

# Mechanisms of Tolerance Induction in Major Histocompatibility Complex Class II-restricted T Cells Specific for a Blood-borne Self-Antigen

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## Summary

Transgenic mice expressing a major histocompatibility complex class II-restricted T cell receptor with specificity for a natural self-antigen, the fifth component of complement, were generated to analyze the mechanism of tolerance induction to a blood-borne self-protein. In the absence of C5 protein thymocytes from T cell receptor transgenic mice develop into mature CD4<sup>+</sup> single positive cells which emigrate into the periphery and mount C5-specific T cell responses upon immunization with C5. In the presence of circulating C5 protein, CD4<sup>+</sup> single positive thymocytes do not develop. Negative selection occurs late in thymic ontogeny leaving the bulk of CD4<sup>+</sup>8<sup>+</sup> thymocytes unaffected. This phenotype may be due to a delay in contact with self-antigen presentation which, under physiological conditions, is inefficient in the cortex of C5<sup>+</sup> mice, and therefore does not affect most immature double positive thymocytes. In contrast, *in vitro* exposure to C5<sup>-</sup>-presenting dendritic cells or *in vivo* injection of C5 peptide results in deletion of double positive thymocytes. C5<sup>+</sup> transgenic mice are tolerant *in vivo*, but contain T cells in spleen and lymph nodes that secrete interleukin 2 and interferon  $\gamma$  in response to C5 activation *in vitro*. When crossed onto a Rag1<sup>-/-</sup> background to prevent endogenous T cell receptor rearrangements, these peripheral potentially autoreactive cells do not appear. This indicates that endogenous T cell receptor rearrangements possibly leading to the expression of two receptors might be a prerequisite for their survival and export into the periphery.

Negative selection of potentially self-reactive T cells is important for induction and maintenance of self-tolerance. A wide range of examples shows that tolerance induction in MHC class I-restricted T cells is to a large degree effected by physical deletion of immature CD8<sup>+</sup>4<sup>+</sup> thymocytes (1–3). In addition, nondeletional mechanisms like down-regulation of coreceptors (4), or TCR (5), and induction of anergy (6) prevent activation of potentially self-reactive cells. Tolerance in MHC class II-restricted T cells has likewise been attributed to the deletion of CD4<sup>+</sup>8<sup>+</sup> thymocytes based on studies in which the cognate peptide was injected into transgenic mice with an ovalbumin-specific, class II-restricted TCR (7). It was unclear how self-tolerance is achieved in the presence of a self-protein that requires internalization, processing, and presentation by MHC class II molecules. To address this question we have generated transgenic mice with a MHC class II-restricted TCR specific for a natural circulating self-protein, the fifth component of complement (C5), and analyzed development of transgenic T cells in the presence or absence of self-antigen. Furthermore, the functional behavior of mature T cells in the periphery of C5<sup>-</sup> or C5<sup>+</sup> TCR transgenic mice was analyzed. The findings are that C5<sup>-</sup> TCR transgenic mice (C5<sup>-</sup>Tg<sup>+</sup>) develop single CD4<sup>+</sup> T cells with high levels of TCR. Mature cells in the periphery of these mice are activated by C5 protein to secrete IL-2 and

IFN- $\gamma$ . C5<sup>+</sup> transgenic mice (C5<sup>+</sup>Tg<sup>+</sup>) do not develop mature CD4<sup>+</sup> single positive cells, but show only a slight reduction in the number of CD4<sup>+</sup>8<sup>+</sup> cells in the thymus. Thymic tolerance induction to this blood-borne self-antigen occurs late in development, probably at the transition to single positive cells, which may be due to delayed exposure to self-antigen presentation. Although C5<sup>+</sup> transgenic mice appear fully tolerant *in vivo*, their splenic T cells can be activated to secrete IL-2 and IFN- $\gamma$  in response to C5 *in vitro*. The presence of anergic, but reactivatable C5-specific T cells is confined to mice that can undergo endogenous rearrangements of alternative TCR genes. C5<sup>+</sup> transgenic mice bred onto a Rag1<sup>-/-</sup> background do not have any T cells in the periphery. These data indicate that, similar to class I-restricted T cells, thymic deletion is the major mechanism for tolerance induction in class II-restricted T cells even if the onset of deletion may be delayed due to limitations in self-antigen presentation. The escape from this mechanism appears to be correlated to the capacity to undergo endogenous TCR rearrangements.

## Materials and Methods

*Animals.* CBA/Ca (C5<sup>+</sup>) and A/J (C5<sup>-</sup>) mice are maintained under specific pathogen free conditions at the National Institute

for Medical Research, Mill Hill. The congenic strain A.C5<sup>+</sup> was kindly provided by Dr. F. Gervais at the Montréal General Hospital Research Institute (Montréal, Canada) (8) and is now bred in Mill Hill. Rag1<sup>-/-</sup> mice were obtained from Dr. E. Spanopoulou (The Rockefeller University, New York) (9).

**Generation of Transgenic Mice.** To generate transgenic mice we used the TCR from clone A18 isolated from an A/J mouse immunized with C5 protein (10). Its variable region is encoded by V $\alpha$ 11.1(a)-J $\alpha$ TA37 and V $\beta$ 8.3-DJ $\beta$ 2.6. The  $\alpha$  chain of the TCR was identified by reverse transcription of total RNA from A18 hybridoma cells and PCR with a panel of primers specific for respective families of V $\alpha$  chains and a C $\alpha$  primer from the constant region (11). The  $\beta$  chain variable region was identified by FACS<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA) staining with antibodies F23.1 (V $\beta$ 8.1, 2, 3) (12) which stained positive, and antibodies KJ16 (V $\beta$ 8.1, 2) (13) and F23.2 (V $\beta$ 8.2) (14) which both did not stain hybrid A18.

An NcoI–NcoI fragment containing the variable region of the  $\alpha$  chain was generated from total RNA by PCR with Pfu polymerase and enzyme digestion. Oligonucleotides used for amplification were the C $\alpha$  primer and the V $\alpha$ 11.1-specific mutational oligonucleotide: 5'-TTG CAG GAC CCA TGG GGA TCA GGT GGA GCA GAGT-3'. Full-length cDNA was reconstituted by ligation with a partial NcoI–NcoI fragment containing leader and constant  $\alpha$  chain sequences of F5 TCR in pATX (15, from D. Kioussis, National Institute for Medical Research, London, UK). Similarly, a PCR fragment encoding leader and variable region of the A18  $\beta$  chain was trimmed with NcoI and ClaI and inserted into a ClaI–NcoI fragment of the F5  $\beta$  TCR chain cDNA in pATX. The cDNA constructs were sequenced and BamHI–XbaI ( $\alpha$ ) and BamHI–EcoRI ( $\beta$ ) fragments were inserted into a blunt-ended EcoRI site of the SalI–BamHI fragment of the human CD2 minigene in pBluescript (16). After a BamHI–XbaI segment with the CD2 LCR was added, both  $\alpha$  and  $\beta$  constructs were isolated as SalI–NotI and SalI–XbaI fragments, respectively, purified by anion-exchange HPLC on a GenFax column (Waters-Millipore, Milford, MA) and mixed together in equimolar quantities. The mixture was used for microinjections into CBA  $\times$  CBA oocytes. Transgenic founders were either maintained on the CBA (C5<sup>+</sup>) background or backcrossed to A/J (C5<sup>-</sup>) or congenic A.C5<sup>+</sup> mice.

**Cell Cultures.** For functional tests of C5 reactivity spleens from transgenic mice or nontransgenic controls were subjected to enzyme digestion with a cocktail of collagenase (1.6 mg/ml CLS4; Worthington Biochemical Corp., Freehold, NJ) and DNase (0.1% Fraction IX; Sigma Chemical Co., Poole, UK) for 60 min at 37°C. Cell suspensions were washed twice and plated into 96-well U-shaped microtiter plates (Costar Corp., High Wycomb, UK) at a density of  $2 \times 10^5$  cells/well in the presence or absence of C5 or C5 peptide. The culture medium was Iscove's modified Dulbecco medium supplemented with 5% heat inactivated FCS,  $2 \times 10^{-3}$  M L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and  $5 \times 10^{-5}$  M mercaptoethanol. Supernatant from cultures incubated for 48 h was tested for the presence of IL-2 by its ability to support the growth of the IL-2-dependent cell line, CTLL. Supernatant from cultures incubated for 72 h was tested for IFN- $\gamma$  activity by a sandwich ELISA as previously described (17). Briefly, antibodies to mouse IFN- $\gamma$  were used for coating ELISA plates, followed by the addition of supernatants to be tested for the presence of IFN- $\gamma$ , capture by a second biotinylated IFN- $\gamma$ -specific antibody, and detection with Streptavidin conjugated with horseradish peroxidase.

**Bone Marrow Dendritic Cells as APC.** Bone marrow-derived

dendritic cells, generated as described previously (18) were used as APC for the results described in Figs. 2 and 6. The source of GM-CSF was supernatant from hypoxanthine–aminopterin–thymidine-sensitive Ag8653 myeloma cells transfected with murine GM-CSF cDNA which was isolated from a T cell clone by PCR and inserted into the vector BCMGSNeo kindly provided by Dr. H. Karasuyama (Basel Institute for Immunology, Basel, Switzerland) (19). Thymic dendritic cells and macrophages were isolated from C5<sup>+</sup> mice as previously described (18).

**C5 Antigen Preparation and C5 Peptide.** C5 was purified from ascites fluid by affinity chromatography as described (20). The C5 epitope recognized by hybrid A18, which donated the transgenic TCR, was identified after digestion of C5 by V8 endoproteinase (Boehringer Mannheim, Mannheim, Germany) and subsequent separation of peptides on a reversed phase HPLC column. A functionally active fraction was sequenced and peptide 107–121 was synthesized by the National Institute of Medical Research (NIMR) peptide synthesis facility.

**In Vitro Apoptosis Set Up (22).**  $2 \times 10^5$  thymocytes per well of a 96-well U-bottomed plate were cultured for 10–12 h with  $2 \times 10^4$ /well dendritic cells in the presence or absence of C5 protein or peptide. Cells were then harvested and analyzed in FACS<sup>®</sup>.

**In Vivo Depletion of Double Positive Thymocytes.** C5<sup>-</sup>Tg<sup>+</sup> mice received daily intraperitoneal injections for 6 d with 250  $\mu$ l of 100  $\mu$ M C5 peptide or with PBS. At various time points after peptide injection mice were killed and the thymus was analyzed by FACS<sup>®</sup>.

**FACS<sup>®</sup> Analysis, Antibodies, and Magnetic Cell Sorting.** Analysis was performed on a FACSCAN (Becton Dickinson & Co.) using three-color staining with antibodies conjugated with FITC, PE, or biotin followed by streptavidin-Tricolor (Caltag Laboratories, San Francisco, CA). The transgenic  $\beta$  chain was detected with antibody F23.1 which reacts with V $\beta$ 8.1,2,3 (12). CD4-PE was obtained from Becton Dickinson & Co., and CD8-FITC was prepared by FITC conjugation of antibody YTS169.4 (21). DNA staining was done with 7-aminoactinomycin D (7-AAD<sup>1</sup>; Sigma Chemical Co.) as follows:  $5 \times 10^5$  cells in V-bottomed plates were first stained for CD8-FITC and CD4-PE, washed once in PBS containing 0.01% sodium azide, 2% FCS and once again in PBS with 0.3% saponin. 7-AAD (4  $\mu$ g/ml in PBS-saponin) was then added and the plates were incubated at room temperature shielded from light for 30 min. Without a further washing step, the samples were then analyzed using linear scale for FL-3 acquisition to assess 7-AAD staining.

Positive selection of T cells expressing V $\alpha$ 2 TCR determinants was done by magnetic cell sorting with the Vario-MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) using biotinylated anti-V $\alpha$ 2 (22) antibody and following the procedure recommended in the manual. Positively selected cells were passed over the selection column twice and the purity of the selected population was between 80 and 90%.

## Results

**Generation of Transgenic Mice with a Class II-restricted TCR Specific for the Serum Protein C5.** The A18 T cell clone that donated the receptor used to generate transgenic mice is a high affinity clone that recognizes very small amounts of C5 protein presented in the context of I-E<sup>k</sup> MHC (10, 18). The C5 epitope recognized is peptide 107–121 close to the NH<sub>2</sub>

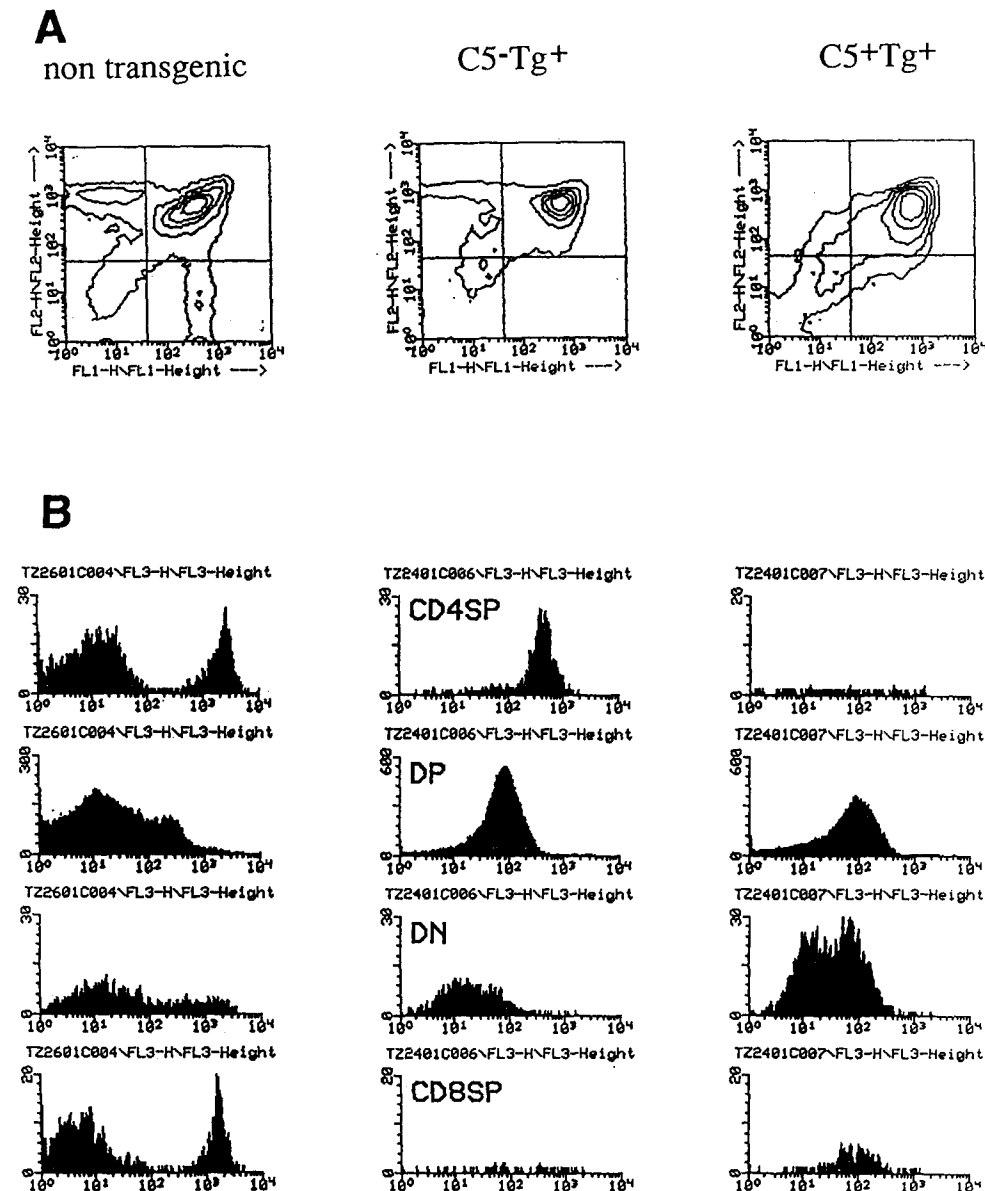
<sup>1</sup> Abbreviation used in this paper: 7-AAD, 7-aminoactinomycin D.

terminus of the C5  $\beta$  chain. The A18 T cell receptor was cloned and cDNAs for the  $\alpha$  and  $\beta$  chains were placed under the control of the human CD2 promoter and LCR region. The human CD2 cassette was previously shown to drive T lymphocyte-specific, copy number-dependent, and integration site-independent expression of the class I-restricted TCR F5 (15). Five founders were identified upon injection of A18  $\alpha$  and  $\beta$  constructs into CBA oocytes, varying in the copy numbers of transgene from 1 to 100. One transgenic line, A18.2, with about 40 copies of the  $\alpha$  transgene and 30 copies of the  $\beta$  transgene, was selected for the following experiments.

**Thymic Development in C5<sup>-</sup> and C5<sup>+</sup> Transgenic Mice.** The transgenic line A18.A was selected for further analysis because its TCR expression in the thymus closely followed normal T cell development in nontransgenic mice as illustrated in Fig. 1. In the absence of C5 (C5<sup>-</sup>Tg<sup>+</sup>) expression

of this class II-restricted receptor results in pronounced skewing of thymocyte development to CD4<sup>+</sup> cells and virtually no generation of CD8<sup>+</sup> cells. The TCR, analyzed by staining with the V $\beta$ 8-specific antibody F23.1 is expressed very low on double negative CD4<sup>-</sup>CD8<sup>-</sup> cells, is upregulated to intermediate levels in the double positive CD4<sup>+</sup>CD8<sup>+</sup> population, and reaches maximum levels upon maturation to single positive CD4<sup>+</sup> cells. This receptor development, particularly the fact that double negative thymocytes are TCR<sup>-/lo</sup>, closely resembles the development in normal mice.

In the presence of C5 (C5<sup>+</sup>Tg<sup>+</sup>) there is no generation of CD4 single positive cells. In contrast to most class I TCR transgenic mice on a deleting background, however, the number of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is only marginally reduced, which is also reflected in similar total cellularity of the thymus from C5<sup>+</sup> transgenic mice compared with C5<sup>-</sup> littermates.

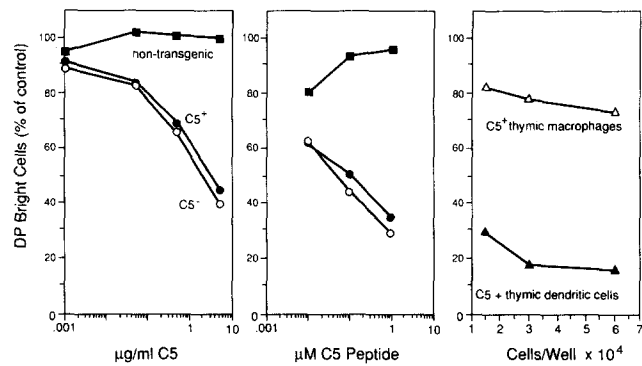


**Figure 1.** Thymocytes from a nontransgenic mouse, a C5<sup>-</sup> TCR transgenic mouse, and a C5<sup>+</sup> TCR transgenic mouse were analyzed for the expression of CD4 and CD8 as well as TCR on gated populations. Staining for TCR was performed with antibody F23.1. (A) Thymic phenotype of transgenic mice in the absence of (C5<sup>-</sup>) or presence (C5<sup>+</sup>) of self-antigen. (B) TCR- $\beta$  chain expression on gated population.

A large population of cells with reduced or absent CD4 and 8 expression appears in the thymus of C5<sup>+</sup> transgenic mice. The apparent TCR expression in the double negative population is probably due to cells that have downregulated TCRs and are in the process of undergoing apoptosis. It appears that tolerance induction to C5 under physiological conditions does not affect the majority of double positive cells, but only those at the transition to single positive cells.

**Induction of Apoptosis in Double Positive Cells In Vitro.** C5 is a circulating serum protein that has to be internalized and processed by MHC class II expressing APC in the thymus for tolerance induction. Previous studies of the C5 presenting capacity of APC populations in the thymus have indicated that the most efficient APC are thymic dendritic cells (18). This APC population resides in the medulla and the cortico-medullary border which means that the bulk of CD4<sup>+</sup>8<sup>+</sup> cortical thymocytes may not have access to efficient C5 presentation; this might explain the failure to delete double positive cells. To address this possibility we set up in vitro suspension cultures in which we exposed all thymocytes to dendritic cells as APC in the presence or absence of C5. As previously reported, the contact of thymocytes in vitro with cognate antigen results in apoptosis, which is first visualized by downregulation of CD4 and CD8 on double positive cells (23). As shown in Fig. 2 it was obvious that thymocytes from C5<sup>-</sup> and C5<sup>+</sup> transgenic mice initiated apoptosis upon contact with dendritic cells and C5 or C5 peptide whereas non-transgenic control thymocytes remained unaffected. The degree of apoptosis was identical in C5<sup>-</sup> and C5<sup>+</sup> thymocytes indicating that both contain the same number of potentially deletable CD4<sup>+</sup>8<sup>+</sup> thymocytes. When efficient APC can gain access to double positive cells in vitro these cells can be deleted; in vivo we believe this access is restricted. Downregulation of CD4 and 8 correlated with a decrease in DNA staining as measured by triple staining for CD4, CD8, and DNA content (data not shown, but see Fig. 3) indicating that the cells had indeed initiated apoptosis. When transgenic thymocytes from C5<sup>+</sup> or C5<sup>-</sup> mice were exposed to macrophages or dendritic cells isolated from thymus of normal C5<sup>+</sup> animals without any further addition of antigen in vitro it was clear that only dendritic cells could induce apoptosis in agreement with previous findings that indicated that only dendritic cells in the thymus were able to present C5.

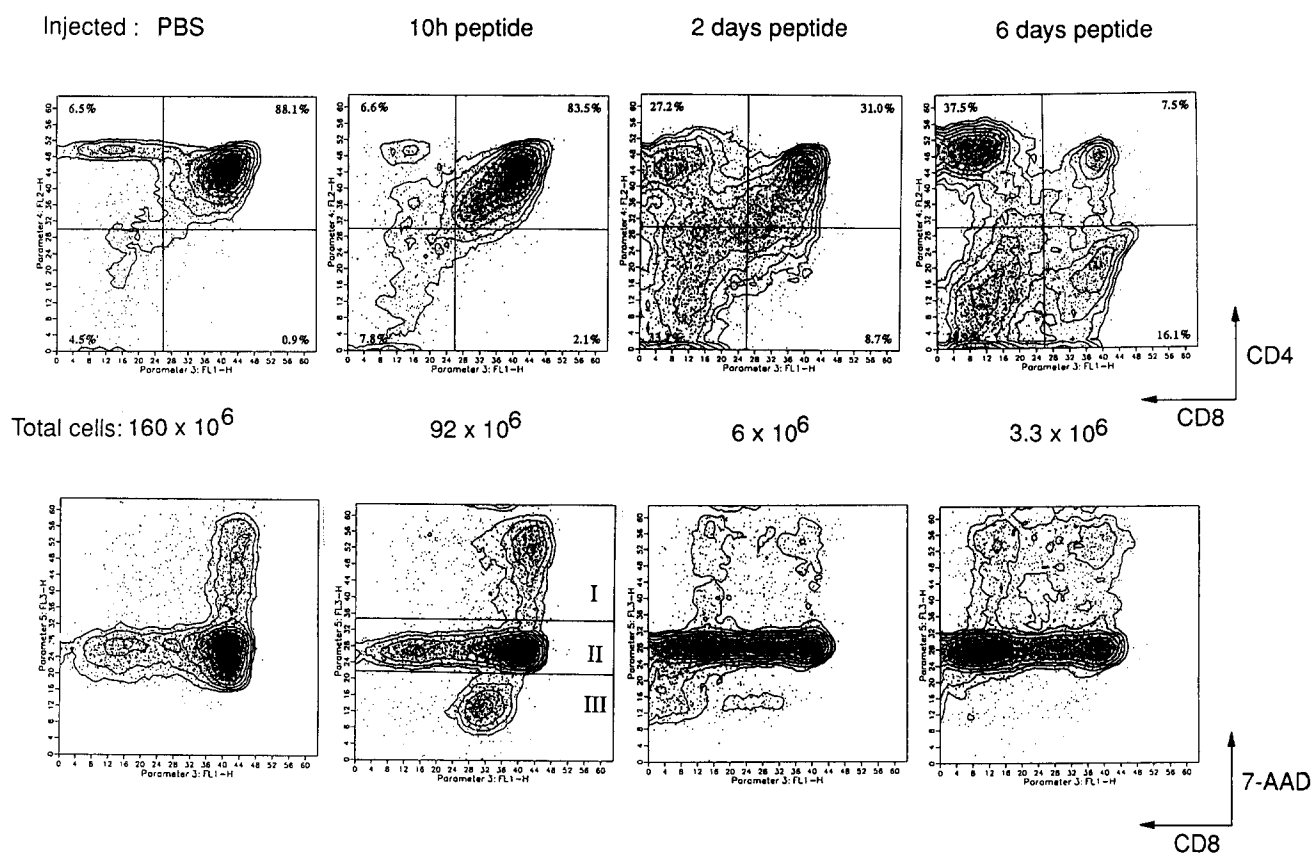
**Apoptosis of Double Positive Cells In Vivo** The in vitro apoptosis results indicate that double positive cells can be deleted under conditions of optimal antigen presentation. To check whether this is true for double positive cells in vivo, we performed the following experiments. Previous studies with transgenic mice bearing a MHC class II-restricted TCR specific for ovalbumin (7) had shown that peptide injection in vivo resulted in deletion of double positive thymocytes. We performed analogous experiments with the C5 peptide 107-121 recognized by the A18 TCR. Fig. 3 *top* shows the pattern of CD4 and CD8 expression at different times after daily intraperitoneal injection of C5 peptide (or PBS in the control group). In addition triple staining with the DNA marker 7-AAD, CD4, and CD8 was performed to visualize apop-



**Figure 2.** Thymocytes from a C5<sup>-</sup> (○), a C5<sup>+</sup> (●) TCR transgenic mouse, and a nontransgenic control (■) were cultured for 12 h with dendritic cells in the absence of C5 or with different doses of C5 protein or C5 peptide. The results shown in the *third panel* were obtained by culturing thymocytes in the presence of different cell numbers of thymic macrophages or dendritic cells isolated from thymus of normal C5<sup>+</sup> mice. CD4 and CD8 expression was analyzed by FACS<sup>®</sup>. The results are expressed as the percentage of bright double positive cells in relation to thymocytes cultured with dendritic cells in the absence of antigen. In the *third panel* the negative control population consisted of thymocytes without any added cells.

toxicity and cell division. Since there are practically no CD8 single positive cells in these mice, all cells that stained positive for CD8 are CD4<sup>+</sup>8<sup>+</sup> and those that do not stain are CD4 single positive and double negative thymocytes. 7-AAD staining is shown on a linear scale and as indicated in Fig. 3, *bottom* the top fraction labeled *I* represents dividing cells in S and M phase with the double content of DNA, fraction *II* represents cells in G1 phase, and fraction *III* contains cells with reduced amounts of DNA due to apoptosis. As early as 10 h after peptide injection a significant downregulation of CD4 and 8 molecules can be observed which is paralleled by the appearance of an apoptotic cell population within the double positive cells (characterized by their reduced 7-AAD staining). After 2 d, double positive cells have virtually disappeared and the size of the thymus is reduced from 160 × 10<sup>6</sup> to 6 × 10<sup>6</sup> cells. No apoptotic cell population is visible by DNA staining anymore, indicating that deletion and apoptosis have been completed by this time. A seemingly CD8 single positive cell population becomes visible on day 2 and more pronounced on day 6, presumably because of its proportional representation within a thymus devoid of double positive cells. We conclude that in a situation where the requirement for antigen internalization and processing is removed, double positive cells are deleted, indicating that MHC class II positive cells in the cortex are able to present C5 for tolerance induction under these conditions. It should be noted, however, that in the late phase after peptide injection, activated mature CD4<sup>+</sup> might exacerbate deletion via secretion of mediators such as TNF.

**Functional Activity of Mature Peripheral T Cells from C5<sup>-</sup> and C5<sup>+</sup> Transgenic Mice.** Spleen cells from C5<sup>-</sup> transgenic mice when cultured with C5 in vitro secrete IL-2 and IFN-γ in response to as little as 10 ng/ml antigen (Fig. 4). We have



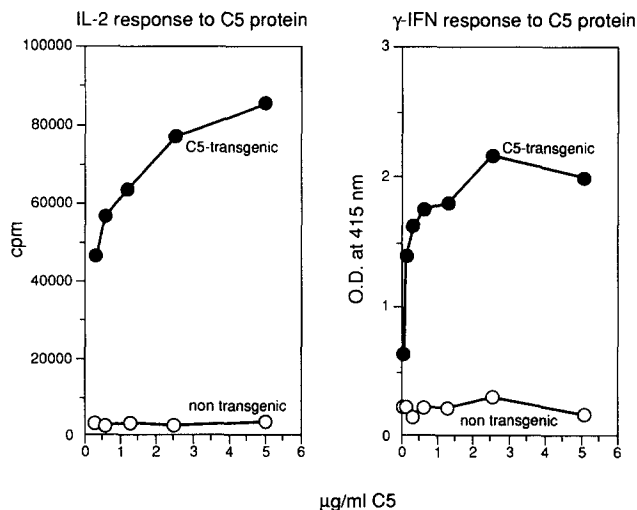
**Figure 3.** C5<sup>-</sup> TCR transgenic mice were injected daily with 250  $\mu$ l of 100  $\mu$ M C5 peptide intraperitoneally for 6 d. At 10 h, 2 d, or 6 d a mouse was killed for analysis of CD4, CD8, and DNA content in thymocytes. The total thymus cell number recovered from each mouse is given. DNA staining by 7-AAD on a linear scale vs. CD8 on a log scale is shown on the bottom. Regions I, II and III refer to cells with double, single, or reduced content of DNA as outlined in Results.

not observed any IL-4 production by these cells. Upon C5 injection, lymph node cells from C5<sup>-</sup> transgenic mice up-regulate CD69 and CD25 (data not shown). Peripheral T cells from C5<sup>+</sup> transgenic mice on the other hand do not show signs of activation in vivo. However, they contain cells that can be induced to secrete IL-2 and IFN- $\gamma$  upon culture with C5 in vitro, indicating that some cells escape tolerance induction in the thymus and are maintained in an anergic state in vivo (Fig. 5). In the absence of a clonotype-specific antibody, we have not been able to identify these cells since endogenous TCR rearrangements that take place in these mice allow accumulation in the periphery of CD4 and CD8 single positive cells that bear nontransgene-derived receptors.

*Is C5 Tolerance Induction Incomplete Due to Limited Amounts of Circulating C5 Early in Ontogeny?* The question arises how C5-specific T cells escape into the periphery of C5<sup>+</sup> transgenic mice. An earlier study investigated the postnatal ontogeny of potentially autoreactive cells specific for an Mls<sup>a</sup>-encoded determinant. Thymocytes bearing V $\beta$ 6 were detectable in Mls<sup>a</sup> mice neonatally and up to 4 d, but rapidly decreased thereafter (24). We considered the possibility that a delay in optimal C5 expression early in ontogeny might likewise allow some C5-specific CD4 cells to escape from thymic tolerance induction.

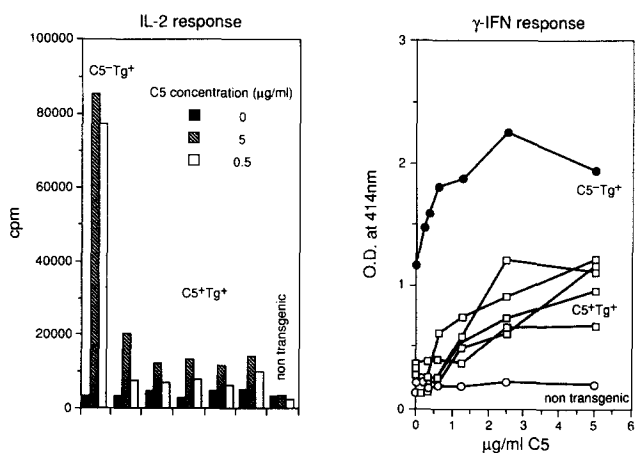
C5 synthesis is demonstrable as early as day 10 of gestation (25). However, adult levels of C5 in the circulation are not reached until several weeks after birth. C5 is a serum protein of medium abundance with average levels of 10<sup>-7</sup> M (about 50  $\mu$ g/ml) in adult male mice, and approximately half those levels in female mice (26). Its expression is governed by a single gene and F1 progeny between a C5<sup>+</sup> and a C5<sup>-</sup> strain harbor half the concentration of C5 (27). It is also important to note that C5 does not cross the placenta. Given the constraints of age, sex, and haplotype for concentration of the antigen, it is unclear what levels of circulating C5 are found at the time of early T cell differentiation and whether they are sufficient to fully prevent the emergence of mature single CD4<sup>+</sup> cells. We reasoned that there might be a window in the ontogeny of C5<sup>+</sup> mice, especially C5<sup>+/-</sup> mice, in which CD4 single positive cells might be allowed to develop due to limited amounts of self-antigen present for tolerance induction. If that were the case one could assume that the peripheral cells from C5<sup>+</sup> transgenic mice which were found to be C5 reactive in vitro might have arisen from such an early wave of CD4 cells that had escaped tolerance induction in the thymus.

To test this hypothesis we isolated thymi from mice at day 19 of gestation and tested them for the presence of C5 reac-



**Figure 4.** Cells from enzyme digested spleen of a  $C5^{-}$  TCR transgenic mouse and a nontransgenic  $C5^{-}$  control mouse were cultured in 96-well plates with different doses of C5 protein. For analysis of IL-2 production, 75  $\mu$ l of culture supernatant were removed after 48 h of culture and tested on the IL-2-dependent CTLL line. Results are expressed as mean cpm (triplicate cultures) of incorporated [ $^3$ H]thymidine. For assessment of IFN- $\gamma$  release 75  $\mu$ l supernatant were removed after 72 h of culture and tested in ELISA. The results are expressed as arbitrary units of OD at 414 nm.

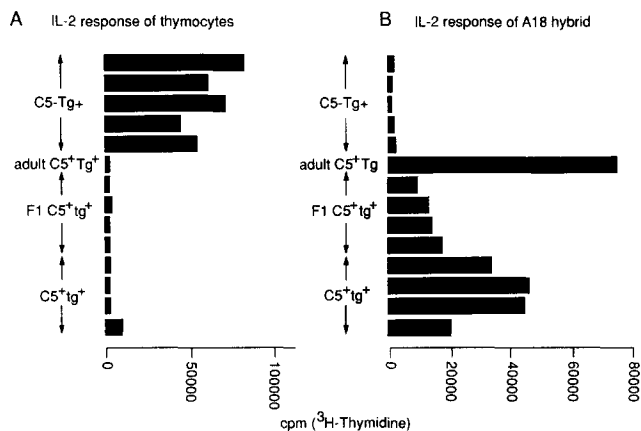
tive cells as well as for C5 presentation capacity which was read out as activation of a C5-specific T cell hybrid. Fig. 6 A shows the IL-2 response of thymocytes from individual day 19 fetuses from  $C5^{-/-}$ ,  $C5^{+/-}$ , and  $C5^{+/+}$  transgenic mice after in vitro culture with dendritic cells and C5. The result unambiguously demonstrates that tolerance induction is complete at day 19 as there is no reactivity in  $C5^{+/+}$  or



**Figure 5.** Cells from enzyme digested spleens of a  $C5^{-}$  TCR transgenic mouse, five individual  $C5^{+}$  TCR transgenic mice and a nontransgenic control were cultured in 96-well plates in the presence or absence of different amounts of C5 as indicated. After 48 h of culture, 75  $\mu$ l supernatant were removed for analysis of IL-2 activity on the IL-2-dependent CTLL line. Results are expressed as mean cpm (triplicate cultures) of incorporated [ $^3$ H]thymidine. For assessment of IFN- $\gamma$  release 75  $\mu$ l supernatant were removed after 72 h of culture and tested in ELISA. The results are expressed as arbitrary units of OD at 414 nm.

$C5^{+/-}$  mice, while C5-specific responses in thymocytes from  $C5^{-/-}$  mice are readily detectable at that time. The reciprocal test for the presence of in vivo processed C5 on day 19 (Fig. 6 B) shows that thymus APC from all  $C5^{+}$  fetuses could be recognized by the C5-specific T cell hybrid A18. There are considerable differences in the levels of stimulation and it is obvious that adult  $C5^{+}$  thymi show higher C5 presentation than day 19 thymi; nevertheless the amounts of C5 present at that time are sufficient to prevent maturation of CD4 single positive cells. This result rules out the possibility that C5-specific T cells in the periphery of  $C5^{+}$  transgenic mice have arisen from an early escape of CD4 single positive cells.

**Phenotype of Thymus and Spleen T Cells in  $C5^{-}$  and  $C5^{+}$  Transgenic Mice Crossed onto the  $Rag1^{-/-}$  Background.** As mentioned previously, endogenous TCR rearrangements that are detectable in  $C5^{+}$  transgenic mice (and to a much lower degree in  $C5^{-}$  littermates) make the identification of potentially autoreactive cells difficult since we do not have a clonotype-specific antibody. In addition we wanted to find out whether suppressor cells are involved in maintenance of C5 tolerance as has been suggested by Cairns et al. (28). We therefore crossed our transgenic mice with mice homozygous for the  $Rag1$  gene deletion to prevent rearrangements of endogenous TCR genes so that the only lymphoid cells in this system would be C5-specific T cells. As shown in Fig. 7,  $C5^{-} Rag^{-}$  transgenic mice show CD4 single positive cells with mature levels of T cell receptor in thymus and the only lymphoid cells found in lymphnodes (and spleen) are CD4 $^{+}$  T cells with transgenic TCRs. These cells can be activated in vivo and in vitro and behave identically to their CD4 coun-

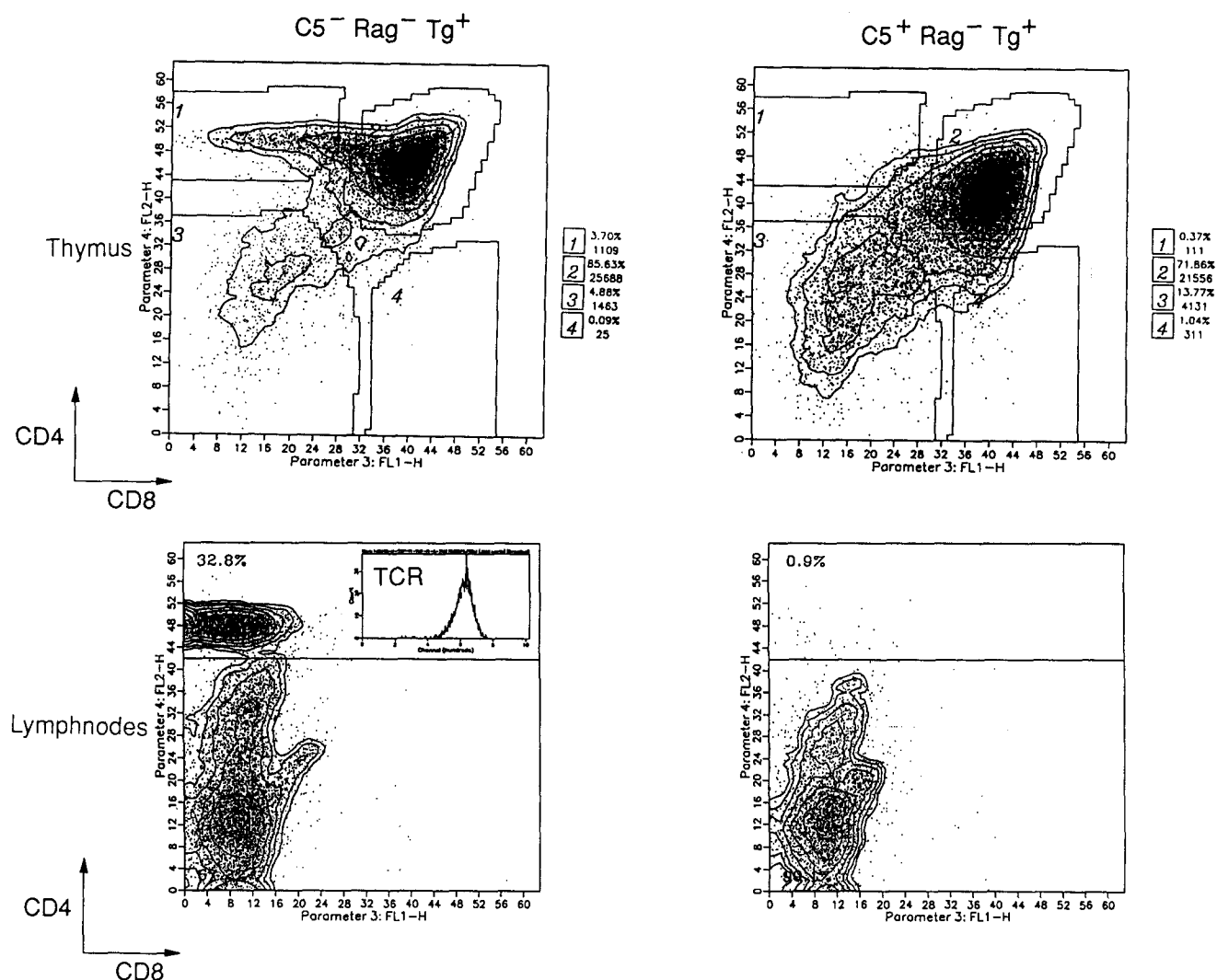


**Figure 6.** Thymus suspensions from day 19 embryos of  $C5^{-} Tg^{+}$  (five embryos),  $C5^{+} Tg^{+}$  (four embryos) or F1 between these two strains (four embryos) and an adult  $C5^{+} Tg^{+}$  control were prepared by enzyme digestion. In A,  $2 \times 10^5$  thymocytes/well of a 96-well plate were cultured with  $2 \times 10^4$  dendritic cells in the presence of 1  $\mu$ M C5 peptide for 48 h. 75  $\mu$ l of supernatant were then removed and tested for IL-2 activity on CTLL cells. In B, the same thymus cell suspensions were irradiated with 200 Gy and cultured at  $2 \times 10^5$  cells/well with  $5 \times 10^4$  cells/well of the A18 T cell hybrid. 24 h later supernatants were transferred to CTLL cells for assessment of IL-2 activity. The results are expressed as mean cpm (triplicate cultures) of incorporated [ $^3$ H]thymidine.

terparts in Rag<sup>+</sup> mice indicating that the absence of other lymphoid cells has not prevented the functional development of transgenic T cells. In the thymus of C5<sup>+</sup>Rag<sup>-</sup> transgenic mice, mature single positive CD4 cells are absent. The number of double positive cells in C5<sup>+</sup>Rag<sup>-</sup> is slightly decreased compared with C5<sup>-</sup> littermates, but the thymic phenotype is very similar to that in young Rag<sup>+</sup>C5<sup>+</sup> mice. Spleens (and lymph nodes) of C5<sup>+</sup>Rag<sup>-</sup> mice do not contain any CD4<sup>+</sup> cells (and in fact no lymphoid cells with expression of Thy1 either) and no functional activity in vitro. These results document that tolerance induction by deletion of MHC class II-restricted T cells specific for circulating C5 is very stringent and that there seems to be no escape of cells through downregulation of TCR or coreceptor.

*C5<sup>+</sup>Rag<sup>+</sup> Transgenic Mice Contain T Cells with Endogenous TCR  $\alpha$  Chains and the Transgenic C5 Reactivity.* The demonstration of C5-specific T cells that appear in the periphery of C5<sup>+</sup>Rag<sup>+</sup> transgenic mice, but not C5<sup>+</sup>Rag<sup>-</sup> transgenic mice implies endogenous TCR rearrangements rescue C5-

specific T cells during selection in the thymus. In this context a crucial question is whether the C5-specific cells in C5<sup>+</sup>Rag<sup>+</sup> transgenic mice carry a single C5-specific endogenous TCR unrelated to the transgenic receptor or have two receptors, the transgenic one and an additional endogenous receptor that mediated thymic selection. The first possibility is unlikely since a single endogenous receptor conferring C5 specificity would have been subject to tolerization as it is in nontransgenic C5<sup>+</sup> mice. Spleen cells from C5<sup>+</sup> transgenic mice mount a rapid primary C5-specific response in vitro in contrast to normal C5<sup>+</sup> or C5<sup>-</sup> mice. It therefore seems reasonable to assume that the transgenic receptor is involved in the response of C5<sup>+</sup>Rag<sup>+</sup> transgenic mice. The most obvious test for this hypothesis would be double staining for the transgenic TCR V $\alpha$  chain and any given endogenously derived V $\alpha$  chain (allelic exclusion for the transgenic TCR  $\beta$  chain is  $\geq 90\%$ ). Unfortunately, the available antibodies against V $\alpha$ 11.1 do not react with the V $\alpha$  haplotype of the A/J strain (29) so that the transgenic TCR V $\alpha$  cannot be



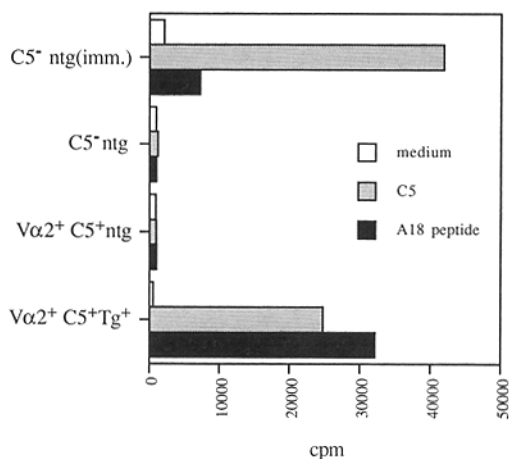
**Figure 7.** Thymus (top) and lymph node cells (bottom) of a C5<sup>-</sup> Rag<sup>-/-</sup> Tg<sup>+</sup> mouse and a C5<sup>+</sup> Rag<sup>-/-</sup> Tg<sup>+</sup> mouse were analyzed for CD4 and CD8 expression. The level of TCR expression in CD4<sup>+</sup> lymph node cells is shown in an inset as assessed by staining with antibody F23.1.

identified by staining. However, the transgenic TCR is easily identified by its reactivity against the A18 peptide epitope of C5. We therefore chose to positively select T cells expressing TCR V $\alpha$ 2 determinants which can be identified with an antibody (22) and test the reactivity of V $\alpha$ 2 expressing T cells to C5 and the A18 peptide in comparison with nontransgenic C5<sup>+</sup> and nontransgenic C5<sup>-</sup> mice.

As shown in Fig. 8, V $\alpha$ 2-expressing T cells, purified from spleen cells of C5<sup>+</sup> transgenic mice by magnetic cell sorting, react to C5 protein and the peptide epitope recognized by the transgenic TCR. No response was detectable in V $\alpha$ 2 T cells from C5<sup>+</sup> nontransgenic mice or in cells from nonimmune C5<sup>-</sup> mice. C5<sup>-</sup> nontransgenic mice immunized with whole C5 protein react to C5 *in vitro*, but only marginally to the transgenic TCR epitope which is not well represented in the random repertoire of C5 reactive T cells. We therefore conclude that a significant proportion of C5-specific T cells detected in C5<sup>+</sup>Rag<sup>+</sup> mice carry V $\alpha$ 2 together with the transgenic receptor (and presumably the transgenic  $\beta$  chain). It is likely that other V $\alpha$  determinants are similarly represented on cells expressing transgenic TCR.

## Discussion

This study analyzes the mechanism of tolerance in mice with a transgenic TCR specific for the serum protein C5. C5 is a natural self-protein that causes complete T cell tolerance in mice that express it, whereas no tolerance is induced in C5-deficient strains of mice which lack C5 in their circulation. The basis of tolerance was unknown and suppressor cells were reported to be involved in its maintenance. C5 TCR transgenic mice allowed us to investigate the mechanisms underlying induction and maintenance of tolerance under physiological conditions of antigen expression *in vivo* by crossing



**Figure 8.** Splens were taken from C5<sup>+</sup> nontransgenic (*ntg*) and C5<sup>+</sup> transgenic (*Tg*<sup>+</sup>) mice and V $\alpha$ 2-bearing T cells were purified by magnetic cell sorting. Splens from nontransgenic C5<sup>-</sup> mice were taken from either untreated animals (*ntg*) or mice primed with C5 in alum 14 d before (*imm.*). Spleen cells were cultured in the presence of 5  $\mu$ g/ml C5 or 1  $\mu$ M A18 peptide (107-121) for 48 h. Supernatants were transferred to CTL cells for assessment of IL-2 activity. The results are expressed as mean cpm (triplicate cultures) of incorporated [<sup>3</sup>H]thymidine.

C5<sup>-</sup> mice that express the transgenic receptor with normal C5<sup>+</sup> mice. The results of these experiments show unequivocally that C5<sup>+</sup> transgenic mice are tolerized by negative selection of CD4<sup>+</sup> single positive thymocytes. Deletion of self-reactive cells obviously occurs late in thymic ontogeny because the CD4<sup>+</sup>8<sup>+</sup> population is not depleted in our mice.

In other class II TCR transgenic mice, as far as thymic tolerance induction has been analyzed, deletion of CD4<sup>+</sup>8<sup>+</sup> thymocytes was observed after injection of cognate peptide. Also transgenic mice bearing a class II-restricted TCR specific for an epitope on the immunoglobulin  $\lambda$  chain showed deletion of double positive cells when these mice were crossed to mice transgenic for the  $\lambda$  chain (30). Since the transgene in the latter mice was expressed under the control of the Ig heavy chain promoter, deletion was attributed to intrathymic synthesis of the  $\lambda$  chain which presumably supplied high local concentrations of antigen rather than to an effect of blood-borne antigen. In fact serum levels of 500  $\mu$ g/ml after repeated injections of  $\lambda$  immunoglobulin were required before deletion of double positive cells in the thymus was detectable.

Several possibilities could account for differences observed in intrathymic stages of deletion. First, the level of TCR expression at different stages of T cell ontogeny in the thymus must play an important role in susceptibility to negative selection. The majority of TCR transgenic mice show high levels of TCR expression already at the double negative stage which is not seen in normal mice. This will clearly enhance the chance of negative selection and certainly contributes to the findings that positive and negative selection can occur simultaneously. We chose a transgenic line for this study in which TCR expression follows that seen in normal mice. However, our data disputes the simple notion that insufficient receptor expression caused the delay in negative selection. Clearly double positive cells could be deleted when cultured with dendritic APC *in vitro* and after injection of the cognate peptide *in vivo*.

Another possibility is that exposure to negatively selecting antigen is delayed in our mice because it requires internalization and presentation by dendritic cells that are found in the thymic medulla, but not the cortex where the bulk of double positive thymocytes resides (31). Our data do not exclude a role for medullary epithelium (32) as APC for negative selection, but we know that thymic macrophages are incapable of presenting exogenous protein because their levels of class II are very low. Cortical epithelial cells, on the other hand, express high levels of class II molecules, yet seem unable to present C5 for negative selection as judged by the fact that double positive cells are present under physiological conditions in C5<sup>+</sup> mice. This failure to present may be due to their relative inefficiency in internalization of exogenous protein, the absence of costimulatory molecules (33, 34), and/or to the possibility that the cortex does not get access to the full amount of C5 present in the blood circulation. Although proteins have been shown to enter the cortex through the transcapillary route (35), this may not be as efficient as the blood supply which carries circulating proteins into the medulla. Thymic nurse cells as representatives of cortical epithelium have been shown to present intravenous injected pro-



teins, but several milligrams of protein had to be injected to detect its presentation by nurse cells (36). It is quite likely, however, that self-proteins that are more abundant than C5 can get presented by cortical epithelial cells, which might result in the deletion of double positive cells. This interpretation is in agreement with the results in  $\lambda$  light chain-specific TCR transgenic mice. These mice show deletion of double positive thymocytes if the serum concentration of  $\lambda$  chain reaches levels  $>500 \mu\text{g/ml}$ , which is about 10-fold more than the average C5 concentration.

The conclusion we would like to draw is that the onset of negative selection in the thymus depends on the overall avidity of interactions that are determined partly by TCR expression, and partly by the number of MHC/peptide complexes that are presented. The latter may rarely be limiting for class I-restricted TCRs because of the abundance of class I-expressing cells in the thymus. If the avidity of interactions is high at an early stage of thymocyte development, negative selection will delete the  $\text{CD4}^+8^+$  compartment. For class II-restricted receptors specific for extracellular antigens, the need for antigen internalization and processing by MHC class II-expressing APC is an obvious limitation to receive high avidity signals for negative selection in the cortical stage of development. When the antigen supply becomes limiting only dendritic cells might be able to provide sufficient MHC/peptide complexes, thus restricting presentation for negative selection to the medulla and cortico-medullary junction and hence a relatively late stage in thymocyte development. Likewise in MHC class I TCR transgenic mice once the antigen density becomes limiting, the elimination of double positive cells is less pronounced (37). Regardless of the delayed onset of negative selection the results presented show that thymic deletion of T cells with C5 specificity is the major mechanism of tolerance induction.

Considering the high incidence of autoimmune disorders with involvement of MHC class II-restricted cells, we had anticipated that negative selection by clonal deletion might be less stringent in this compartment compared with MHC class I-restricted T cells. The inherent limitations of presentation for exogenous antigens, such as threshold concentrations that have to be reached and the need for internalization and processing by MHC class II positive APC raised the possibility that some T cells might escape thymic negative selection. Despite the fact that C5 levels are low early in development and adult amounts of C5 in the circulation are not found until several weeks after birth, there was sufficient self-antigen present to prevent maturation of  $\text{CD4}$  single positive cells at day 19. Preliminary experiments using fetal thymic organ cultures have indicated that a day 14 fetal thymus from a  $\text{C5}^+$  transgenic mouse can generate  $\text{CD4}^+$  single positive cells after 10 d of culture indicating that the C5 levels at that time are not high enough for sustained presentation in vitro

in the absence of ongoing C5 supply. Thus, between days 14 and 19 C5 synthesis increases sufficiently to supply the levels of C5 needed for tolerance induction. The demonstration of C5-specific T cells in these mice which are nonresponsive in vivo, but are activatable in vitro at face value suggested leakiness of the thymic tolerance process. In view of the results with  $\text{Rag}^- \text{C5}^+$  transgenic mice in which we do not find any evidence of T cells escaping into the periphery, however, this notion has to be modified. The findings rule out a number of possibilities which could have accounted for the presence of potentially self reactive cells in  $\text{C5}^+$  mice. First, they show that clonal deletion is complete and that there is no significant leakage into the periphery of cells with downregulated receptors or coreceptors, at least up to the relatively young age of 8 wk. Second, the results rule out the contribution of suppressor T cells to the tolerant state since their absence in  $\text{Rag1}^-$  mice should have resulted in accumulation of autoreactive cells. Third, our findings strongly suggest that endogenous rearrangements might be involved in creating a potentially autoreactive repertoire of cells in the periphery.

One possibility is that endogenous rearrangements have created receptors composed of the transgenic  $\beta$  chain with an endogenous  $\alpha$  chain that by chance convey C5 specificity. The reason why this possibility seems relatively unlikely is that C5-specific cells detected in  $\text{C5}^+$  transgenic mice have the same fine specificity as the correct transgenic receptor recognizing peptide 107-121, whereas the random repertoire of C5-specific T cells is diverse and does not focus onto this particular epitope. An alternative possibility is that autoreactive cells might carry two receptors, the transgenic receptor and an additional receptor using a different  $\alpha$  chain. Incomplete allelic exclusion of the  $\alpha$  chain has been demonstrated to result in productive rearrangement of two different  $\alpha$  chains in T cell clones, although technical difficulties prevented their detection on the cell surface (38). Recently, the expression of two different TCRs on cells from transgenic mice was described (39). This phenomenon is not restricted to transgenic mice or T cell clones, but appears to occur frequently in normal T cells as indicated by a study that detected two receptors on about 30% of normal human T cells (40). In our study T cells of  $\text{C5}^+$  transgenic mice carrying a TCR- $\alpha$  chain unrelated to the transgenic TCR were shown to react with the fine specificity of the transgenic receptor. This supports the assumption that they express a second receptor that allows their positive selection and exit into the periphery. The signals involved in this process and the mechanisms that (a) keep the transgenic receptor unreactive in vivo and (b) allow its activation in vitro are presently unknown, but their elucidation should give important insight into the generation of autoimmunity.

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