4$^+$ CD45RO$^+$ and 20 clones from the CD4$^+$ CD45RA$^+$ T cells of patient 1. Seventeen clones were derived from the CD4$^+$ CD45RO$^+$ and four clones from the CD4$^+$ CD45RA$^+$ T cells of patient 2. All of these clones proliferated in response to *D. pteronyssinus* (Fig. 4). The level of proliferation was comparable regardless of whether the clones were derived from CD4$^+$ CD45RA$^+$ or CD4$^+$ CD45RO$^+$ precursors. All clones were CD3$^+$ CD4$^+$ CD45RO$^+$. Production of IL-4 and IFN-$\gamma$ was determined following stimulation of the clones with PMA and ionomycin. This stimulus was chosen because it yields larger amounts of cytokines, permitting a more complete analysis of the clones. There was no difference in the overall cytokine profile of CD4$^+$ CD45RA$^+$-derived and CD4$^+$ CD45RO$^+$-derived clones and a comparable proportion of Th1-(IFN-$\gamma^+$, IL-4$^-$), Th2- (IFN-$\gamma^-$, IL-4$^+$) and Th0-like (IFN-$\gamma^+$, IL-4$^+$) clones was observed (Fig. 5). Production of IL-4 and IFN-$\gamma$ was also determined using freshly isolated CD4$^+$ CD45RA$^+$ and CD4$^+$ CD45RO$^+$ T cells activated with PMA and ionomycin (Fig. 6a). Although proliferation of CD45RA and CD45RO cells was comparable (Fig. 6b), little IL-4 or IFN-$\gamma$ could be detected in the supernatants of the CD4$^+$ CD45RA$^+$ cells compared with CD4$^+$ CD45RO$^+$ T cells.

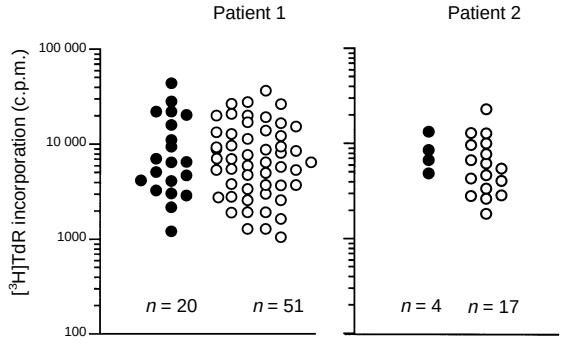


**Figure 4.** The proliferative response of CD45RA (closed circles) and CD45RO (open circles) -derived clones to *D. pteronyssinus*. Clones were seeded in 96-well plates at $1 \times 10^5$/well together with 10 $\mu$g/ml of *D. pteronyssinus* and $1 \times 10^5$ autologous irradiated feeder cells, without IL-2. After 30 h the plates were pulsed with [$^3$H]TdR and harvested at 48 h. Results are expressed as net [$^3$H]TdR incorporation (c.p.m.) after subtraction of the counts obtained from cultures to which no *D. pteronyssinus* had been added.

### The specificity of CD45RA$^+$- and CD45RO$^+$-derived *D. pteronyssinus*-specific CD4$^+$ T-cell clones

The ability of the clones to respond to an independent antigen was investigated with an extract of *Tubercule bacillus* PPD. None of 20 CD4$^+$ CD45RA$^+$ and none of 20 CD4$^+$ CD45RO$^+$ T-cell clones responded to PPD (cut-off $2 \times$ background) (examples shown in Table 2). In addition, 40 of the clones from patient 1 were tested for their ability to proliferate in response to one of the major dust mite allergens, *Der p* 1. Of 40 clones tested from patient 1, 6/20 derived from CD4$^+$ CD45RA$^+$ precursors, and 13/20 derived from CD4$^+$ CD45RO$^+$ precursors, responded to purified *Der p* 1 antigen (Fig. 7).

### V$\beta$ usage by CD45RA$^+$ and CD45RO$^+$-derived *D. pteronyssinus*-specific CD4$^+$ T-cell clones

V$\beta$ gene usage was determined in 20 CD4$^+$ CD45RA$^+$- and 20 CD4$^+$ CD45RO$^+$-derived clones from patient 1 by

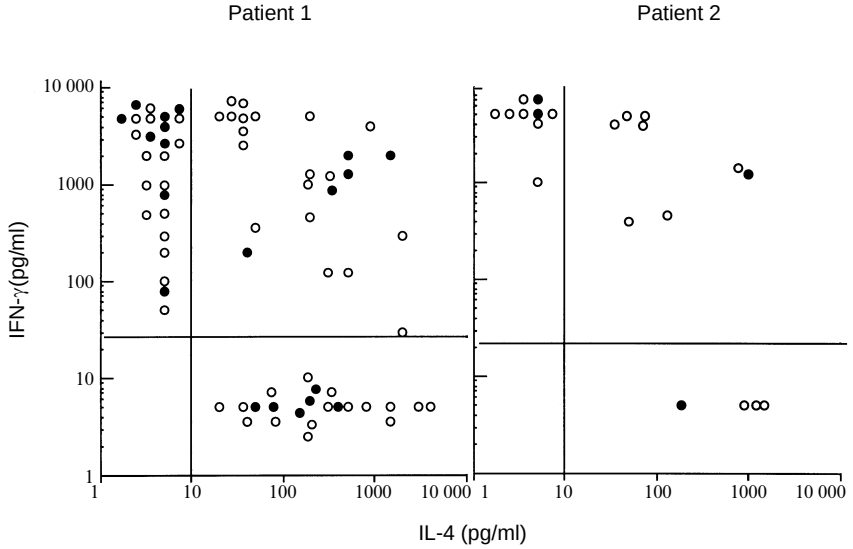


**Figure 5.** Cytokine profiles of CD45RA (closed circles) and CD45RO (open circles) -derived clones. Cells were cultured in 24-well plates at $2 \times 10^4$/ml and stimulated with PMA (10 ng/ml) and ionomycin (400 ng/ml) for 24 h. The cell supernatants were then harvested and assayed for IL-4 and IFN-$\gamma$ by ELISA. Results are expressed as pg/ml. Both CD45RA- and CD45RO-derived clones exhibited a mixture of Th1, Th2 and Th0 cytokine profiles, as defined by IL-4 and IFN-$\gamma$ production.

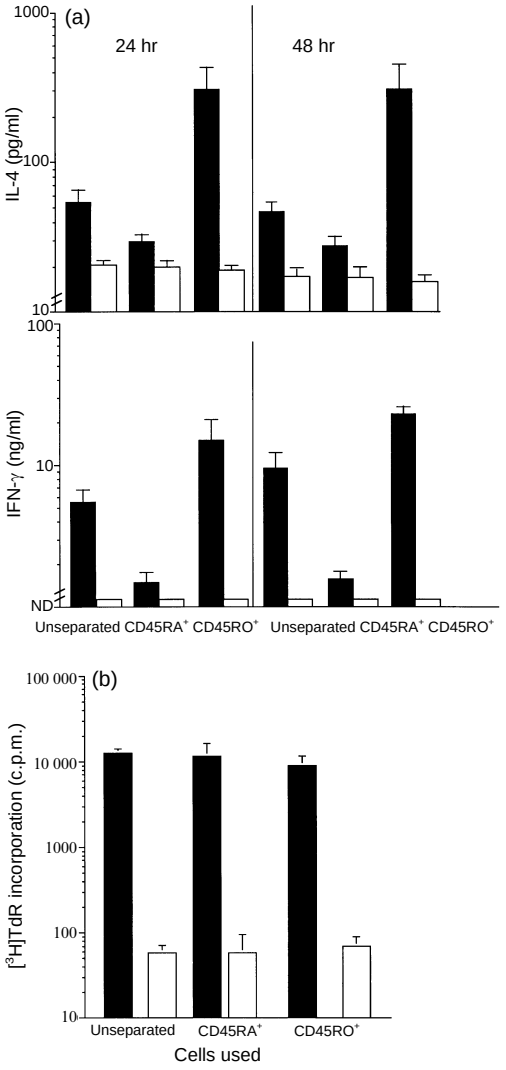


**Figure 6.** (a) Cytokine secretion and (b) proliferation of freshly isolated PMA and ionomycin-stimulated human CD45RA$^+$ and CD45RO$^+$ (stimulated closed bars, unstimulated open bars) T cells. Pure CD45RA$^+$ or CD45RO$^+$ CD4$^+$ T cells were cultured in 24-well plates at $1 \times 10^4$ cells/ml and stimulated with PMA (10 ng/ml) and ionomycin (400 ng/ml) for 24 h. The cell supernatants were then harvested and assayed for IL-4 and IFN-$\gamma$ by ELISA. The results shown are the mean $\pm$ SD of four experiments.

**Table 2.** Antigen specificity of CD4$^+$ T-cell clones

| Clone | Source | Stimulus | | |
|---|---|---|---|---|
| | | *D. pteronyssinus* | PPD | Medium |
| Patient 1 | | | | |
| DM8 | RO | 23263 | 466 | 151 |
| DM13 | RO | 15711 | 260 | 371 |
| DM20 | RO | 24532 | 301 | 208 |
| DM7 | RA | 17504 | 516 | 192 |
| DM17 | RA | 4353 | 142 | 338 |
| PPD9 | RO | 503 | 24370 | 430 |
| PPD11 | RA | 122 | 4210 | 108 |
| PPD12 | RA | 338 | 3777 | 270 |
| Patient 2 | | | | |
| DM1 | RO | 15721 | 407 | 370 |
| DM4 | RO | 5600 | 160 | 210 |
| DM5 | RO | 17493 | 250 | 178 |
| DM17 | RO | 10200 | 1020 | 940 |
| DM19 | RO | 7000 | 750 | 1000 |
| DM20 | RO | 15000 | 230 | 242 |
| PPD2 | RO | 300 | 15300 | 270 |
| PPD1 | RO | 200 | 7200 | 245 |

Antigen specificity of representative *D. pteronyssinus* and PPD clones derived from CD45RA$^+$ and CD45RO$^+$ bulk cultures from two patients. Cloned cells ($2 \times 10^5$) and autologous irradiated peripheral blood MNC ($2 \times 10^5$) were stimulated with 10 $\mu$g/ml *D. pteronyssinus* or 10 $\mu$g/ml PPD as detailed in the Materials and Methods. The data represent the geometric mean of triplicate cultures.

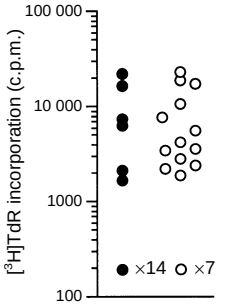


**Figure 7.** The proliferative response of CD45RA (closed circles) and CD45RO (open circles)-derived clones to *Der p* 1. Clones were seeded in 96-well plates at $1 \times 10^5$/well together with 10 $\mu$g/ml of *Der p* 1 and $1 \times 10^5$ autologous irradiated feeder cells, without IL-2. After 30 h the plates were pulsed with [$^3$H]TdR and harvested at 48 h. Results are expressed as net [$^3$H]TdR incorporation (c.p.m.) after subtraction of the counts obtained from cultures to which no *Der p* 1 had been added.

RT-PCR. A wide range of V$\beta$ genes was expressed by both sets of clones. These included V$\beta$2, V$\beta$3, V$\beta$4, V$\beta$5·1, V$\beta$5·2, V$\beta$6, V$\beta$8, V$\beta$10, V$\beta$15, V$\beta$17, V$\beta$18, V$\beta$21 and V$\beta$24. Comparison of the TCR V$\beta$ genes used by CD4$^+$ CD45RA$^+$- and CD4$^+$ CD45RO$^+$-derived T-cell clones indicated that 60% of the clones used the same pool of V$\beta$ genes. However, there were clones from both precursors that expressed V$\beta$ not used by the opposite subset (Table 3).

## DISCUSSION

In this study we have tested the hypothesis that immune memory resides in peripheral CD45RA$^+$ as well as CD45RO$^+$ CD4$^+$ T cells. We set out to determine whether *D. pteronyssinus*-reactive CD4$^+$ CD45RA$^+$ T cells could be detected in human peripheral blood, whether these cells could be cloned, and if so whether they used the same V$\beta$ genes and produced the same cytokines. By limiting dilution analysis, the precursor frequency of peripheral *D. pteronyssinus*-reactive CD4$^+$ CD45RA$^+$ T cells in atopic donors was found to be only six- to eightfold lower than that of comparable CD4$^+$ CD45RO$^+$ T cells. This cannot be explained by contaminating CD45RO$^+$ cells as the CD4$^+$ CD45RA$^+$ T-cell preparations used contained less than 1 : 1000 CD45RO$^+$ and less than 1 : 200 CD45RA$^+$ CD45RO$^+$ T cells. The frequency of *D. pteronyssinus*-responder CD4$^+$ CD45RA$^+$ from atopic donors (mean 1/9673 $\pm$ 2300) was similar to that seen with

**Table 3.** Vβ expression

| | $CD45RA^+$ derived | $CD45RO^+$ derived |
|---|---|---|
| Vβ1 | | |
| Vβ2 | | 2 |
| Vβ3 | | 1 |
| Vβ4 | | 1 |
| Vβ5.1 | 3 | 2 |
| Vβ5.2 | 2 | 1 |
| Vβ6 | 1 | 4 |
| Vβ7 | | |
| Vβ8 | 3 | |
| Vβ9 | | |
| Vβ10 | | 4 |
| Vβ11 | | |
| Vβ12 | | |
| Vβ13 | | |
| Vβ14 | | |
| Vβ15 | 5 | 1 |
| Vβ16 | | |
| Vβ17 | 3 | |
| Vβ18 | | 1 |
| Vβ19 | | |
| Vβ20 | | |
| Vβ21 | 2 | 3 |
| Vβ22 | | |
| Vβ23 | | |
| Vβ24 | 1 | |
| Total | 20 | 20 |

Vβ expression was determined by RT-PCR in 20 representative CD45RA-derived and 20 CD45RO-derived *D. pteronyssinus*-specific $CD4^+$ T-cell clones. Two micrograms of total RNA were reverse transcribed using AMV reverse transcriptase. cDNA was then aliquotted into 25 PCR tubes together with Cβ 3′ primer and one of the Vβ(1–24) 5′ primers. Between 24 and 27 amplification cycles were then performed and the products run on a 3% agarose ethidium bromide gel. A single band in one Vβ channel together with a single band in the control Cα channel was taken to indicate clonality.

$CD4^+$ $CD45RO^+$ T cells from non-allergic, but exposed, subjects (mean $1/11\,136 \pm 771$).

The clones derived from the two starting cell populations were compared for their ability to secrete cytokines, for antigen-specificity and Vβ gene usage. The cytokine profile of the clones generated from $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ precursors was comparable and a similar proportion of Th1-, Th2- and Th0-like clones was obtained. The Vβ genes used were identified in 40 of the clones, 20 from $CD4^+$ $CD45RA^+$ and 20 from $CD4^+$ $CD45RO^+$ precursors. Thirteen different Vβ genes were represented by the 40 clones. This spectrum is wider than has been reported for other *D. pteronyssinus*-specific T-cell clones, generated with purified *Der p* 1, where a smaller Vβ repertoire was used.[24] For 60% of cases, common Vβ genes were used by $CD4^+$ $CD45RA^+$- and $CD4^+$ $CD45RO^+$-derived T-cell clones.

The likelihood that the $CD4^+$ $CD45RA^+$ T-cell response to *D. pteronyssinus* described in this study could have been the consequence of a primary *in vitro* immune response was considered. Cell culture conditions that were able to induce *in vitro* human T-cell responses to antigens to which the T-cell donor was not known to have been exposed have been described. These antigens include keyhole limpet haemocyanin (KLH), pigeon cytochrome c and malarial antigens.[25–27] The T cells responding in these experiments have not been cloned and their specificity has not been confirmed. Furthermore, the antigen we have used did not produce strong T-cell responses in the non-allergic but exposed individuals tested and we prepared clones in which specificity could be demonstrated. Evidence that there may be immune memory in human $CD4^+$ $CD45RA^+$ T cells is supported by experiments in which these cells, following stimulation with mitogen *in vitro*, were able to transfer antigen-specific memory to tetanus toxoid in SCID mice.[28]

If immune memory is also related to the state of readiness as well as the number of antigen-specific T cells, we may expect that $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ cells would respond to produce different amounts of cytokines when stimulated. This proved to be so, and freshly isolated $CD4^+$ $CD45RA^+$ cells stimulated with PMA and ionomycin produced *c.* 30-fold less IL-4 and IFN-γ than comparable $CD4^+$ $CD45RO^+$ cells. $CD45RA^+$ cells are reported to require greater cross-linking of both CD3 and CD28 than $CD45RO^+$ cells.[29,30] IL-2 gene expression in $CD45RA^+$ T cells is controlled by both a silencer and activator transcription factor.[31] The silencer factor has been lost by CD45RO cells and IL-2 expression is controlled by an activating factor. Overcoming the role of the silencer may be an important step in activating $CD45RA^+$ T cells. Stimulation of $CD45RA^+$ cells with anti-CD3 and anti-CD45RA is reported to switch to the CD45RO phenotype.[32] This response is enhanced in the presence of IL-2. Our cloning procedure should facilitate the growth of $CD4^+$ $CD45RA^+$ as well as $CD4^+$ $CD45RO^+$ cells. Transforming growth factor-β (TGF-β) has also been shown to be an important co-stimulus for $CD45RA^+$ T cells stimulated with anti-CD3/TCR;[33] again this was dependent on exogenous IL-2 being present. Addition of irradiated, activated, CD45RO T cells to CD45RA T cells caused the down-regulation of CD27 and the up-regulation of CD25;[34] again mimicked by the conditions in our cultures in which there would have been irradiated $CD4^+$ $CD45RO^+$ cells.

In this study we have sought to determine whether T-cell memory resides in $CD45RA^+$ $CD4^+$ T cells as well as $CD45RO^+$ $CD4^+$ T cells. The fact that so many $CD45RA^+$ T cells responded to antigen and could be cloned from atopic compared with non-atopic donors strongly suggests that memory to *D. pteronyssinus* does indeed reside in $CD4^+$ $CD45RA^+$ as well as $CD4^+$ $CD45RO^+$ T cells. Recent data from Bunce & Bell[35] on revertant $CD45RC^+$ rat T cells from previously sensitized animals show that 2 months after immunization, and in the absence of further antigenic stimulation, immune memory immune memory can be found in cells with a 'naive' phenotype.

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