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POSTTHYMIC DEVELOPMENT OF CD4+ AND CD8+ T CELLS IN RATS

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INTRODUCTION

Peripheral T cells in rats differ in their expression of the membrane determinants CD4, CD8, CD45RC, RT6 and Thy-1 (*Brideau* et al., 1980; *Ely* et al., 1983; *Spickett* et al., 1983; *Thiele* et al., 1987). By combining these markers it becomes possible to identify a large number of phenotypically different subsets of peripheral T cells (*Kampinga* et al., 1992, 1997).

The presence or absence of CD4, CD8, CD45RC or RT6 expression have been associated with differences in T cell functions (for references see *Kampinga* et al., 1997). Such an association has not been found for Thy-l (*Golub*, 1988).

Besides indicating differences in function, these determinants have also been proposed to mark different T cell lineages or different stages of maturation (*Mojcik* et al., 1988; *Powrie* and *Mason*, 1988; *Thiele* et al., 1987). Relating to this subject numerous studies have been performed with CD45RC. So far however, they have generated rather confusing and conflicting data. Some authors claim that the loss of CD45RC is a uni-directional event and marks a subset of memory cells developing from naive CD45RC T cells after activation by antigen (*Powrie* and *Mason*, 1989). Others suggest that the expression of CD45RC is bi-directional and that this marker identifies T cells that exist transiently in different functional or activated stages (*Bell* and *Sparshott*, 1990; *Sparshot* et al., 1991). A similar situation exists for Thy-1 and RT6, for which it is also still not clear whether cells expressing these markers represent different T cell lineages and/or different functions, and whether losing, respectively gaining of these markers is a uni-directional maturation and/or differentiation effect or just reflecting temporary stages of activation (*Mojcik* et al., 1988).

Theoretically, based on the different possibilities of presence or absence of Thy-1, RT6 and CD45RC expression on T cells, 8 different subsets can be created. This number is increased to 27 when also the level of expression intensity (i.e. dull and bright is taken into account. In previous experiments (*Kampinga* et al., 1992, 1997) we have shown that at least 11 of these latter possibilities can be identified in the peripheral lymphoid organs. To establish the relationships between these 11 different subsets, we used the method of vascular thymus transplantation in RT7 congenic rats (*Kampinga* et al., 1990a), and followed the phenotypic changes of donor thymic emigrants during their sojourn in the lymphoid organs. Based on these and additional short-time thymectomy experiments a putative pathway of postthymic T cell development was postulated (*Kampinga* et al., 1997).

In this article we propose a similar, but more simplified diagram, of post

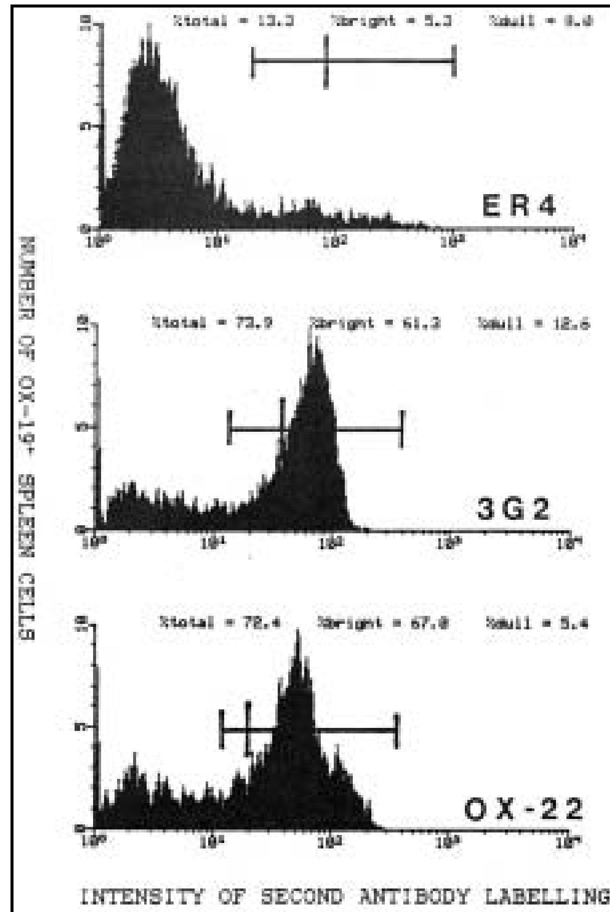

Figure 1: FACS analysis of different T cell subsets (Experiment I).

thymic T cell development and provide additional data, which further strengthens the validity of our hypothesis on the relationships of the different T cell subsets. In addition, new data will be presented showing that $CD4^+$ T cells follow the developmental pathway as originally postulated for all T cells, but that $CD8^+$ T cells take a slightly different route.

MATERIAL AND METHODS

Experiment I

Using standard labelling procedures (*Kampinga* et al., 1997), spleen cells of two SPF 13 wk old male PVG rats were double labelled with FITC conjugated OX-19 (anti-CD5, used as marker to define T cells; *Dallman* et al., 1984) in combination with the biotinylated subset monoclonal antibodies (mab's) ER4 (anti-Thy-1; *Vaessen* et al., 1985), 3G2 (anti-RT6; *Butcher*, 1987) and OX-22 (anti-CD45RC; *Ely* et al., 1983) respectively. The same was done with biotinylated 3G2 and FITC conjugated ER4 and OX-22 respectively. In addition, allo-phyco-cyanine conjugated

Table 1: calculation method used for establishing the percentages of total T cells for each of the 15 different T cell subsets found by OX-22 labelling

Subset phenotype	% of total T cells in spleen	
	FACS	Microscope
Thy-1+ T cells:		
Thy-1^d/RT6^-/CD45R^-	4.0 (1.2)	4.5 (1.7)
Thy-1^d/RT6^d/CD45R^-	1.9 (0.1)	<1
Thy-1^d/RT6^b/CD45R^-	1.8 (0.7)	0
Thy-1^b/RT6^-/CD45R^-	3.4 (0.4)	3.2 (0.6)
Thy-1^b/RT6^d/CD45R^-	0.7 (0.1)	0
Thy-1^b/RT6^b/CD45R^-	1.1 (0.1)	0
Thy-1-/RT6+ T cells:		
Thy-1^-/RT6^d/CD45R^-	2.1 (0.5)	1.2 (1.1)
Thy-1^-/RT6^d/CD45R^d	0.7 (0.1)	1.9 (1.1)
Thy-1^-/RT6^d/CD45R^b	8.3 (0.7)	10.8 (0.9)
Thy-1^-/RT6^b/CD45R^-	4.5 (0.3)	1.5 (0.3)
Thy-1^-/RT6^b/CD45R^d	3.1 (0.1)	2.9 (0.9)
Thy-1^-/RT6^b/CD45R^b	51.3 (1.2)	61.2 (2.6)
Thy-1-/RT6- T cells:		
Thy-1^-/RT6^-/CD45R^-	8.0 (0.1)	9.8 (1.7)
Thy-1^-/RT6^-/CD45R^d	1.5 (0.2)	0
Thy-1^-/RT6^-/CD45R^b	8.1 (2.8)	4.1 (2.8)

OX-19 was used in combination with FITC conjugated ER4 and biotinylated OX-22. R-PE conjugated Streptavidin was used as a second-stage reagent. For analysis a FACS 440 (Becton Dickinson), equipped with an argon and helium-neon laser, in combination with Lysis (Becton Dickinson) software was used. Lymphocytes were gated according forward and sideward scatter profiles.

Based on the shape of the histograms of both labelled spleen cell populations, and the experience of similar previous FACS studies, three arbitrary gates were established for each of the subset mab's separately (Figure 1). In this way OX-19^+ cells were divided in negative, dull and bright labelled cells, for each of the subset mab's respectively. The same gates were used for the double labelling with 3G2 in combination with ER4 and OX-22 respectively. T cells with bright 3G2 staining were gated and within these gated cells the percentage cells with bright ER4 respectively bright OX-22, and dull ER4 respectively dull OX-22 labelling was established. This was repeated for dull 3G2 stained cells. For the triple staining, OX-19^+ cells positive for ER4 staining were gated and checked for OX-22 labelling.

The calculation method used for establishing the percentages of total T cells for each of the 15 different T cell subsets found by this labelling procedure is mentioned in Table 1.

Experiment II

Spleen cells and Peyer's patches (Pp's) cells (pooled from 4 Pp's of each rat) obtained from two SPF 13 wk old male PVG rats were labelled as in experiment I. Analysis of the cells was as in experiment I, but cells were now only checked for the presence or absence (and not the intensity) of labelling.

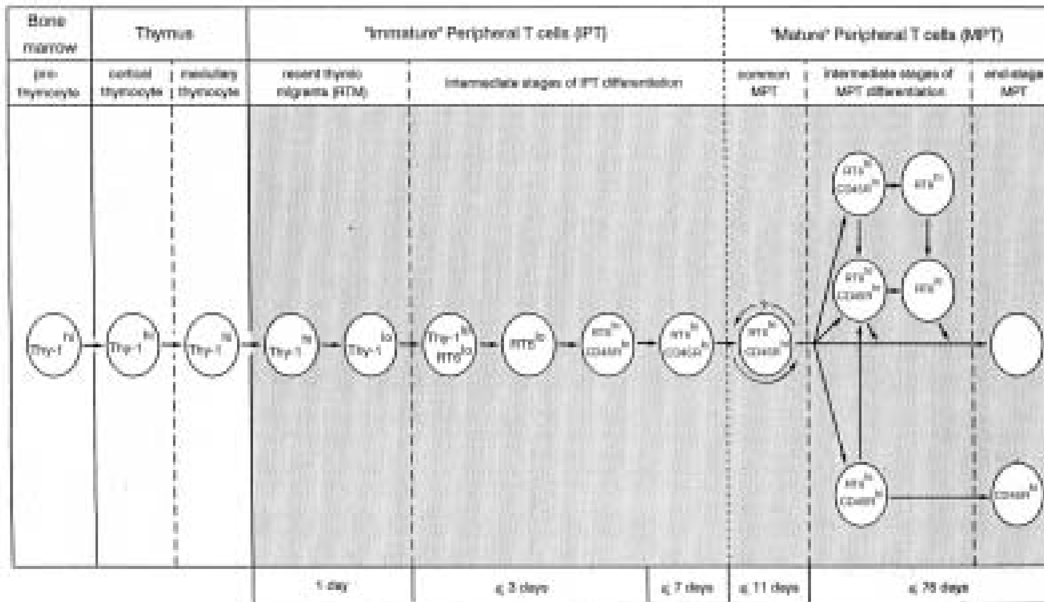

Figure 2: Postthymic T cell differentiation.

Experiment III

Cervical lymph node (CLN) cells (pooled from 4 nodes of each rat) were obtained from two 112 wk old male PVG rats. These rats were kept under SPF conditions for the first 8 wk of life, whereafter they were thymectomised and kept under conventional conditions. Cells were labelled with the same mab combinations as mentioned in experiment I, but instead of using FITC conjugated antibodies, now non-conjugated mab's were used in combination with isotype specific second-stage reagents (*Kampinga* et al., 1997). This method gives a stronger fluorescence signal, which makes it easier to define the difference between negative and weakly positive stained cells. This was especially important, because large numbers of cells lacking Thy-l, RT6 and/or CD45RC expression were expected in this experiment. Pilot experiments had shown that the results of these two staining methods did not differ much from each other when used for T cell subset analysis in young adult rats. Analysis of the cells was as in experiment II.

Experiment IV

Blood leukocytes obtained from 8 wk, 17 wk and 75 wk old male PVG rats (each group n=2) were isolated, using standard procedures, from blood obtained from the tail vein or heart. The same was done for 29, respectively 42 wk old male PVG rats (each group n=2), that were thymectomised at 6 wk after birth (housing conditions for all rats were similar as in the above experiments). Leukocytes were triple labelled using allo-phyco-cyanine conjugated to OX35 (anti-CD4; *Brideau* et al., 1980), respectively OX8 (anti-CD8; *Brideau* et al., 1980) together with the combination of biotinylated 3G2 and FITC-conjugated ER4, and the combination of biotinylated 3G2 and FITC-conjugated OX-22 respectively. For analysis an Elite cell flow cytometer (Coulter), equipped with an argon and helium-neon laser, in combination with Coulter's analysis software was used.

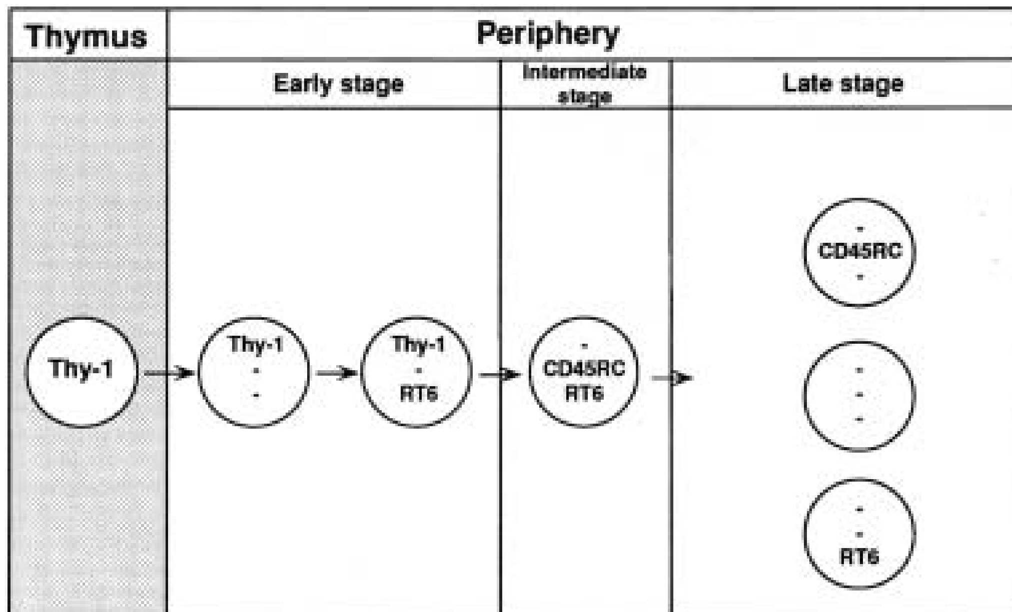

Figure 3: The hypothesis of postthymic T cell development in a more simplified form.

Lymphocytes were gated. In addition, $CD4^+$ respectively $CD8^+$ cells were gated, and within these gated T cell lineages the frequency of the different subsets were analysed for both combinations of subset mab's.

RESULTS AND DISCUSSION

T cell subset analysis by FACS supports the previous hypothesis about postthymic T cell development

The previous hypothesis (see Figure 2) on postthymic T cell development was based on labelling studies using immunofluorescence microscopy (*Kampinga* et al., 1997). With this method 11 different T cell subsets were identified (Table 1). However, counting cells under a microscope may not be very accurate, because the number of cells that can be checked is rather limited and the judgement of whether a cell has a bright or dull labelling is not very objective. Therefore, it was decided that for future studies FACS analysis should be used. However, before doing so it was felt that repeating part of the previous study was needed to see whether the FACS data are comparable with the microscope data, and to find out whether the previous hypothesis still holds despite changing the method.

The results of experiment I (Table 1) show that all subsets identified by microscopy were also found by FACS analysis, and that no major differences in the percentages were found. With FACS analysis 4 additional "new" subsets could be identified. Their respective percentages however, were less than 2% and therefore around the limits of the accuracy of the used method for FACS analysis. But even when they really exist, their presence does not interfere with the validity of the original hypothesis.

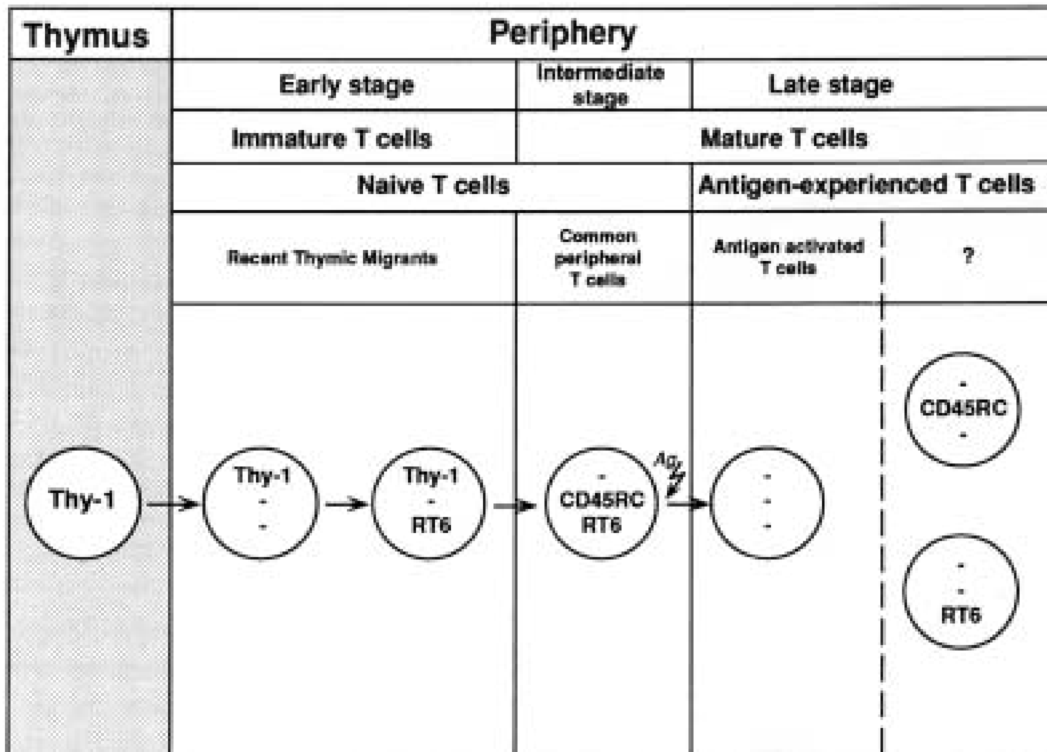

Figure 4: Both Thy-1$^+$ and Thy-1$^-$/RT6$^+$/CD45RC$^+$ T cells are not part of the late stages of postthymic T cell development.

The hypothesis of postthymic T cell development in a more simplified form (Figure 3)

The large number of different T cell subsets shown in Figure 2 makes it very complicated to further unravel the process of T cell development. From our previous experiments (*Kampinga* et al., 1997) it has become clear that distinguishing dull from bright labelled cells is not essential. Although their presence in the diagram of postthymic T cell development gives a certain refinement, they do not seem to be necessary for understanding the important steps in the pathway of postthymic T cell development. All the different T cell subsets mentioned in Table 1 can be accommodated within the following 6 main subsets:

Thy-1$^+$/RT6$^-$/CD45RC$^-$,
Thy-1$^+$/RT6$^+$/CD45RC$^-$,
Thy-1$^-$/RT6$^+$/CD45RC$^+$,
Thy-1$^-$/RT6$^+$/CD45RC$^-$,
Thy-1$^-$/RT6$^-$/CD45RC$^+$, and
Thy-1$^-$ /RT6$^-$/CD45RC$^-$.

Using the previous hypothesis, the relationships between these main subsets can be drawn in its most simplified form as shown in Figure 3. The validity of this basic diagram will be tested and the contents extended with the following experiments.

Both Thy-1$^+$ and Thy-1$^-$/RT6$^+$/ CD45RC$^+$ T cells are not part of the late stages of postthymic T cell development (Figure 4)

In the previous study (*Kampinga* et al., 1997), it was shown that all thymo-

cytes that leave the thymus express Thy-1 in the absence of CD45RC and RT6 expression. Already within one week the majority of these recent thymic migrants (RTM's) become Thy-l$^-$ and start to express both maturity markers CD45RC and RT6. These latter cells are called common peripheral T cells (CPT's). After a while, CPT's start to loose one or both maturity markers, and become part of the last stages of postthymic T cell development.

One way of explaining this route is that all RTM's "automatically" and quickly develop into CPT's (as part of an extra-thymic and last maturation step for thymocytes), but that CPT's have to wait for getting triggered by antigen recognition, before they differentiate into the late-stage compartment.

This explanation would predict, that (i) when the production of thymocytes is stopped for a short period of 2 wk, Thy-1$^+$ T cells should be nearly absent, whereas the frequency of CPT's should be hardly influenced. Furthermore, this would predict (ii) that in the absence of thymocyte production for a long period of time the CPT frequency should severely decrease. The first prediction has been shown to be true in our previous experiments (*Kampinga* et al., 1997). More recently we have shown that 2 years after thymectomy the frequency of CPT's has been halved compared to the adult situation.

Antigen-activated and proliferating T cells are Thy-1$^-$/RT6$^-$/ CD45RC$^-$, and antigen recognition is likely to be involved in the transition from CPT to late-stage T cells (Figure 4)

In contrast to spleen, Pp's will contain large numbers of (gut) antigen-activated and proliferating T cells. So, comparison of the subset frequencies between spleen and Peyer's patches should reveal the phenotype of activated/proliferating T cells. Furthermore, if our idea that CPT's have not experienced previous antigen contacts is true and that, in contrast to the postulated maturation step between RTM's and CPT's, transfer to the late-stage compartment only happens after activation by antigen, the expected increase of activated T cells in Pp's should be accompanied by a decrease of a similar magnitude within the CPT population, but should hardly effect the frequency of RTM's. We have shown that activated/proliferating T cells are likely to be Thy-l$^-$/RT6$^-$/CD45RC$^-$, and that the enormous increase of the frequency of this subset is almost exclusively at the expense of the CPT frequency. Our proposed phenotype for activated/proliferating T cells *in vivo* is in agreement with studies showing that antigen or mitogen activated T cells loose CD45RC and RT6 expression *in vitro* (*Bell*, 1992; *Powrie* and *Mason*, 1989, 1990).

Thy-1$^-$/RT6$^+$/CD45RC$^-$ and Thy-1$^-$/RT6$^-$/CD45RC$^+$ T cells are likely to be resting memory T cells (Figure 5A and 5B)

Memory T cells with specificity for a certain antigen originate from T cells that have been previously activated by the same antigen. Since further development (besides a temporary state of [re-]activation when a memory T cell encounters the same antigen) of memory T cells is unlikely, memory T cells are seen as the end-stages of postthymic T cell development (*Freitas* et al., 1986; *Jerne*, 1984; *Stutman*, 1986).

All antigen-activated/proliferating T cells are likely to be Thy-l$^-$/RT$^-$/ CD45RC$^-$ T cells (see above). In addition, if one conversely assumes that all Thy-l$^-$/RT6$^-$/CD45RC$^-$ T cells are in a state of activation, than only the remaining two other phenotypes of the late-stage compartment can be consid-

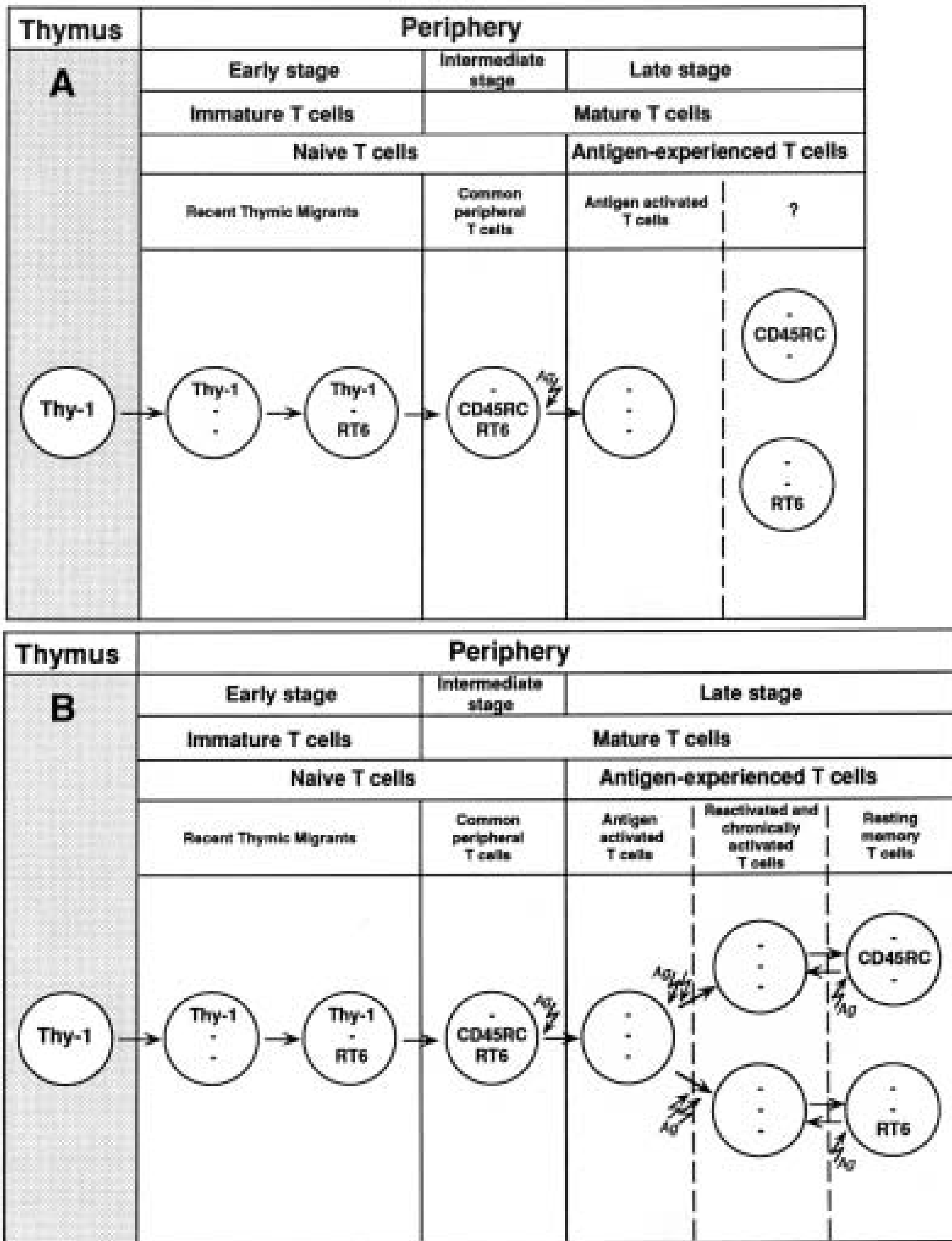

Figure 5: Thy-1$^-$/RT6$^+$/CD45RC$^-$ and Thy-1$^-$/RT6$^-$/CD45RC$^+$ T cells are likely to be resting memory T cells.

ered as being resting memory T cells. A consequence of this idea is that, similar to the findings of the group of Bell (*Bell* and *Sparshott*, 1990; *Bell*, 1992), the

loss of CD45RC (and RT6) is reversible, and that for the majority of T cells the presence or absence of CD45RC may only indicate whether a T cell is resting or activated respectively.

Powrie and *Mason* (1989) have found that memory T cells that help B cells in a secondary response are $CD45RC^-$. According to our diagram, these memory T cells are likely to be of the $Thy\text{-}1^-/RT6^+/CD45RC^-$ phenotype. Whether cells with the $Thy\text{-}1^-/RT6^-/CD45RC^+$ phenotype are involved in different memory T cell functions as, for instance, DTH reactions ($CD4^+$ T cells) and/or secondary graft rejections ($CD8^+$ T cells) is not clear from the literature.

Besides acutely antigen-activated T cells also chronically antigen-activated T cells may exist. It has for instance been suggested that *in vitro* activated Th0 cells, that have developed from naive T cells due to a single addition of antigen, can further differentiate into activated Th1 or Th2 cells when they are chronically stimulated by the same antigen (*de Jong* et al., 1992; *H. Savekoul*, personal communication). Rather than directly developing from activated Th0 cells, we feel it to be more likely that resting memory T cells develop from these chronically activated Th1 and Th2 cells. Both acutely and chronically antigen-activated T cells are likely to be $Thy\text{-}1^-/RT6^-/CD45RC^-$. In the human situation acutely and chronically activated late-stage T cells (i.e. $CD45RO^+$) differ in their expression of CD27 (*Loenen* et al., 1991), a marker that unfortunately is not available in rats. In addition, *Gray* (1991) has suggested that for the maintenance of a particular memory T cell repertoire, repeated reactivation (possibly due to the proposed continuous presence of a small amount of antigen left in the body) of these memory T cells is necessary. So it may well be that chronic activation not only leads to the initial forming of memory T cells, but that this is also a prerequisite for maintaining them. So we would expect a high turnover of T cells in the late-stage T cell compartment. Recent observations of the group of Beverly (*Michie* et al., 1992), showing that, in contrast to CPT's, late stage T cells are short-lived, fit nicely with this prediction.

Possibilities for maintenance of the CPT pool after thymectomy (Figure 6)**:**

An unexpected result from the long-term thymectomy experiment was that CPT's, although strongly decreased in percentage, were still present. One possible explanation is that the remaining CPT's have still not encountered their antigen trigger for further development. Although this can not be excluded, it is unlikely since we waited two years after thymectomy before analysing the cells, which should be enough to enable a rat housed under conventional conditions to encounter a vast number of different antigens. In addition, according to the work of *Stutman* (1986), it is likely that the remaining CPT's that have not received such an antigen-trigger during this period would have died as part of a peripheral repertoire selection process. Our finding that large numbers of T cells die soon after they have left the thymus (*Kampinga* et al., 1988, 1997) fits nicely with *Stutman*'s idea.

Another explanation, which we favour, is that CPT's might have self-renewal capacity. From other studies (*Freitas* et al., 1986; *Stutman*, 1986) it has become clear that within the T cell pool more cells die than are emigrating from the thymus. *Stutman*'s experiments suggest that the peripheral T cell pool as a whole is maintained by the progeny of a special subset of T cells with precursor activity (the so called postthymic T cell precursors [PTP's]

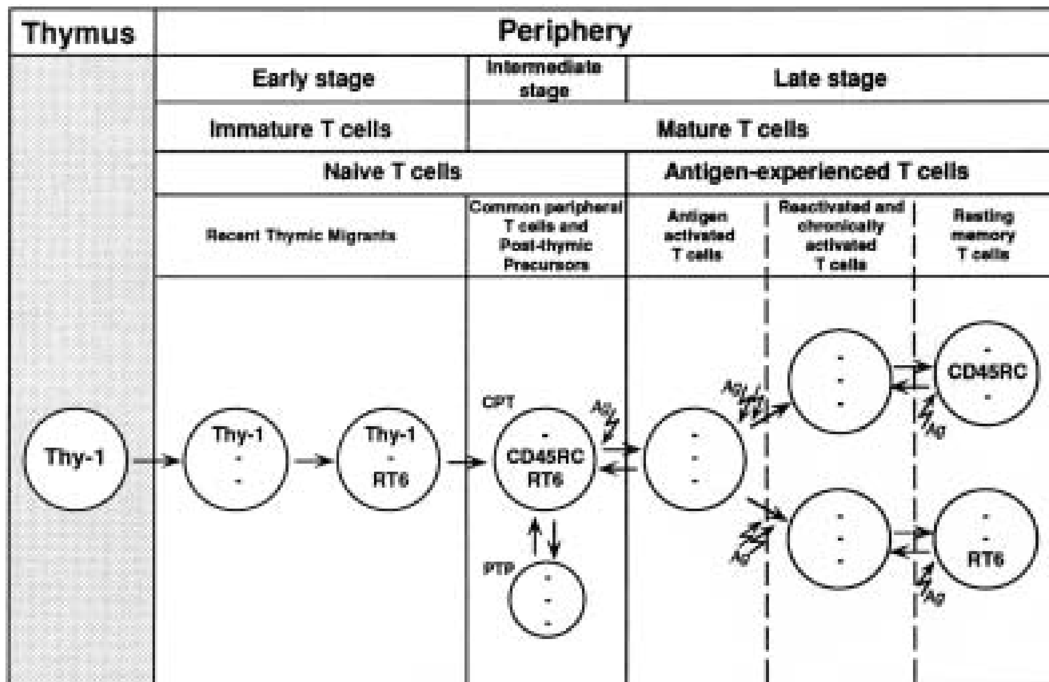

Figure 6: Possibilities for maintenance of the CPT pool after thymectomy.

that proliferate in the absence of an antigen trigger; for references see: *Stutman*, 1986). If one assumes that the number of T cells in the peripheral T cell pool is solely maintained by PTP's, and the unexpected large number of remaining CPT's after thymectomy is a result from the self-renewing capacity of these PTP's, than at least part, if not all, of the PTP activity must derive from cells situated in the intermediate stage. In the absence of thymic output one might expect that a substantial part of these PTP's are proliferating. From the results of experiment II one can expect that proliferating PTP's lack both RT6 and CD45RC expression. In conclusion, our data suggest that active PTP's are Thy-1$^-$/RT6$^-$/CD45RC$^-$ and resting PTP's are Thy-1$^-$/RT6$^+$/CD45RC$^+$. This also fits nicely with the work of *Sparshott* and colleagues (1991), who showed that potential PTP capacity is present in both CD45RC$^+$ and CD45RC$^-$ T cell subsets (when transferred to athymic nude rats, small numbers of both these cell populations were shown to expand very rapidly by an apparently non-antigen driven mechanism, resulting in normal T cell numbers after a few months). Whether partial PTP activity is also present amongst cells of the late-stage compartment can not be excluded from our experiments. *Stutman*'s experiments, however, strongly suggest that PTP activity is only present in the early stages of T cell development (*Stutman*, 1986).

A third explanation, favoured by the groups of Bell (*Bell*, 1992) and Rocha (*Freitas* et al., 1986; *Rocha*, 1987), is that cells from the late-stage compartment can return to the intermediate stage. The idea is that all T cell divisions necessary to maintain the peripheral lymphocyte pool are antigen driven. This would mean that part of the antigen activated Thy-1$^-$/RT6$^-$/CD45RC$^-$ T cells

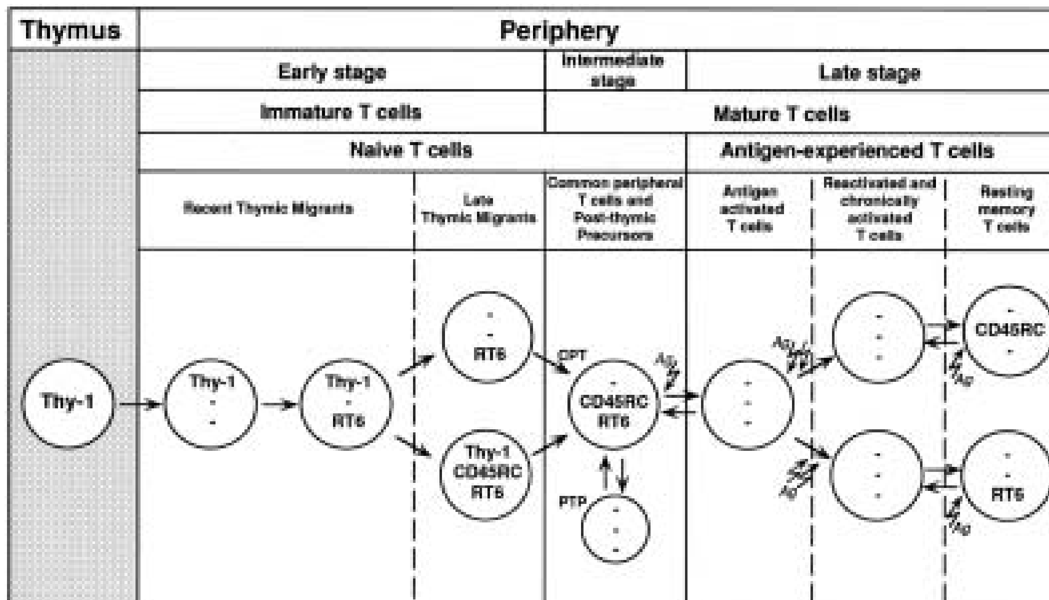

Figure 7: Additional maturation steps in the early-stage compartment.

will return to a resting state and become Thy-1$^-$/RT6$^+$/CD45RC$^+$ again. Although such a mechanism explains why the vast majority of CD45R$^-$ rat T cells rapidly changes in CD45RC$^+$ T cells when maintained *in vitro* in the absence of antigen (*Bell*, 1992), but does not explain why memory T cells have not been found amongst CPT's. A possibility that might explain both observations is that only activated Th0-like cells are able to return to the intermediate-stage compartment, whereas cells that are already further differentiated into chronically activated T cells have changed in such a way that they have lost this capacity and irreversibly will become memory T cells when they enter the resting state. Conversely, this change or commitment might be such that upon reactivation of resting memory T cells, the cells can also not enter the intermediate stage anymore. By definition it is of course not possible to regard cells that have been activated by antigen and returned to the Thy-l$^-$/RT6$^+$/CD45RC$^+$ phenotype as being "antigen naive". Functionally however, they can be regarded as being naive, since, at least so far, only primary responses have been found amongst Thy-1$^-$/RT6$^+$/CD45RC$^+$ T cells.

In conclusion, one could argue that in the absence of thymic output the pool of CPT's is partially maintained by both antigen and non-antigen driven cell divisions. Since it is assumed that the non-used naive repertoire vanishes as part of a peripheral selection process, both antigen and non-antigen driven proliferation eventually will lead to a severely decreased diversity of the intermediate-stage compartment's repertoire, and the remaining naive repertoire is likely to become oligoclonal in nature upon ageing.

Additional maturation steps in the early-stage compartment (Figure 7)**:**

In the previous experiments using the model of vascular thymus transplantation (see Figure 1) it was suggested that Thy-l$^+$/RT6$^+$/CD45RC$^-$

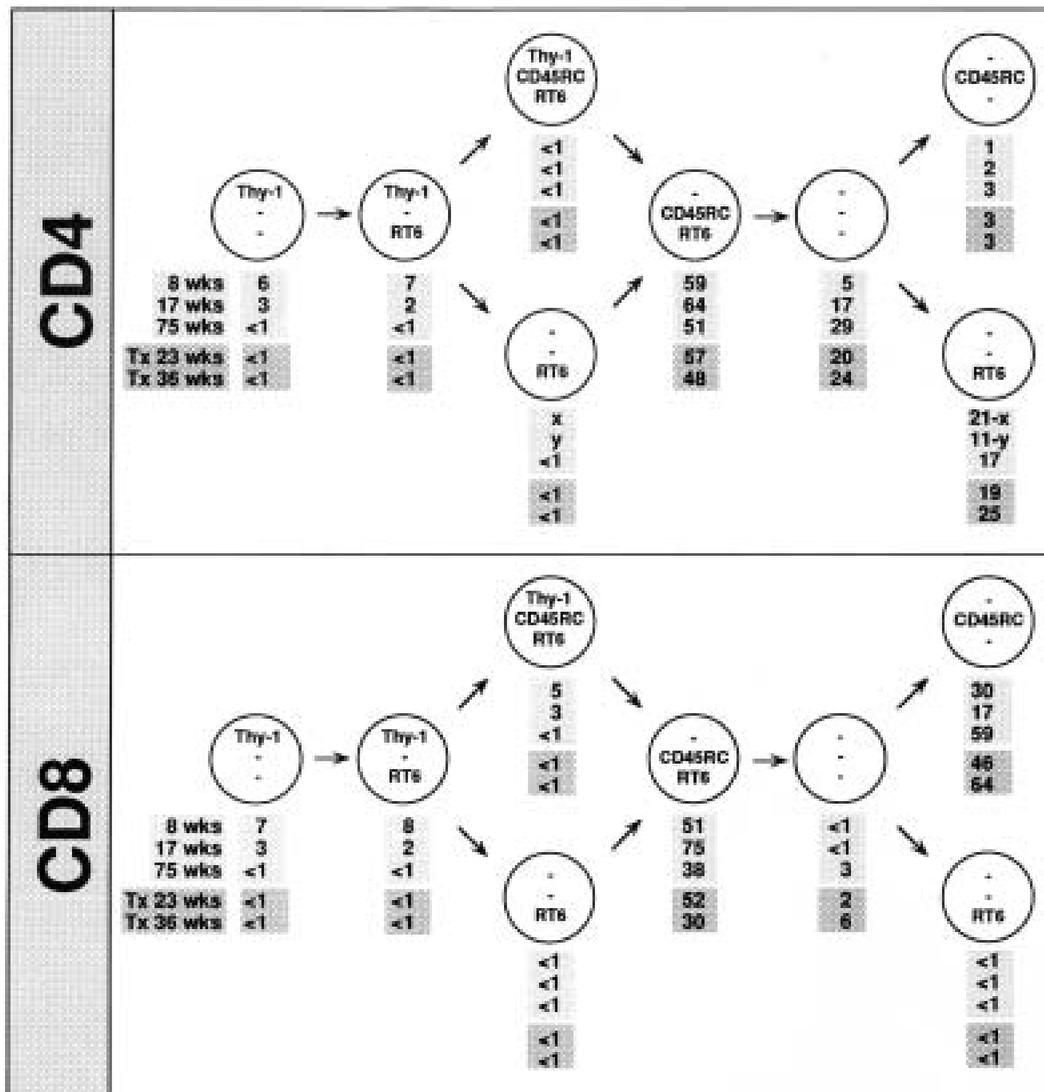

Figure 8: Postthymic T cell development of CD4$^+$ and CD8$^+$ T cells (1).

RTM's before maturing into CPT's did pass a stage in which they had lost Thy-1 expression, but had not yet gained CD45RC on their membrane (phenotypically this subset of late thymic migrants does not differ from the Thy-1$^-$/RT6$^+$/CD45RC$^-$ resting memory cells!). Recently, by analysing T cell subsets in neonatal rats, we have found more evidence for the existence of this intermediate subset. In the first two weeks after birth high percentages (up to 40%!) of Thy-l$^-$/RT6$^+$/CD45RC$^-$ T cells could be found, whereas during this period the frequency of Thy-l$^-$/RT6$^+$/CD45RC$^+$ T cells was very low (<10%). These data are in agreement with recent studies of *Thiele* and colleagues (1987). Although, formally we can not exclude the possibility that all of these Thy-l$^-$/RT$^+$/CD45RC$^-$ are in fact resting memory T cells, it is very unlikely that such a large number of T cells would have had time enough in the first two weeks

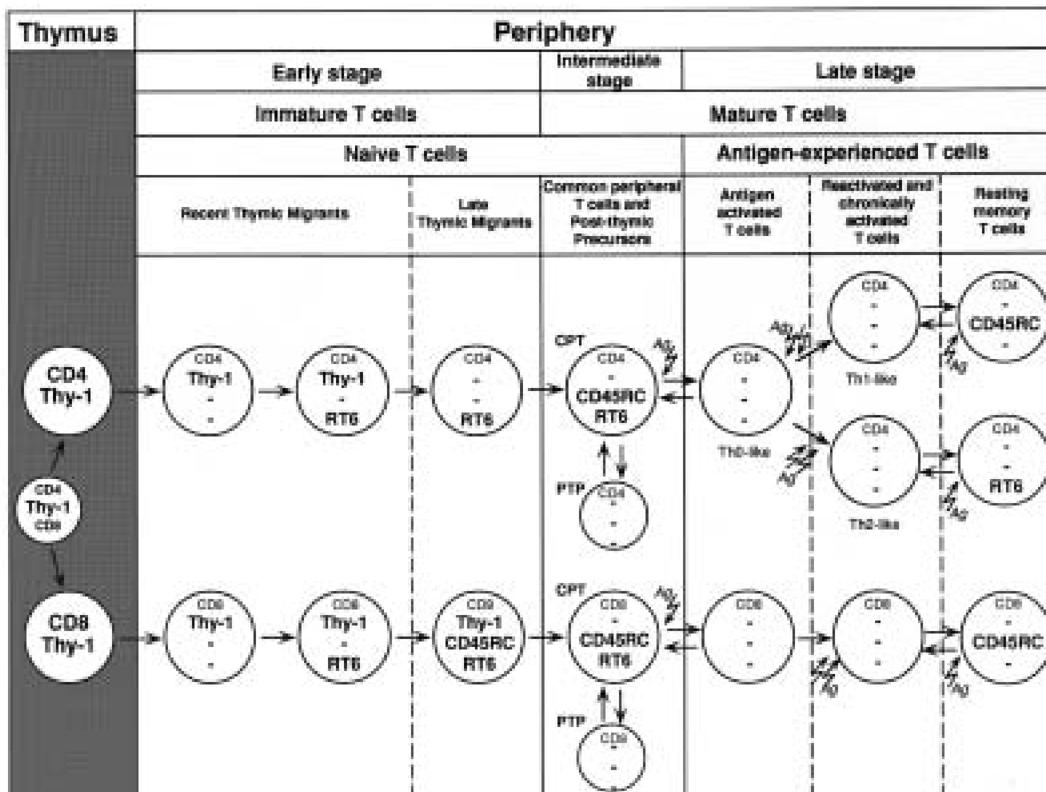

Figure 9: Postthymic T cell development of $CD4^+$ and $CD8^+$ T cells (2).

of life to follow the sequence of maturing into CPT's, getting activated by antigen, passing the chronically activated stage and ending as resting memory T cells. Therefore, we feel that in neonatal and young adult animals, which both have a high output of recent thymic migrants (see Figure 8), a large part, if not all, of the $Thy\text{-}1^-/RT6^+/CD45RC^-$ subset are in fact thymic migrants rather than resting memory T cells.

Although not found in adult and aged rats, occasionally a subset of T cells with the $Thy\text{-}1^+/RT6^+/CD45RC^+$ phenotype has been noticed in very low frequencies (around 1%) in young adult rats, and has been found to be increased in neonatal rats (around 3%). Due to the expression of Thy-l it is likely that these T cells are late thymic migrants, which are just about to become CPT's.

In conclusion, apparently two different subsets of T cells seem to be located between RTM's and CPT's. Because it is difficult to see in which sequence these more mature thymic migrants should be drawn in our diagram of postthymic T cell differentiation, they have been located above each other (Figure 7).

Postthymic T cell development of $CD4^+$ and $CD8^+$ T cells (Figures 8 and 9):

So far, our present hypothesis on postthymic T cell differentiation is based on data from the total population of T cells. Whether the T cell developmental pathways are similar or different within de $CD4^+$ respectively $CD8^+$ T cell lineages is subject of our present studies, from which the data of a first set of pre-

liminary experiments will be shown here.

In PVG rats about 80% of the peripheral T cells are $CD4^+$. So it is likely to expect that the majority, if not all, of our findings for the development of the total T cell pool will also apply for $CD4^+$ cells. Experiments from *Mason*'s group (*Powrie* and *Mason*, 1988), showing that all $CD8^+$ T cells are nearly found to be $CD45RC^-$ is already an indication that a similar sequence is not necessary the case for $CD8^+$ T cells.

Previous experiments from our own group have shown that both $CD4^+$ and $CD8^+$ T cells are Thy-1^+ when they leave the thymus, and that the RTM's of both lineages are likely to pass the intermediate Thy-1^-/$RT6^+$/$CD45RC^+$ stage before entering the late-stage compartment (*Kampinga* et al., 1997). In addition, *in vitro* activation of both subsets resulted in the loss of CD45RC and RT6 (*D. Mason,* and *H-G. Thiele*, personal communication). Based on these previous findings it looks like as if the major steps of postthymic maturation and differentiation are similar for both subsets. Therefore, it was decided to use the present hypothesis as a temporary template to analyse the development of $CD4^+$ and $CD8^+$ T cells separately.

Using triple-staining, it was first analysed whether all the 7 phenotypically different subsets mentioned in Figure 7 could also be found within $CD4^+$ respectively $CD8^+$ blood T cells, derived from young adult (8 wk) and adult (17 wk) rats. As shown in Figure 8, it was found that within $CD4^+$ T cells only the Thy-1^+/$RT6^+$/$CD45RC^+$ phenotype could not be identified, whereas within $CD8^+$ T cells only the Thy-1^-/$RT6^+$/$CD45RC^-$ phenotype was lacking. This strongly suggests that $CD4^+$ RTM's mature into CPT's with Thy-1^-/RT^+/$CD45RC^-$ as an intermediate step, whereas $CD8^+$ RTM's seem to use Thy-1^+/$RT6^+$/$CD45RC^+$ as an intermediate step. Furthermore, the complete absence of Thy-1^-/$RT6^+$/$CD45RC^-$ cells suggests that, in contrast to $CD4^+$ cells, $CD8^+$ cells might only have one phenotype (i.e. (Thy-1^-/$RT6^-$/$CD45RC^+$) indicating resting memory T cells.

Another test for showing that the proposed sequence of postthymic development for the total T cell pool is, at least for the most part, also applicable for the $CD4^+$ and $CD8^+$ T cells separately, is to compare within these two lineages the frequencies of the different phenotypes during ageing of the T cell pool. It is generally assumed that aged rats have hardly any thymic function left (e.g. *Greenstein* et al., 1987; *Ezaki* et al., 1990). Therefore, the prediction would be that, compared to 17 wk old adult rats (young adult rats can not be used, since the presumed [see above] presence of high numbers of late thymic migrants in these rats makes the interpretation of the data very complicated), 75 wk old aged rats should have a severely decreased frequency of phenotypes that mark thymic migrants and CPT's, whereas an increase is to be expected for T cells expressing markers that are supposed to be indicative for resting memory T cells. Furthermore, since repeated activation is associated with the maintenance of the memory T cell pool (see above), an increase in the frequency of memory T cells should be accompanied with an increase of activated T cells exhibiting the proposed Thy-1^-/$RT6^-$/$CD45RC^-$ phenotype. Similar findings are to be expected by comparing 17 wk old rats with adult rats that have been thymectomised at 6 wk of age, and by comparing rats that differ in the length of the period after thymectomy. Figure 8 clearly show that all of these expectations are met.

Based on these results a new diagram of postthymic T cell development can now be drawn for the separate $CD4^+$ and $CD8^+$ T cell lineages (Figure 9).

CONCLUSIONS AND FUTURE EXPERIMENTS

The above experiments have further strengthened the key parts of our previous hypothesis on postthymic T cell development. Furthermore, some minor alterations have been added, and suggestions have been put forward to explain how the CPT pool maintains a reasonable size during ageing. In addition, a new hypothesis has been postulated about the sequence of postthymic T cell development separately for $CD4^+$ and $CD8^+$ T cells (Figure 9).

This new hypothesis is mainly based on indirect evidence, as changes in the frequencies of the different subsets during ageing and after thymectomy. Additional experiments, in which the different T cell subsets are sorted, transferred to congenic (nude) rats and checked for phenotypic changes at different intervals (*Bell* and *Sparshot*, 1990; *Sparshot* et al., 1991; *Fowell* et al., 1991), are needed to formally proof the postulated relationships. Furthermore, new ageing and thymectomy experiments are needed to investigate whether the changes in phenotype we have found upon ageing are due to some kind of special feature of recirculating T cells in blood, or whether they are representative for the whole T cell pool.

Another point that need being studied is our assumption that all ($Thy\text{-}l^-$) T cells that lack both RT6 and CD45RC expression are always activated/ proliferating T cells. Besides classical BrdU incorporation studies, activated/ proliferating cells can also be identified using HIS45, an antibody against QCA-l, because this antigen is thought to be only present on non-activated, non-proliferating quiescent cells (*Kampinga* et al., 1990b). This marker might therefore be a useful tool to investigate whether within the $Thy\text{-}l^-/RT6^-/\ CD45RC^-$ subset a small non-dividing "new" subset is hidden. Similar studies are planned using OX39 and OX-40, which are antibodies specific for different activation molecules (*Fowell* and *Mason*, 1993; *Paterson* et al., 1987).

In the diagram, we not only postulate a sequence of phenotypic changes, but also connect purely speculative, certain phenotypes with certain T cell functions. Sorting of, for instance, the proposed Th1-like and Th2-like resting memory T cells followed by activation *in vitro* is needed to establish their expected cytokine production profiles (Th1: IL-2 and IFN-γ; Th2: IL-4, 5 and 10) (*Fowell* et al., 1991). In addition, functional studies will be needed to investigate whether the proposed Th1-like memory T cells are involved in typical functions as DTH, help for CD8 cytotoxic T cells, macrophage activation and direct cytotoxicity, and whether the proposed Th2-like memory T cells are involved in typical functions as B cell help, initiation of IgE production and regulation of eosinophils/mast cells (for references: see *Swain* et al., 1991).

Factors suggested to be involved in influencing activated Th0-like cells in deciding whether to differentiate in either Th1-like or Th2-like subsets are, amongst others, type of APC, concentration of presented antigen and it's structure, route of antigen challenge, and the presence of certain cytokines made by other leukocytes (for references: see *Swain* et al., 1991).

One can imagine that these circumstances may differ from one rat strain to the other, resulting in rats that preferable react on antigens in a Th1-like and others in a Th2-like fashion, and it is likely that such preference can be indirectly read from the ratio between the frequencies of the two resting $CD4^+$ memory T cell subsets. As a consequence of these differences, the former

rats might be prone to get spontaneous or easily induced cellular autoimmune diseases, whereas the latter rats might be prone for humoral autoimmune diseases and IgE based allergies. In a first pilot experiment, we have indeed found some indications for this idea. BB and adult Lewis rats were found to lack completely our proposed Th2-like resting memory ($Thy\text{-}1^-/RT6^+/CD45RC^-$) T cell subset, and these animals are known to be models for cellular autoimmune diseases like diabetes, thyroiditis, multiple sclerosis and rheumatoid arthritis (for references: see *Groen* et al., 1989). On the contrary, adult BN rats were found to lack completely our proposed Th1-like resting memory ($Thy\text{-}1^-/RT6^-/CD45RC^+$) T cell subset, and these animals are used as an autoantibody induced nephritis model and known for their increased production of IgE antibodies (for references: see *Aten*, 1992). Control rat strains like AO and PVG, that are not prone to develop autoimmune diseases, were found to contain both of the proposed resting memory T cell subsets. Obviously these intriguing findings need confirmation, and a large number of different rat strains should be incorporated in the follow-up studies. When these findings are confirmed, one might try to explore the possibility of developing new therapies for auto-immune diseases based on influencing the factors involved in directing activated Th0-like cells in their differentiation to Th1- or Th2-like cells, an idea already previously suggested by Mason's group (*Day* et al., 1992).

Pilot studies have shown that our results in thymectomised rats do not markedly differ from euthymic 2,5 year old rats. This not only further confirms that aged thymi do not produce RTM's in traceable numbers anymore (see Figure 8), but also suggest that the presence or absence of thymic hormones do not seem to be of importance in regulating the pathway of postthymic T cell development. Furthermore, our data indicate that a large population of T cells in aged rats are already antigen experienced, and that the diversity of repertoire amongst the remaining (and during ageing continuously further declining number of) naive CPT's is severely decreased. Due to the absence of input from thymic derived "fresh" repertoire, holes in the peripheral T cell repertoire will eventually develop and the naive repertoire will become oligoclonal in nature. As a consequence of this, aged rats are in a situation in which they may encounter antigens for which highly specific T cell receptors are not available anymore. This situation might, at least partly, be responsible for the impairment of immune functions found in ageing immune systems (for references: see *Makinodan* and *Kay*, 1980). Regeneration of thymic function in aged persons might solve this problem. Such a therapy has become a realistic option for the near future due to the fact that growth hormone, which has a potent rejuvenating effect on aged thymi (*Kelley* et al., 1986), has become readily available in a recombinant format (*Vance*, 1990).

An important issue about animal studies is whether the data generated from such experiments reflect the human situation. Unfortunately, it is unknown whether human T cells express RT6 antigens, although the genetic code seems to be present (*H-G.Thiele*, personal communication). In addition, CD45RC has not yet been identified in humans. However, the expression pattern of the human CD45RB antigen has been suggested to be similar to the rat's CD45RC (*Fowell* et al., 1991). Based on our new hypothesis, we have started to perform a few initial experiments on analysing human $CD4^+$ T cell subsets in the late-stage compartment (i.e. $CD45R0^+/CD45RA^-$). Using additional

markers for "acute" activation ($CD38^{+}$) and chronic activation (CD27) (*Loenen* et al., 1991), preliminary results show that in the late-stage compartment the following $CD4^{+}$ T cell subsets can be identified: $CD27^{+}/CD38^{+}/CD45RB^{-}$ (possibly representing Th0-like activated cells), $CD27^{-}/CD38^{+/-}/CD45RB^{-}$ (possibly representing chronically activated Th1- and Th2-like cells), $CD27^{-}/CD38^{+}/CD45RB^{-}$, and $CD27^{+}/CD38^{-}/CD45RB^{-}$. To our opinion, the latter two are likely to represent the proposed rat analogues for resting Th1- and Th2-like memory T cells respectively. Similar ageing and thymectomy (Myasthenia Gravis patients!) studies, as have been performed in rats, are presently in the process of being carried out in humans. These and additional studies on cytokine profiles of sorted human T cell subsets will help to further unravel the phenotypes of human memory (and other important) T cell subsets, and might also answer the question whether experiments in animals are useful to understand the complexity of the human immune system.

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