# Targeted Complementation of MHC Class II Deficiency by Intrathymic Delivery of Recombinant Adenoviruses

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# Summary

De novo differentiation of CD4<sup>+</sup> T cells was provoked in mice lacking major histocompatibility complex (MHC) class II molecules by intrathymic injection of adenovirus vectors carrying class II genes. This permits a new approach to questions concerning the dynamics of CD4<sup>+</sup> T cell compartments in the thymus and peripheral lymphoid organs. Here two issues are explored. First, we show that mature CD4+CD8- cells reside in the thymus for a protracted period before emigrating to the periphery, highlighting the potential importance of, and our ignorance of, the postselection maturation period. Second, we demonstrate that the survival of CD4<sup>+</sup> cells in peripheral lymphoid organs is markedly curtailed when class II molecules are absent and is not further reduced in the absence of both class II and class I molecules, raising the possibility that MHCmediated selection may continue in the periphery.

#### Introduction

Key to an understanding of the immune system is an appreciation of the population dynamics of the different lymphocyte compartments, both in the resting state and after confrontation with antigen. The immune system must accommodate two conflicting needs: the constant requirement for a repertoire containing a broad range of specificities in order to meet a diversity of antigenic challenges, and the sporadic requirement for rapid mobilization of a select subset of specificities to respond effectively to particular challenges. Since the total number of circulating lymphocytes is limited in the adult, the production of cells in the primary lymphoid organs and their export to the periphery need to be constrained, and the composition of peripheral pools needs to be regulated-the homeostasis of naive cells, expansion of antigen-stimulated cells, and the maintenance of a memory population. These controls are exerted largely through regulation of lymphocyte lifespans (reviewed by Freitas and Rocha, 1993).

The dynamics of T cell differentiation in the thymus

have been studied by several groups, and there is general agreement on most of the major points (Egerton et al., 1990; Huesmann et al., 1991; Lucas et al., 1993; Ernst et al., 1995). However, a consensus has not yet emerged on the behavior of mature thymocytes, once they have attained the single-positive CD4+CD8- or CD4-CD8+ stage expressing high levels of T cell receptor (TCR) and have migrated from the cortex into the medulla (see discussion by Scollay and Godfrey, 1995; Tough and Sprent, 1995b). It has been reported that they remain there for a prolonged period before exiting to the periphery, from one to two weeks on average (Egerton et al., 1990). Indeed, a progressive further maturation of singlepositive thymocytes can be observed, as a gradual change in expression levels of several differentiation markers (e.g., heat shock antigen, Qa-2, and CD69 [Vernachio et al., 1989; Nikolic-Zugic and Bevan, 1990; Ramsdell et al., 1991; Lucas et al., 1994]), and it has been estimated that reaching full maturity according to such criteria requires an average of 5 or 6 additional days (Lucas et al., 1994). However, conflicting results have also been presented, contending that thymocytes exit to the periphery almost immediately once they have attained the single-positive stage (Tough and Sprent, 1994) and demonstrating that maturation as defined by the same differentiation markers is also characteristic of the population of recent thymic emigrants (Kelly and Scollay, 1990). In addition, no matter how long the average residence of single-positive cells in the medulla, it is not yet known whether this merely reflects probabilistic behavior and the exit of a fixed fraction every day, irrespective of the transit time of individual cells, or an incompressible time of stay in the medulla (Scollay and Godfrey, 1995; Tough and Sprent, 1995b).

The dynamics of T cell compartments in the periphery has also been an area of active study (reviewed by Tough and Sprent, 1995a). Although initial debate was heated (Freitas and Rocha, 1993; Rajewsky, 1993), a consensus seems to have emerged more recently that most naive T cells are quiescent and can persist for long periods in the absence of intentional antigenic stimulation (Sprent et al., 1991; von Boehmer and Hafen, 1993; Tough and Sprent, 1994; Bruno et al., 1995). Some memory T cells are similarly long-lived, but there is also a component of this population that is dividing and that has a significantly shorter lifespan (Tough and Sprent, 1994; Bruno et al., 1995; Bruno et al., 1996). How critical stimulation by persisting antigen is to the maintenance of the memory population is still controversial (Ahmed and Gray, 1996). Naive and activated memory T cell numbers appear to be regulated independently (Tanchot and Rocha, 1995; Bruno et al., 1996); while at present we have little understanding of how this is achieved, it has recently been shown that naive and memory cells do respond differently to certain growth factors, such as the  $\alpha/\beta$  interferons (Tough et al., 1996). Clearly, much needs to be learned about the cellular and molecular interactions that permit the cells in both compartments to persist for such long periods.

Several approaches have been employed over the

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years to study T cell population dynamics. These have often entailed manipulations (such as irradiation, thymus transplantation, cell transfer, treatment with cytostatic drugs, or incorporation of labeled DNA precursors) that could potentially bias experimental results. Here we describe an alternative approach that can be used to address some of the outstanding questions, providing an independent perspective. It involves the use of adenovirus vectors carrying major histocompatibility (MHC) class II genes to complement the defect of mutant mice created by homologous recombination. When injected intrathymically, these recombinant viruses provoke expression of class II molecules on thymic stromal cells, selection of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from their blocked CD4<sup>+</sup>CD8<sup>+</sup> precursors, and eventual export of fully mature CD4<sup>+</sup> cells to the peripheral lymphoid organs. A synchronized cohort of cells is thereby generated, and its behavior during differentiation and survival upon maturity can be readily monitored.

# Results

### **Generation of Recombinant Viruses**

Recombinant adenovirus genomes directing expression of different murine MHC class II genes under a class II gene promoter were generated by homologous recombination between the Ad5dl324 genome and pNV4based plasmids. Ad5dl324 is a replication-deficient, double-deletion mutant lacking most of the E1 and part of the E3 regions (Figure 1A). pNV4 is a derivative of the published pRSVnlslacZpIX construct (Stratford-Perricaudet et al., 1990) and carries, in order: residues 1-455 of the adenovirus genome, essential for virus packaging; the promoter/enhancer region of the murine  $E\alpha$  gene, known to drive expression of linked cDNAs in all of the usual class II-positive cells in mice (Kouskoff et al., 1993); a segment of the rabbit  $\beta$ -globin gene containing splice donor and acceptor sites; the SV40 polyadenylation sequence; and residues 3362-6502 of the adenovirus genome, the target for homologous recombination



(Figure 1B). A cDNA of interest can be inserted at the unique EcoRI site within the  $\beta$ -globin gene segment; in this case, the murine  $A\beta^k$  and  $E\alpha^k$  cDNAs were introduced. Cotransfection of Clal-digested Ad5*dl324* and one of the linearized pNV4-based plasmids into 293 cells yielded a high percentage of plaques containing recombinant virus. Recombinants were plaque-purified and their structures and purity monitored by restriction enzyme mapping and Southern blotting. The final recombinant viruses carrying MHC class II cDNAs were named AdcII-Abk and AdcII-Eak (Figure 1C).

Infection of large cultures of 293 cells and purification of the viruses by ultracentrifugation on density gradients routinely yielded stocks titering at  $5 \times 10^{10}$ – $5 \times 10^{11}$  infectious units (iu)/ml. The results described below were routinely verified with independent virus stocks.

# Expression of Adenovirus-Delivered MHC Class II Genes

The expression of MHC class II cDNAs carried within the adenovirus vector was studied by infecting class II-deficient (II<sup>0</sup>) or class I, class II double-deficient (I<sup>0</sup>II<sup>0</sup>) mice (Koller et al., 1990; Cosgrove et al., 1991; Chan et al., 1993) with the different recombinant viruses. II<sup>o</sup> animals have an engineered mutation of the  $A\beta$  gene and a natural deletion in the  $E\alpha$  gene; in the absence of both class II isotypes, only a few CD4<sup>+</sup> T cells mature, and these have an atypical phenotype (Cosgrove et al., 1991; Cardell et al., 1995). It should be possible to complement all major features of the class II-deficient phenotype by reintroducing either an  $A\beta$  or  $E\alpha$  wild-type gene (Cosgrove et al., 1992). IºIIº animals have a mutation of the  $\beta_2$ -microglobulin gene as well, resulting in additional defects in the expression of class I molecules and development of mature CD8<sup>+</sup> T cells (Chan et al., 1993). As discussed below, complementation of the class II deficiency in I<sup>0</sup>II<sup>0</sup> double-deficient mice can be especially useful for studying induced changes in thymocyte and thymic stromal cell compartments.

> Figure 1. Construction of Adenovirus Vectors Expressing MHC Class II Molecules

> (A) The Ad5*dl324* replication-deficient adenovirus used to derive recombinant viruses. Deletions in E1 and E3 regions are detailed; the numbers in parentheses correspond to positions of the wild-type adenovirus type 5 genome.

> (B) The homologous recombination event taking place between the Clal-digested, righthand segment of the Ad5*d*1324 genome and the basic pNV4 plasmid. The sequences shared by *d*1324 and pNV4, which direct homologous recombination, are shaded. The pNV4 plasmid contains the MHC class II expression cassette taken from the pDOI-5 plasmid (Kouskoff et al., 1993) and contains adenovirus sequences (open bars);  $E\alpha$  promoter/enhancer (hatched bars); rabbit  $\beta$ -globin gene (solid bars); and plasmid backbone (solid line). ITR, inverted terminal repeat of the adenovirus genome.

(C) The final AdcII–MHC cDNA vector resulting from homologous recombination, and carrying either  $E\alpha$  or  $A\beta$  cDNAs.

The expression of class II cDNAs was examined after injection of recombinant virus directly into the thymus. Polymerase chain reaction and S1 nuclease analyses revealed class II transcripts as early as 24 hr after injection, and they reached a maximum level about 48 hr later (data not shown). Class II proteins were readily detected on thymus sections after immunofluorescent staining with anti-class II monoclonal antibodies (MAbs) (compare Figure 2A, panels 1 and 2). A series of such sections on cohorts of animals confirmed the early onset of protein expression: peaking at 10 days after viral delivery, beginning to wane after 20 days, and only weak and scattered by 35 days (data not shown). To estimate the proportion of the injected thymus expressing the transduced class II gene, we analyzed sets of serial sections taken at 100 µm intervals across the entire organ (data not shown). Not more than 20% of an injected thymus expressed class II molecules at any given time, positive cells being grouped into large regions as shown in Figure 2A, panel 2. At peak times, the levels of class II molecule expression on positive cells routinely reached those on sections from control B10.BR mice. (Note that the system has a built-in ceiling; even if the virus-encoded chains were overproduced, levels of surface class II molecules would be limited by the availability of endogenous  $A\alpha$  or  $E\beta$  chains.)

The pattern of immunofluorescent staining by anti-MHC class II reagents suggested that the cells expressing virally delivered class II genes were primarily epithelial cells in the cortex, with little labeling of medullary stroma. To confirm this possibility, we performed a confocal microscopic study of doubly stained sections using fluorescein isothiocyanate (FITC)-conjugated anticlass II MAbs and Texas red-conjugated ER-TR4, a MAb known to specifically label cortical epithelial cells (Van Vliet et al., 1984). As illustrated in Figure 2A, panels 3-5, class II-positive cells also stained with ER-TR4. A predominant expression on cortical epithelial cells is also consistent with our examination of V $\beta$ 17a<sup>+</sup> T cells in SJL mice injected intrathymically with AdcII-Eak. We never observed the expected superantigen-mediated clonal deletion (Kappler et al., 1987), nor did we see reduced TCR levels on or unresponsiveness to anti-CD3 stimulation by peripheral V $\beta$ 17a<sup>+</sup> cells, indicating that AdcII gene delivery does not target the medullary stromal cells of bone marrow origin, principally responsible for superantigen-mediated negative selection of Vβ17a<sup>+</sup> cells (Ramsdell and Fowlkes, 1990 and references therein).

We looked repeatedly for leakage of virus to other organs. Using either polymerase chain reaction or immunofluorescence assays, viral DNA and class II molecules were never detected in the spleen or lymph nodes or even in the adjacent, uninjected, lobe of the thymus (data not shown).

By now, we have injected many MHC-deficient mice with recombinant viruses carrying class II molecules. Significant variation in the efficiency of viral delivery was observed from one mouse to the next, even with experienced injectors. Taking this variability into account, we have not seen any obvious differences in the intensity or pattern of thymus staining with viruses expressing different class II isotypes or haplotypes. In short, we conclude that direct intrathymic injection of adenovirus vectors carrying MHC class II genes provokes the display of class II molecules on cortical epithelial cells in a segment of the injected thymus. Expression is rapidly induced and reaches levels found in normal mice, but is transient.

## Rescue of CD4<sup>+</sup> T Cell Compartments

To demonstrate the functionality of MHC class II molecules encoded by the recombinant adenoviruses, we examined primary and secondary lymphoid organs from I<sup>0</sup>II<sup>0</sup> mice injected intrathymically with the AdcII-Eak virus. As shown in Figure 2B (top), a substantial population of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes could be observed 18 days after virus delivery. The population was clearly larger than that in I<sup>0</sup>II<sup>0</sup> animals infected with control virus, but somewhat smaller than that in C57BL/6 (B6) positive controls. In the many experiments performed, the CD4<sup>+</sup>CD8<sup>-</sup> compartment that appeared after injection of an AdcII virus ranged in numbers from negative-control (IºIIº) levels to wild-type (B6) levels (consistent with the variable proportion of transduced stromal cells noted above); no significant and reproducible differences were seen after injection of the AdcII-Abk and AdcII-Eak viruses.

The maturity of the newly selected CD4<sup>+</sup>CD8<sup>-</sup> thymocytes was confirmed by four-color cytofluorimetry, staining with an anti- $\alpha\beta$ TCR MAb and peanut agglutinin (PNA) in addition to anti-CD4 and -CD8 reagents. The new CD4<sup>+</sup>CD8<sup>-</sup> cells attained the fully mature  $\alpha\beta$ TCR<sup>hi</sup>PNA<sup>lo</sup> status (Figure 2B, top), comparable with observations in normal B6 mice. CD4+CD8- cells from AdcII-injected IºIIº animals also displayed usual levels of heat-stable antigen and CD69 (data not shown). Finally, immunohistological analysis revealed CD4<sup>+</sup> cells in the medullary regions of AdcII- but not control virus-injected IºIIº animals (data not shown). This last point is consistent with patterns of staining by the ER-TR5 MAb, which labels medullary epithelial cells (Van Vliet et al., 1984). As described previously (Chan et al., 1993), only rudimentary medullary foci were observed in I<sup>o</sup>II<sup>o</sup> thymi (Figure 2C, middle), severely reduced in comparison with thymi from wild-type animals (left). Normal medullary development is known to require mature  $\alpha\beta$ T cells (reviewed by Ritter and Boyd, 1993). Injection of an AdcII virus and the subsequent expression of class II molecules and selection of fully mature CD4+CD8- T cells restored a nearnormal pattern of ER-TR5 positivity (Figure 2C, right).

Thymocytes that matured in response to AdcII virus injection were exported to the periphery, as evidenced by the development of a significant population of CD4<sup>+</sup> T cells in the lymph nodes (Figure 2B, bottom left) and spleen (data not shown). Most of these cells were CD44<sup>lo</sup>, like the CD4<sup>+</sup> population from uninjected B6 control animals (Figure 2B, bottom right), although there remained some cells with a TCR<sup>int</sup>CD44<sup>int/hi</sup> phenotype, similar to the small CD4<sup>+</sup> population from control virus-injected I<sup>0</sup>II<sup>0</sup> animals. The bulk of CD4<sup>+</sup> cells in AdcII-injected mice also differed from the residual CD4<sup>+</sup> population in II<sup>0</sup> or I<sup>0</sup>II<sup>0</sup> animals in their localization. The former accumulated normally in the T cell areas of the paracortex, while the latter were enriched in the B cell follicles,



Figure 2. Thymic Expression of the Transduced Genes and Its Consequences

(A) Intrathymic injection of I<sup>0</sup>II<sup>0</sup> animals with a virus containing an MHC class II expression cassette results in the expression of class II molecules. Thymus sections were taken 15 days postinjection. Control virus- (panel 1) or AdcII-Eak- (panels 2-5) injected I<sup>0</sup>II<sup>0</sup> mice were

as has been described (data not shown and Cosgrove et al., 1991). As in the thymus, peripheral reconstitution varied from animal to animal, ranging from 0%–100% of wild-type levels; and, again, no meaningful differences were observed after injection of the AdcII-Abk and AdcII-Eak viruses.

# Peripheral Emergence of Newly Selected CD4<sup>+</sup> T Cells

As described above, injection of AdcII viruses into MHC class II-deficient mice provoked the differentiation of mature CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from immature CD4<sup>+</sup>CD8<sup>+</sup> precursors arrested in development because of the absence of class II molecules. This permits monitoring of the dynamics of a synchronized cohort of CD4<sup>+</sup> T cells, providing a new approach to address some of the controversial issues about their life cycle.

The first question we tackled was how long newly generated CD4<sup>+</sup>CD8<sup>-</sup> thymocytes reside in the medulla before emigration to the periphery. One study that followed the flow of [<sup>3</sup>H]thymidine into thymocyte subpopulations suggested that their stay is quite prolonged (Egerton et al., 1990), but contradictory conclusions have also been reported (Tough and Sprent, 1994). In addition, there is mounting evidence that at least some mature thymocytes undergo division, complicating experiments involving labeled DNA precursors (e.g., Scollay and Godfrey, 1995; Akashi and Weissman, 1996). Thus, it is imperative to reexamine this question by alternative techniques.

To this end, we injected I<sup>0</sup>II<sup>0</sup> mice with an AdcII virus; since the results with AdcII-Eak and AdcII-Abk proved identical, we do not differentiate between them here. At different times after injection, a few animals were killed and the percentage of CD4<sup>+</sup>TCR<sup>hi</sup> cells in the thymus and lymph nodes determined. In Figure 3, data from several animals are pooled, each point representing the mean percentage for three to six animals. CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>hi</sup> cells were not detectable in the thymus for the first 6 days postinjection; afterward, numbers gradually augmented for about 10 days and then slowly declined. New CD4<sup>+</sup> T cells were not measurable in the lymph nodes for the first 12 days after injection; their numbers also increased gradually over a 10-day period and then began to decrease. Thus, in this experimental system, mature CD4+CD8- thymocytes spend, on average, about 6-7 days in the medulla before emigrating to the periphery.



Figure 3. Kinetics of Appearance of De Novo–Selected CD4 $^+$  Cells in the Thymus and Periphery

A large cohort of I°II° mice was injected intrathymically with virus and individuals killed at different times after injection. Single cell suspensions from thymus or lymph nodes were stained for CD4, CD8, and  $\alpha\beta$ TCR, and the proportion of CD4+TCR<sup>th</sup> cells measured by cytofluorimetry (percentages of CD4+TCR<sup>th</sup> cells rather than numbers are given because of individual variability in lymph node size). Each point corresponds to a mean percentage obtained from three to six mice.

# Survival of Peripheral CD4 $^{\scriptscriptstyle +}$ T Cells in the Absence of MHC Molecules

Another question of current interest concerns the lifespan of CD4<sup>+</sup> T cells in the periphery. The bulk of evidence indicates that naive cells are long-lived in the absence of intentional antigen stimulation (Sprent et al., 1991; von Boehmer and Hafen, 1993; Tough and Sprent, 1994; Bruno et al., 1995). It remained possible, however, that the cells monitored in these studies were subject to low-level stimulation resulting from reactivity to MHC molecules presenting environmental or endogenous antigens, or from loose reactivity to MHC molecules irrespective of their bound peptides. Indeed, Takeda et al. (1996) have provided evidence that CD4<sup>+</sup> T cells survive less well in the absence of MHC class II molecules but, as is discussed below, several features of their experimental design made the issue worth examining by an alternative strategy.

Thus, we asked how long CD4<sup>+</sup> T cells can survive when released into an environment devoid of MHC class II molecules. Initially, a set of II<sup>0</sup> mice was injected intra-thymically with AdcII virus, and the kinetics of appearance and decay of the CD4<sup>+</sup> T cell population in the blood were monitored. Data for individual animals are

stained with an FITC-conjugated anti-E reagent. Panels 3–5, Sections counterstained with the ERTR-4 MAb (red). Stained sections were examined by confocal microscopy. Panels 1 and 2,  $10 \times$  objective. Panels 3–5 ( $40 \times$  objective) represent the same section examined using different filters, revealing the class II-specific stain (panel 3) and the cortex-specific stain (panel 4). Panel 5 is a computer-generated superposition of panels 3 and 4, the yellow color indicating green and red overlaps.

<sup>(</sup>B) Transduction of class II genes into the thymus of  $|^0||^0$  animals restores the CD4<sup>+</sup> populations of thymus and lymph nodes. (Top row) single cell suspensions obtained from thymi of B6 control mice or from  $|^0||^0$  animals 18 days after injection of either control virus or recombinant vector were analyzed by four-color cytofluorimetry detecting surface CD4, CD8,  $\alpha\beta$ TCR, and PNA receptor. The CD4/CD8 profiles are shown for each mouse (left plots); the TCR/PNA profile (right plots) correspond to the CD4<sup>+</sup>CD8<sup>-</sup> population (gate D). (Bottom row) The CD4<sup>+</sup> populations in the lymph nodes 25 days after injection, analyzed by four-color cytofluorimetry with anti-CD4, -CD8,  $\alpha\beta$ TCR, and -CD44. The CD4/CD8 profiles (left plots) are shown for an identical number of lymphocytes for all samples; the TCR/PNA profiles (right plots) correspond to the CD4<sup>+</sup> population.

<sup>(</sup>C) The thymic medulla is restored concomitantly with positive selection. Thymi were obtained from normal animals (B6) or from I<sup>0</sup>II<sup>0</sup> mice 15 days after injection of control or AdcII-Eak virus. Cryostat sections were stained with the MAb ERTR-5, specific for medullary epithelial cells, and were examined by fluorescence microscopy.



Figure 4. Dynamics of the CD4<sup>+</sup> Population Appearing in II<sup>0</sup> Animals

(A) Percentage of  $CD4^+$  T cells in the blood of injected animals. Mice were injected intrathymically with recombinant or control virus on day 0 and bled sequentially to monitor circulating  $CD4^+$  cells by staining blood lymphocytes for CD4 and CD8. The shaded area represents the background of  $CD4^+$  cells found in II<sup>0</sup> mice (Cosgrove et al., 1991; Cardell et al., 1995).

(B) Decay rate of CD4<sup>+</sup> cells in the lymph nodes of AdcII-injected, thymectomized animals. A cohort of II<sup>0</sup> mice were injected intrathymically with recombinant virus at day -30, and the animals were thymectomized 30 days later (day 0). At the same time, one axillary lymph node was surgically removed and its content of CD4<sup>+</sup> cells determined. Two other time points were obtained for every animal shown; the second point was generated by surgically removing an axillary lymph node, and the third from inguinal lymph nodes after killing the animal. The horizontal line represents the percentage of CD4<sup>+</sup> cells from control virus-injected animals (1.42% ± 0.35%; shaded area) (n = 15). Closed circles, mice thymectomized 30 days after AdcII-Eak intrathymic injection; open squares: unthymectomized mice injected and tested in parallel.

plotted in Figure 4A. A significant delay in the accumulation of cells was again observed. The percentage of CD4<sup>+</sup> cells generally peaked between days 20 and 30 and decreased slowly thereafter. Already by 75 days, the CD4<sup>+</sup> population had declined to background levels, suggesting that most of the newly generated cells were able to survive less than 2 months in a class II-deficient environment.

To obtain a more accurate estimate of the decay rate, we instituted two protocol modifications. First, the mice were thymectomized 30 days after AdcII injection in order to halt further emigration of cells from the thymus as well as to prevent cells from circulating back to the thymus and encountering class II molecules encoded by the adenovirus vector. Second, lymph nodes were removed surgically from individual animals in a sequential fashion, to reduce the scatter inherent in analyses of blood-borne cells, while still permitting multiple data points to be obtained from each animal. The evolution of the CD4<sup>+</sup> populations for a large number of mice is depicted in Figure 4B. The values at time 0 represent the percentage of CD4<sup>+</sup> cells in lymph nodes at the time of thymectomy, mostly ranging between 5% and 10%. With time, the CD4<sup>+</sup> population decreased in all of the animals. The data appeared to follow classic exponential decay kinetics; indeed, fitting of the data by nonlinear regression analysis matched well a simple exponential curve (r = 0.8), with a half-life of 42 days. This is similar to observations with CD4<sup>+</sup> cells in the blood of euthymic AdcII-injected class II-deficient mice (Figure 4A) and is indistinguishable from the decay rate of the CD4<sup>+</sup> population in lymph nodes of AdcII-injected animals in which the thymus was left intact (Figure 4B). However, it is distinctly different from what has been reported for T cells in mice normally expressing MHC molecules (Sprent et al., 1991; von Boehmer and Hafen, 1993; Tough and Sprent, 1994; Bruno et al., 1995; and data below).

It remained possible that low-level stimulation of naive CD4<sup>+</sup> cells by MHC class I molecules influenced their survival in class II-deficient animals, especially since it is known that some T cells can recognize both class I and II molecules (e.g., Robey et al., 1991). Thus, we performed an experiment of identical design except that I°II<sup>0</sup> animals were the AdcII recipients. As illustrated in Figure 5A, the CD4<sup>+</sup> population also persisted for some time in this environment and then slowly decayed, the half-life of the CD4<sup>+</sup> population (45 days) being very similar to that measured (above) for the equivalent population in II<sup>0</sup> animals. In contrast, CD4<sup>+</sup> cells persisted at largely unchanged levels in thymectomized control I<sup>0</sup>II<sup>+</sup> mice (Figure 5B).



Figure 5. Influence of MHC Class I Molecules on the Decay Rate of  $\mathsf{CD4^+}$  Cells

(A) I<sup>0</sup>II<sup>0</sup> animals were injected with AdcII virus at day -30. On day 0, mice were thymectomized, one lymph node taken, and the proportion of CD4<sup>+</sup> cells determined. For each mouse, two later points were also obtained, as described in the legend to Figure 4. The shaded area represents the background of CD4<sup>+</sup> lymph node cells in I<sup>0</sup>II<sup>0</sup> mice.

(B) As a control,  ${}^{10}\mathrm{II^+}$  mice were thymectomized and analyzed over time by the same protocol.

However, one difference was evident between the two experiments: consistently, there was a more efficient reconstitution of the CD4<sup>+</sup> compartments in thymus (data not shown) and lymph nodes (compare Figures 4B and 5A) of the double-deficient mice. This was true when calculated as numbers as well as percentages: on average, twice the number of CD4<sup>+</sup> cells was found in the axillary lymph nodes of I<sup>0</sup>II<sup>0</sup> (2 × 10<sup>5</sup>) compared with II<sup>0</sup> (1.1 × 10<sup>5</sup>) animals.

# Cycling Status of Newly Generated Peripheral Cells

To better interpret the above results, it was important to evaluate the cycling status of the newly selected CD4<sup>+</sup> T cells residing in the periphery of MHC-deficient mice. Thirty days after virus injection, I<sup>0</sup>II<sup>0</sup> animals were fed bromodeoxyuridine (BrdU) in their drinking water for 3 days; lymph nodes were then removed and cells stained with anti-CD4, -CD8, - $\alpha\beta$ TCR, and -BrdU reagents. The CD4<sup>+</sup> population in wild-type control mice was TCR<sup>hi</sup> and had a low percentage of cycling cells (Figure 6, top), while CD4<sup>+</sup> cells in uninjected (data not shown) or control virus-injected (Figure 6, middle) I<sup>0</sup>II<sup>0</sup> animals expressed slightly lower levels of TCR and cycled more frequently. This is similar to results reported for the equivalent cells in II<sup>0</sup> animals (Cardell et al., 1995) and may reflect activation since these cells also express



Figure 6. Cycling Status of Peripheral CD4<sup>+</sup> Cells

Uninjected wild-type (B6), control virus-injected l<sup>0</sup>II<sup>0</sup>, or AdcII-injected l<sup>0</sup>II<sup>0</sup> animals were given BrdU in their drinking water for 3 days. Lymph node cells were stained with anti-CD4, -CD8, - $\alpha\beta$ TCR, and -BrdU MAbs. The CD4<sup>+</sup> population was divided into TCR<sup>II</sup> (gate D) and TCR<sup>III</sup> (gate E) populations. The proportions of BrdU<sup>+</sup> cells in these populations are shown in the histograms.

high levels of several activation markers, in particular CD44 (Cardell et al., 1995, and Figure 2B). The CD4<sup>+</sup> compartment of AdcII-injected mice appeared to harbor both components (Figure 6, bottom) the TCR<sup>hi</sup> subset probably representing newly generated cells and the TCR<sup>int</sup> subset mostly derived from preexisting cells. The cycling properties of these two components in AdcII-injected mice were, as usual, lower in the TCR<sup>hi</sup> than TCR<sup>int</sup>.

### Discussion

# Manipulation of T Cell Selection via Adenovirus-Mediated Delivery of Genes to the Thymus

Our goal at the outset of these experiments was to develop a means of interfering with T cell selection that would be a cheaper, faster alternative to transgenesis. A promising approach seemed to be targeted delivery of genes of interest by intrathymic injection of a viral vector carrying cDNAs driven by a promoter active in thymic stromal cells. We chose to work with an adenovirus vector because of a number of attractions (reviewed by Berkner, 1988; Ali et al., 1994), perhaps the most important of which are the following.

(1) Adenovirus vectors can accomodate relatively large extraneous inserts—up to 7.5 kb.

(2) Adenovirus can be grown to high titers, routinely 10<sup>10</sup>–10<sup>11</sup> iu/ml. This is particularly important for intrathymic delivery, permitting small injection volumes to minimize damage to and altered function of the thymus.

(3) Unlike most retroviruses, adenovirus can infect nondividing cells, a critical consideration if one wishes to deliver genes to thymic stromal cells.

(4) Adenovirus has a relatively broad cell tropism.

(5) Unlike the situation with transgenesis or retroviral vectors, a gene transduced by adenovirus remains episomal, avoiding variable expression due to random integration as well as potential disruption of genes in the target cell genome.

We have used an adenovirus vector to deliver MHC class II cDNAs driven by a class II gene promoter to the thymus of class II-deficient mice. In just days, class II molecules were expressed at the surface of epithelial cells in the cortex of a chunk of the thymus, and they remained detectable for about 1 month. This provoked positive selection of a significant population of CD4+CD8- thymocytes, which eventually exited the thymus and colonized the peripheral lymphoid organs. Hence, this system is valuable both for dissecting the mechanism of positive selection and for exploring phenomena that critically depend on positive selection. The temporal control provided by a provoked and transient virus infection is a particular advantage for addressing certain guestions. Here we have exploited the system to study the dynamics of newly selected cohorts of CD4<sup>+</sup> T cells; in contemporary experiments, a modification of the system was employed to examine the role of peptide in positive selection (Nakano et al., 1997). In future studies, the system or modifications of it could be used to evaluate the importance of diverse proteins in positive selection or in preceeding differentiation events dependent on thymocyte-epithelial cell interactions, such as adhesion molecules (intercellular adhesion molecule and very late antigen [Crisa et al., 1996; Kishimoto et al., 1996]), costimulatory receptors (the B-7s [Kishimoto et al., 1996]), or other less understood proteins (CD81 [Boismenu et al., 1996], tumor necrosis factor and tumor necrosis factor receptor [Zuniga-Pflücker and Lenardo, 1995], c-Kit/steel [Rodewald et al., 1995], and others [Anderson et al., 1993]).

## Positively Selected T Cells Remain in the Thymus for a Prolonged Period before Exiting to the Periphery

The first issue we tackled with class II–expressing adenoviruses was how long mature, single-positive thymocytes stay in the thymus before emigrating to the peripheral lymphoid organs. By comparing the kinetics of accumulation of CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>hi</sup> thymocytes and CD4<sup>+</sup> lymph node cells, we estimated that, on average, a mature CD4<sup>+</sup> thymocyte remains 6–7 days in the medulla. A clear lag of at least 6 days was seen in the accumulation curve for lymph node cells, without detectable export up to the day 12 time point. These observations are difficult to reconcile with a stochastic emigration process whereby a certain percentage of cells would exit daily irrespective of their seniority and are more suggestive of a set time of medullary residence. They are in general agreement with the prolonged thymic retention indicated by experiments monitoring the distribution of [<sup>3</sup>H]thymidine in thymocyte subpopulations during continuous labeling protocols (Egerton et al., 1990). The latter estimate of residence time was somewhat longer, at about 12 days, but may be complicated by recent descriptions of the complexities of the double-positive to single-positive thymocyte transition (Akashi and Weissman, 1996; Lucas and Germain, 1996) as well as by recent data indicating that mature thymocytes can undergo division (e.g., Scollay and Godfrey, 1995; Akashi and Weissman, 1996). Alternatively, there might be a longer average residence when the medulla is already full of cells than when it is essentially empty, as with IºIIº mice. Our findings are in excellent accord with the estimated time of 5-6 days that it takes for a singlepositive thymocyte to make the full transition from its immediate CD4<sup>+</sup>CD8<sup>+</sup> precursor to a fully mature cell expressing differentiated levels of markers such as heat shock antigen, Qa-2, and CD69 (Lucas et al., 1994). In contrast, they disagree with the suggestion of Tough and Sprent (1994) that single-positive cells exit from the thymus almost immediately after formation.

The extended stay of single-positive cells in the thymic medulla raises two important questions. First, what are they doing during this time? This 7-day residence represents about a third of the time a differentiating T cell spends in the thymus. Yet we know very little about what is going on during this period, beyond the observations that expression levels of some differentiation markers change (Nikolic-Zugic and Bevan, 1990; Ramsdell et al., 1991; Lucas et al., 1994), that some but not all of the cells undergo division (Huesmann et al., 1991; Scollay and Godfrey, 1995), and that the cells acquire enhanced capacity to respond to antigen challenge or other TCR crosslinking (Nikolic-Zugic and Bevan, 1990; Ramsdell et al., 1991; Dyall and Nikolic-Zugic, 1995) and to survive and expand in the peripheral organs (Dyall and Nikolic-Zugic, 1995). With regard to the last point, it may be that a certain amount of time is required before positively selected cells have "recovered" from positive selection and reset their signaling machinery to permit a productive response to TCR engagement.

Second, what is it that finally signals the competence for emigration? We are just as ignorant on this subject. It has been suggested that cell division might be the trigger for exodus (Scollay and Godfrey, 1995), but this cannot be universal because cells with certain specificities do not divide in the medulla (Huesmann et al., 1991). Interestingly, emigration from the thymus was blocked in thymocytes that expressed a transgene encoding pertussis toxin, suggesting a regulated process almost certainly involving a Gi protein (Chaffin and Perlmutter, 1991). Clearly, then, more attention needs to be focused on this poorly understood window of T cell differentiation.

# Naive T Cells Require Interactions with MHC Molecules for Long-Term Survival in the Periphery

The second issue we addressed with the adenovirus vector system was whether the lifespan of naive T cells in the periphery is influenced by MHC-mediated excitation, independent of stimulation by antigen. Several groups have shown that naive T cells persist for a long time in the absence of intentional antigenic stimulation (Sprent et al., 1991; von Boehmer and Hafen, 1993; Tough and Sprent, 1994; Bruno et al., 1995), but it remained possible that the cells in these experiments (or at least a fraction of them) were unintentionally stimulated by crossreactivity to environmental or self antigens. This caveat was underlined recently by the finding that a monoclonal T cell population displaying a virus hemaglutinin-specific receptor contained a significant component of activated and dividing cells in the absence of immunization (Bruno et al., 1996).

Our data show that newly emergent CD4<sup>+</sup> T cells have a significantly shorter half-life when MHC class II molecules are absent. The observed half-life (42 days) corresponds to a true half-life of approximately 26 days when corrected for the contribution of cycling cells (see Experimental Procedures). This value is quite similar to the value (about 50 days without correction for cycling) that can be calculated from the data of Takeda et al. (1996) from experiments involving transplantation of a B6 fetal thymus into a host lacking both MHC class II molecules (II<sup>-/-</sup>) and, due to a mutation in a recombination-activating gene (RAG<sup>-/-</sup>), T cells. However, other findings with the two systems clearly diverged-most notably, with the latter there were somewhat higher levels of reconstitution ( $\sim$ 2-fold), a much greater proportion of cycling cells at early stages of reconstitution (>10-fold), and an evolution in the expression of certain activation markers. These differences could potentially be due to physiological peculiarities of fetal thymuses, to recirculation of peripheral T cells back to the class II-expressing thymus (which may be exaggerated with the fetal organ (Surh et al., 1993), to migration of class II-positive hematopoietic cells from the grafted thymus to the periphery, or to a mild graft-versus-host reaction due to genetic differences in the B6 background of the thymus donor and the mixed 129/B6 background of the II<sup>-/-</sup>RAG<sup>-/-</sup> recipient. Considering such differences in experimental design, it is comforting that the two systems gave such similar values for the half-life of naive CD4<sup>+</sup> T cells in a class II-negative environment. Our data provide the additional information that crossreactivity to class I molecules does not significantly influence the turnover of the population of CD4<sup>+</sup> cells.

It seems clear, then, that MHC class II molecules somehow prolong the lifespan of CD4<sup>+</sup> T cells, probably by providing some form of stimulation through their TCRs, although "tickling" through CD4 is also theoretically possible. This interaction appears neither to overtly activate the cells nor to induce their proliferation, since the phenotypes of naive cells in the CD4<sup>+</sup> compartments of class II-positive and class II-negative mice were indistinguishable with regard to these features. Perhaps this type of low-level excitation in the periphery is akin to positive selection in the thymus: low- to moderateaffinity interactions that fend off programed cell death but do not productively activate the cells. It is imperative to see how such interactions are reflected in the mobilization of various components of the signaling network and how this translates into a denial of cell death. It will then be of interest to follow the evolution of signaling and cell death effector molecules during the decay of the CD4<sup>+</sup> population in class II-negative animals: why does it take as long as 26 days for these cells to die?

### **Experimental Procedures**

#### Mice

Mutant mice used in this study included MHC class II–deficient mice (II<sup>0</sup>) (Cosgrove et al., 1991) at the tenth generation of backcross to B6; β-microglobulin–deficient mice (I<sup>0</sup>; Koller et al., 1990), and double-deficient animals, with both mutations (I<sup>0</sup>II<sup>0</sup>; Chan et al., 1993) on a mixed 129 × B6 background. All mutant and inbred strains were bred under specific-pathogen–free conditions. After virus injection, the animals were kept in segregated isolator conditions, according to EEC guidelines.

#### Plasmids

To construct an adenovirus vector directing expression of MHC class II genes, we modified the previously described pRSVnlslacZpIX plasmid (Stratford-Perricaudet et al., 1990). Its EcoRI sites were eliminated, and its promoter and reporter segments were replaced by cloning the 3.4 kb Xbal-Xho I fragment from the pDOI-5 MHC class II expression cassette (Kouskoff et al., 1993) in lieu of its Bam HI-BamHI segment. The resulting vector pNV4 contains a unique EcoRI site, into which we ligated the  $A\beta^k$  (Landais et al., 1986) and  $E\alpha^{k}$  (flanked by artificial EcoRI sites introduced 33 bp upstream of the ATG codon and 40 bp downstream of the termination codon) cDNAs to produce pNV4Ab and pNV4Ea. In the pNV4 construct, the expression cassette is flanked by two adenovirus sequences: the upstream element includes the first 455 nucleotides of the adenovirus genome and contains packaging sequences and the E1A enhancer; the downstream element spans nucleotides 3362-6502 of the adenovirus genome, which allows homologous recombination.

#### **Cells and Viruses**

The Ad5dl324 parental virus (originally provided by M. Perricaudet, Villejuif, France) and all recombinant viruses were obtained, grown, and amplified by infecting or transfecting the 293 human embryonic kidney carcinoma cell line (ATCC CRL 1573). We have noticed that 293 cells lose their capacity to adhere efficiently to the culture dish when they are kept for more than a certain number of passages, resulting in poor infection and transfection efficiencies. To avoid these problems, only 293 cells at less than passage 60 were used in all experiments (obtained from the American Type Culture Collection at passage 30). Transfections, purifications, titrations, and small-scale production of virus were done on low-passage, adherent 293 cells maintained in minimal essential medium supplemented with 10% fetal calf serum (FCS). Large-scale virus production for injectable stocks was in suspension culture of 293 cells maintained in Joklik medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

#### **Recombinant Viruses**

Recombinant viruses were obtained by transfecting low-passage, adherent 293 cells, seeded at  $1\times10^6$  in 6 cm culture dishes 24 hr prior to transfection. Medium was changed 4 hr before DNA addition. Two micrograms of Clal-digested Ad5*d*/324 DNA was cotransfected with 2  $\mu$ g of Eag I–linearized pNV4Ea or pNV4Ab DNA by the CaPO4 method (according to standard protocols [Sambrook et al., 1989]). Twenty-four hours later the medium was removed and the cell mono-layer covered with Dulbecco's medium containing 5% FCS and a

1:1 mixture of Noble agar and low-melting-point agar at a final total concentration of 1%. Nine days later, 12 viral plaques were picked with a Pasteur pipette, and each was resuspended in 100 µl of medium, which was used to infect an individual well of a 24-well plate confluent with 293 cells. When the cytopathic effect of each . individual well reached 100% (from 1-4 days, depending on plaque size), cells and supernatant were collected and briefly spun. The clarified supernatant was frozen at -80°C while the total DNA of the corresponding cell pellet was extracted, precipitated, and resuspended in 50 µl TE (10 mM Tris HCI [pH 8], 1 mM EDTA). Recombination events were screened by Southern blotting of the appropriate restriction enzyme digests using probes for the 5' adenovirus sequences and for the MHC class II cDNA insert. One or two of the supernatants corresponding to viruses with correct genomic organization were serially diluted (10<sup>-3</sup>-10<sup>-6</sup>), and 50 µl of each dilution was added to each of 48 wells of a 96-well plate, confluent with 293 cells. After 7 days of culture, supernatants from wells of the highest dilution showing cytopathic effect were used to infect confluent wells of a 24-well plate (150 µl supernatant/well). Infected wells were rescreened as described above, and positive clones were subjected to a second serial dilution. Finally, only one clone, showing the proper genomic organization, was kept and amplified to high titers and used in reconstitution experiments.

#### High-Titer Virus Stocks, Titration, and Viral DNA Extraction

One clone of each virus strain was first amplified by infecting, with 1 ml of virus suspension, a 75 cm<sup>2</sup> culture flask confluent with 293 cells. After 3 days in culture, cells and supernatant were collected; frozen and thawed once; and spun at 3000 rpm for 10 min at 4°C (final volume approximately 30 ml). Six 125 cm<sup>2</sup> confluent culture flasks were each exposed to 5 ml of the clarified supernatant for 1 hr, after which fresh medium was added to a final volume of 50 ml and the cultures returned to 37°C. When a 100% cytopathic effect was seen, virus was harvested by pooling the infected cells from the six flasks by centrifugation. The cell pellet was resuspended in approximately 10 ml of medium and subjected to two freeze-thaw cycles. The supernatant was rid of cell debris by centrifugation (3000 rpm, 10 min, 4°C) and used to infect large cultures. Two liters of suspension culture-adapted 293 cells, at a density of 5  $\times$  10<sup>5</sup> cells/ ml, was spun (2000 rpm, 15 minutes, 15°C) and resuspended in 200 ml of complete Joklik medium to which 10 ml of virus preparation was added. Under such conditions, the multiplicity of infection is about 10. Cells were incubated with virus particles for 1 hr at 37°C with agitation, diluted in 4 liters of complete Joklik medium, and further incubated for 48-60 hr at 37°C with agitation. They were spun (2000 rpm, 15 minutes, 15°C), supernatant removed, and the cell pellet washed once in 50 ml phosphate-buffered saline. The pellet was resuspended in 10 ml of 20 mM Tris HCI (pH 7.8), cooled on ice, and subjected to 3 rounds of sonication (Vibracell 72434. 80% power, 13 mm diameter probe) of 20 sec each. The cell lysate was extracted twice with an equal volume of trichlorotrifluoroethane, the aqueous phase weighed, and one half its weight of CsCl was added. The viral suspension was spun in a Vti65.2 rotor at 58,000 rpm for 1 hr at 10°C. The virus band was extracted and dialyzed for 1 hr at room temperature against 1 liter of 500 mM NaCl, 20 mM Tris (pH 7.8) followed by an overnight dialysis against 2 liters of the same buffer but at 4°C with agitation. Virus stocks were aliquoted in small volumes and stored at -80°C. The genomic organization of the virus stocks was verified again as described above. In parallel, the stock was titered by serial dilution (10<sup>-8</sup>-10<sup>-12</sup>) and by plating these dilutions in 96-well plates as described above for limitingdilution purification procedure: titers in the stock were extrapolated back from these dilutions. Virus stocks having a titer lower than 5 imes10<sup>10</sup> iu/ml were discarded.

# Intrathymic Injections, Thymectomy, and Lymph Node Sampling

Intrathymic injections were performed as described (Scollay et al., 1980). In brief, 4- to 7-week-old mice were anesthetized and were immobilized face up. The skin was opened with a short longitudinal incision from the apex of the mandibula to the middle of the sternum. The skin was pushed sideways to free therib cage. The submandibular salivary glands were displaced toward the head of the animal.

One blade of a blunt scissor was inserted in the ribcage from the suprasternal notch such that the tip of the scissor reached the sternal angle (second rib). A sharp, upward movement was done as the manubrium was snipped opened. A small opening was made in the pleura and was kept open with forceps. Each thymic lobe received 10  $\mu$ l of virus solution injected with a 50  $\mu$ l Hamilton syringe fitted with a short, no-dead-volume needle (SPE RN L20MM .31/.16 94). Stocks were diluted so that each lobe would receive the same amount of infectious virus, corresponding to about 3  $\times$  10<sup>8</sup> iu/ml. The surgical opening was closed by gluing it with Histoacryl. The same procedure was used for thymectomy except that both thymus lobes were extracted with straight, narrow forceps.

To follow the CD4 populations in lymph nodes of individual animals, axillary nodes were taken surgically. A short (0.5–1 cm) incision was made in the skin on the side of the animal, from which the axillary lymph node was taken and immediately transferred to cold medium. The surgical wound was sutured. Three data points were thus obtained from each mouse: the first from one axillary node at the time of thymectomy; the second axillary node some time later; and a third point from inguinal nodes at the time of sacrifice.

#### Flow Cytometry and Population Analysis

Single cell suspensions from blood, lymph nodes, or thymus were stained with MAbs of the following specificities: FITC- or biotinconjugated KT3 (anti-CD3e) or H57-597 (anti-TCR $\alpha$ ; $\beta$ ); FITC-conjugated IM-7 (anti-CD44), anti-CD69, PNA, or anti-CD8; phycoerythrin (PE)-conjugated-GK1.5 (anti-CD4); and red-613-conjugated anti-CD8. Biotin-conjugated antibodies were revealed with streptavidinconjugated PE-Cy5 (for references to MAbs and basic staining procedure, see Chan et al., 1993). Labeled cells were analyzed on a Coulter (Hialeah, FL) Elite flow cytometer.

The half-life of T cells in the periphery of the intrathymically reconstituted I<sup>0</sup>II<sup>0</sup> mice was evaluated as follows: from the data points of Figure 4B, an exponential decay curve was fitted by regression analysis (Statistica 4.1, StatSoft). To take into account the effect of cycling within the population, one can pose that the kinetics of the CD4<sup>+</sup> population can be approximated by

### $A(t) = Aoe^{-kt}$ and k = k' - c

where A(t) is the number of CD4<sup>+</sup> cells at time t; Ao the starting number of CD4<sup>+</sup> cells; k the observed decay constant of the population; k' the true rate of loss of CD4<sup>+</sup> cells; and c the rate of cycling. From Figure 6, c = 0.0098, and from Figure 4B, k = 0.016; thus k' = 0.0258, for a half-life of 26 days.

#### BrdU Labeling

Dividing cells were marked with BrdU and stained basically as described (Gilfillan et al., 1994). Mice were given 5% glucose and 1 mg/ml BrdU in their drinking water for 3 days. They were killed and their lymph nodes removed. Cells were stained with the following antibody combination: anti-CD4-PE, anti-CD8-red613, and H57-957-biotin followed with streptavidin Tricolor (Caltag). The cells were then fixed in 70% ethanol, washed, and permeabilized by treatment with 1% paraformaldehyde, 0.1% Tween-20 in phosphate-buffered saline. The cell suspension was exposed to a brief DNAse treatment and stained with anti-BrdU-FITC (Becton-Dickinson).

#### Immunohistology

Organs were snap-frozen in optimal cutting temperature medium and cryostat sections stained as previously described (Cosgrove et al., 1991). Antibodies used were FITC-14.4.4 (anti-E), FITC-10.2.16 (anti-A<sup>t</sup>), and ER-TR4 or ER-TR5 (for references, see Ozato and Sachs, 1981; Van Vliet et al., 1984; Landais et al., 1986), revealed with a Texas red-conjugated goat-anti-rat-IgG, and analyzed by confocal microscopy.

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