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Expression of a second receptor rescues self-specific T cells from thymic deletion and allows activation of autoreactive effector function

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ABSTRACT **Allelic exclusion at the T-cell receptor α chain locus is incomplete resulting in the generation of T cells that express two T-cell receptors. The potential involvement of such T cells in autoimmunity has been suggested [Padovan, E., Casorati, G., Dellabona, P., Meyer, S., Brockhaus, M. & Lanzavecchia, A. (1993) *Science* 262, 422–424; Heath, W. R. & Miller, J. F. A. P. (1993) *J. Exp. Med.* 178, 1807–1811]. Here we show that expression of a second T-cell receptor can rescue T cells with autospecific receptors from thymic deletion and allow their exit into the periphery. Dual receptor T cells, created by constitutive expression of two transgenic T-cell receptors on a Rag1$^{-/-}$ background, are tolerant to self by maintaining low levels of autospecific receptor, but self-reactive effector function (killing) can be induced through activation via the second receptor. This opens the possibility that T cells carrying two receptors in the periphery of normal individuals contain putatively autoreactive cells that could engage in autoimmune effector functions after recognition of an unrelated environmental antigen.**

Self tolerance is established through clonal deletion of self-reactive T cells in the thymus (1–5). In addition, there are nondeletional means of maintaining tolerance in the periphery, such as down-regulation of co-receptors (6, 7) or T-cell receptors (TCR, ref. 8) and induction of anergy (9).

The presence of T cells expressing low levels of a particular T-cell receptor clonotype can be indicative for the expression of an additional T-cell receptor by these cells, as shown originally by Heath and Miller (10). TCR transgenic mice crossed with a transgenic line expressing the target antigen deleted thymocytes with high levels of clonotype but contained cells in the periphery that expressed low levels of clonotype, many of which carried an additional T-cell receptor. Endogenous rearrangements in TCR transgenic mice have been shown to provide at least a limited repertoire for unrelated antigens, some of which is carried by T cells with two receptors (11). The existence of T cells with two T-cell receptors is now widely demonstrated in mice and men (11–13). Potentially, such cells have dual specificity and may thus play a role in autoimmunity.

We have used transgenic mice expressing a major histocompatibility class II restricted, transgenic T-cell receptor (A18 TCR) with specificity for a natural self antigen, the serum protein C5, to address the question of whether expression of a second T-cell receptor allows escape from thymic deletion and activation to autoreactive effector function.

A18 TCR transgenic mice develop CD4$^+$ T cells when crossed onto the C5-deficient A/J background. In contrast, when crossed with C5$^+$ strains, where circulating C5 protein is present, thymocytes are deleted at the point of transition from double positive to CD4 single positive cells (5). However, thymic deletion in the presence of self antigen is not absolute. Peripheral T cells from such mice can be activated by C5 stimulation *in vitro*, although they are functionally tolerant *in vivo*. In contrast, such T cells are absent from C5$^+$ A18 TCR transgenic mice carrying a disrupted *Rag-1* gene to prevent endogenous T-cell receptor rearrangements. This indicates that endogenous rearrangements in normal Rag1$^+$ C5$^+$ transgenic mice might be involved in creating a potentially autoreactive repertoire of cells in the periphery. We show here that endogenous rearrangements produce T cells expressing a second T-cell receptor in addition to the transgenic receptor, which may allow exit of self-specific T cells into the periphery.

To examine the fate of dual receptor T cells in the absence or presence of self antigen, we crossed two T-cell receptor transgenic mouse strains, both bred onto a Rag1$^{-/-}$ background; in such mice, all T cells constitutively express two T-cell receptors. Our data provide evidence that cells with low levels of a self-specific receptor can escape negative selection in the thymus provided they express a second, non-self-specific receptor. Furthermore, we show that self-specific receptors can mediate their effector function even if they themselves do not participate in the initial activation of the cell.

MATERIALS AND METHODS

Transgenic Mice. A18 TCR transgenic Rag-1$^{-/-}$ C5$^-$ mice recognize the serum protein C5 in the context of H-2E^k using a receptor composed of Vβ8.3 (BV8S3, ref. 14) and Vα11.1^a (ADV11S5, ref. 14). BM3 Rag-1$^{-/-}$ mice carry an anti-H-2K^b TCR composed of Vβ2 (BV9S1, ref. 14) and ADV17S2 (14) (recognized by clonotypic antibody 98 (15), which is positively selected by H-2^k major histocompatibility class. BM3 Rag1$^{-/-}$ mice were back-crossed onto the C5$^-$ A/J background, which expresses positively selecting determinants for the A18 and the BM3 receptors; positive selection of CD8 T cells proceeds in BM3 mice irrespective of the presence or absence of C5 protein. To obtain dual TCR offspring, A18 TCR Rag1$^{-/-}$ mice were crossed with BM3 TCR Rag1$^{-/-}$ mice. C5$^-$ dual TCR transgenic as well as single TCR transgenic littermates were obtained with BM3 Rag1$^{-/-}$ TCR mice back-crossed to A/J (C5$^-$), whereas C5 expressing (C5$^+$) dual TCR transgenic mice were obtained by crossing A18 single TCR Rag1$^{-/-}$ (C5$^-$) mice with BM3 Rag1$^{-/-}$ mice on the C5$^+$ CBA (H-2^k) background. All mice are kept in conventional animal facilities in the National Institute for Medical Research.

Flow Cytometry Analysis. Peripheral blood lymphocytes were isolated by centrifugation through Lympholyte–Mammal (Cedarlane Laboratories). Cells were stained with fluorescein isothiocyanate-conjugated anti-CD8 (YTS169.4, ref. 16), phy-

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Abbreviations: TCR, T-cell receptor; IL, interleukin.

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coerythrin-conjugated anti-CD4 (Boehringer Mannheim), and biotinylated 7G8.2 anti-Vβ8.3 TCR antibody (17) or the clonotypic antibody Ti98 (15) against the BM3 receptor. Negative controls were stained with isotype-matched F23.2 anti-Vβ8.2 (18) control antibody followed by streptavidin-Red670 (Gibco).

Magnetic Cell Sorting. Vα2-expressing cells were positively selected on the Vario-MACS (Miltenyi Biotech, Camberley, Surrey, United Kingdom) after labeling with biotinylated antibody B20.1 (19) followed by incubation with streptavidin-coupled magnetic beads. Positively selected cells were passed over the selection column twice, and their purity was between 80% and 90%.

Cell Cultures. Cells were stimulated in round-bottomed 96-well plates (2×10^5 cells/well) with 2×10^4/well dendritic cells from bone marrow cultures with granulocyte macrophage colony-stimulating factor (20). Spleen cells from dual TCR-expressing mice were depleted of CD8 cells with biotinylated anti-CD8 antibody YTS169 followed by sheep anti-rat IgG coupled Dynabeads and a further depletion step with streptavidin-MACS beads and passage through a magnetic cell sorter (Miltenyi Biotech). Fluorescence-activated cell sorting analysis prior to culture showed <0.7% CD8-positive cells. After culture for 48 h, aliquots of supernatants were removed (the cells were cultured further) and tested for IL-2 content in serial dilutions on IL-2-dependent CTLL cells.

Cytotoxicity Assay [JAM test (21)]. On day 4 of culture (see above), the cells were harvested, and CD8 depletion was repeated before the cytotoxic assay. Targets were labeled with 5 μCi of [^{3}H]thymidine for 12 h prior to the assay. Effector and target cells were incubated for 3 h. Target cells without effector cells were used as background control. This assay measures intact DNA from living cells; killed cells degrade their DNA into small fragments that are washed through the fiberglass filters. Specific lysis values are calculated according to the formula:

$$\frac{\text{cpm of background} - \text{cpm experimental}}{\text{cpm of background}} \times 100\ [\%]$$

The standard errors of the mean of duplicate (A) or triplicate (B) cultures were all within 20% of the mean.

Limiting Dilution Assays. For assessment of C5-specific T cell precursors, serial dilutions of spleen cell suspensions (24 replicates for each cell concentration) were cultured in the presence of 3×10^4 bone marrow-derived dendritic cells and 10 μM C5 peptide 107–121. Control wells received T cells and dendritic cells, but no peptide. After 48 h of culture, 100 μl of supernatants were transferred to IL-2-dependent CTLL cells, and CTLL proliferation was assessed by [^{3}H]thymidine incorporation over 18 h. Reactions were considered positive in wells that exhibited proliferation greater than the mean plus three times the standard deviation value of control wells. The frequency of antigen specific cells was calculated by regression analysis of the number of negative wells at each dilution of responder cells. All points had χ^2 values of <12.

RESULTS

C5$^+$ Rag1$^+$ Transgenic Mice Contain T Cells with Two Receptors. A18 TCR transgenic mice, when crossed onto a Rag1$^{-/-}$ background, develop CD4 T cells in the absence of C5, whereas in the presence of C5, such cells are deleted in the thymus and no T cells reach the periphery. In contrast, as shown previously (5), thymic deletion in C5$^+$ A18 TCR mice, which can undergo endogenous rearrangements (Rag1$^+$ mice), is not complete. Peripheral T cells from such mice can be activated by C5 stimulation *in vitro*, although they are functionally tolerant *in vivo*. Theoretically, the capacity to rearrange endogenous TCR α chains, which can associate with transgenic β chains, could create a pool of low affinity C5 peptide-specific T cells. To address this possibility, we analyzed precursor frequencies of C5 specific T cells in C5$^+$ mice transgenic for the A18 TCR β chain alone compared with those from C5$^+$ TCR α$^+$β$^+$, C5$^-$ TCR α$^+$β$^+$, and nontransgenic mice (Table 1) About 1 in 100 spleen cells from C5$^-$ TCR α$^+$β$^+$ mice reacted with C5 peptide 107–121. This frequency was approximately 100-fold lower in C5$^+$ TCR α$^+$β$^+$ mice, whereas mice transgenic for the A18 TCR β chain alone did not contain more C5 peptide-specific precursor cells than nontransgenic mice. Thus, it seems that specificity for C5 peptide 107-121 depends on the presence of the complete transgenic TCR and cannot be recreated by fortuitous endogenous rearrangements.

This suggests that C5-specific T cells in C5$^+$ Rag1$^+$ transgenic mice carry two receptors, one composed of transgenic αβ chains and one functionally unrelated receptor formed by combinations of endogenous α with transgenic β chains. In support of this assumption, we found that a substantial proportion of T cells from C5$^+$ Rag1$^+$ transgenic mice, carrying an endogenous TCR α chain (Vα2), unrelated to the transgenic TCR, reacted with C5 peptide (Table 1). The apparently increased precursor frequencies in spleen cells from C5$^+$ TCR α$^+$β$^+$ mice sorted for Vα2 expression compared with unselected spleen cells can be attributed to the fact that positive selection for Vα2 expressing cells increases the proportion of CD4 cells in the starting population (19).

Phenotype of Dual Receptor T Cells from Crosses of Two TCR Transgenic Strains. To test the possibility that expression of a second receptor allows exit of autospecific T cells into the periphery, we crossed two transgenic mouse strains both bred onto a Rag1$^{-/-}$ background and each carrying a distinct, constitutively expressed TCR. One strain carries the H-2E^k restricted, C5 specific A18 receptor, the other a class I restricted T-cell receptor specific for the alloantigen K^b (BM3), which is also positively selected in H-2^k mice (22). Normally, these two strains, when back-crossed to Rag1$^{-/-}$ mice, express only CD4 cells or CD8 cells, respectively. On a C5-deficient (C5$^-$) background, dual TCR transgenic mice develop CD4 and CD8 cells that co-express C5-specific and anti-K^b specific T-cell receptors (Fig. 1 *A* and *B*). Compared with C5$^-$ mice carrying only A18 receptors, we observe improved positive selection to mature CD4 thymocytes in C5$^-$ dual TCR transgenic mice. On a C5$^+$ background, where A18 single TCR transgenic thymocytes undergo complete deletion, the presence of the BM3 receptor in dual TCR transgenic mice rescues a proportion of CD4 cells and allows their exit into the periphery (Fig. 2 *A* and *B*). Their numbers are decreased when compared with C5$^-$ dual TCR transgenic mice, and their A18 TCR levels, in contrast to the levels of BM3 TCR, are very low. This indicates that substantial numbers of CD4 cells with higher levels of the C5 specific receptor were deleted in the thymus.

Table 1. Precursor frequencies of C5 specific T cells in spleen

Exp.	Nontransgenic	C5$^-$ TCR α$^+$ β$^+$	C5$^+$ TCR α$^+$ β$^+$	C5$^+$ TCR β$^+$	C5$^+$ TCR α$^+$ β$^+$ Vα2 + ve
1	1/56,500	1/150	1/8,000	1/95,000	1/2,300
2	1/67,000	1/72	1/14,200	1/70,000	1/3,600

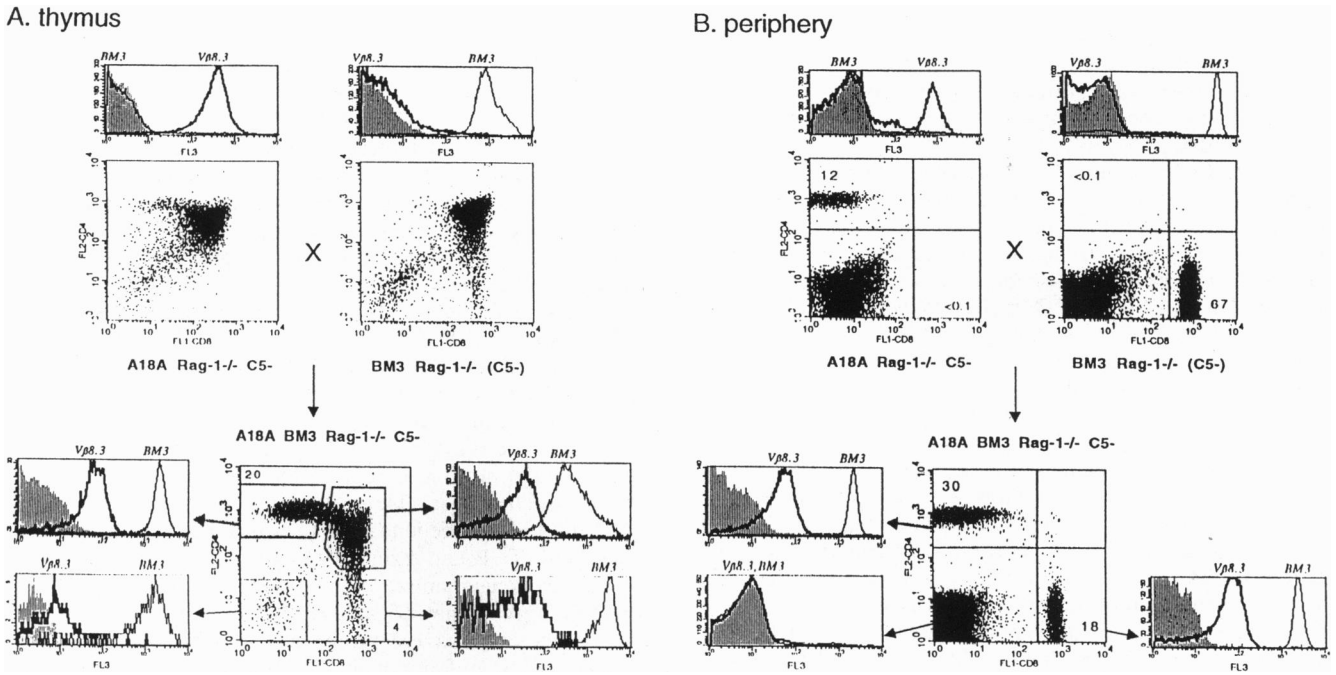

FIG. 1. Flow cytometric analysis of TCR Rag-$1^{-/-}$ transgenic mice crossed onto a C5- background (nondeleting for the single A18 TCR). Three color staining for CD8, CD4, and TCR of thymocytes (*A*) and peripheral lymphocytes (*B*) from single TCR transgenic Rag$1^{-/-}$ mice (upper dot plot panels) and their dual TCR littermates, A18A × BM3 (lower dot plot panels) are shown. Numbers in the quadrants represent percent of gated subpopulations. TCR expression (FL3) is presented on histograms for gated subpopulations (dual TCR) or total cells (single TCR). Staining for the A18 TCR is shown by thick lines, and staining for the BM3 TCR by thin lines. Gray filled histograms represent negative controls (stained with isotype matched control antibody and streptavidin-670 as third color). Total cell numbers from thymus were 1.5×10^8 for single A18 TCR (C5-) and 2×10^8 for dual TCR and BM3 single TCR mice.

Activation and Effector Function of T Cells from Dual Receptor Transgenic Mice. *In vitro* activation tests showed that purified CD4 T cells from dual TCR transgenic mice on a $C5^-$ background can be activated to produce IL-2 by C5 stimulation as well as by stimulation with H-2^b cells (Fig. 3*A*). This indicates that both receptors can be functionally competent on the same cell. In contrast, CD4 T cells from tolerant $C5^+$ dual TCR transgenic mice cannot be activated with C5. We ascribe this to the low levels of C5 specific receptors that prevent the triggering threshold for activation of the cells being reached so that functional tolerance upon contact with self antigen *in vivo* is ensured. However, these

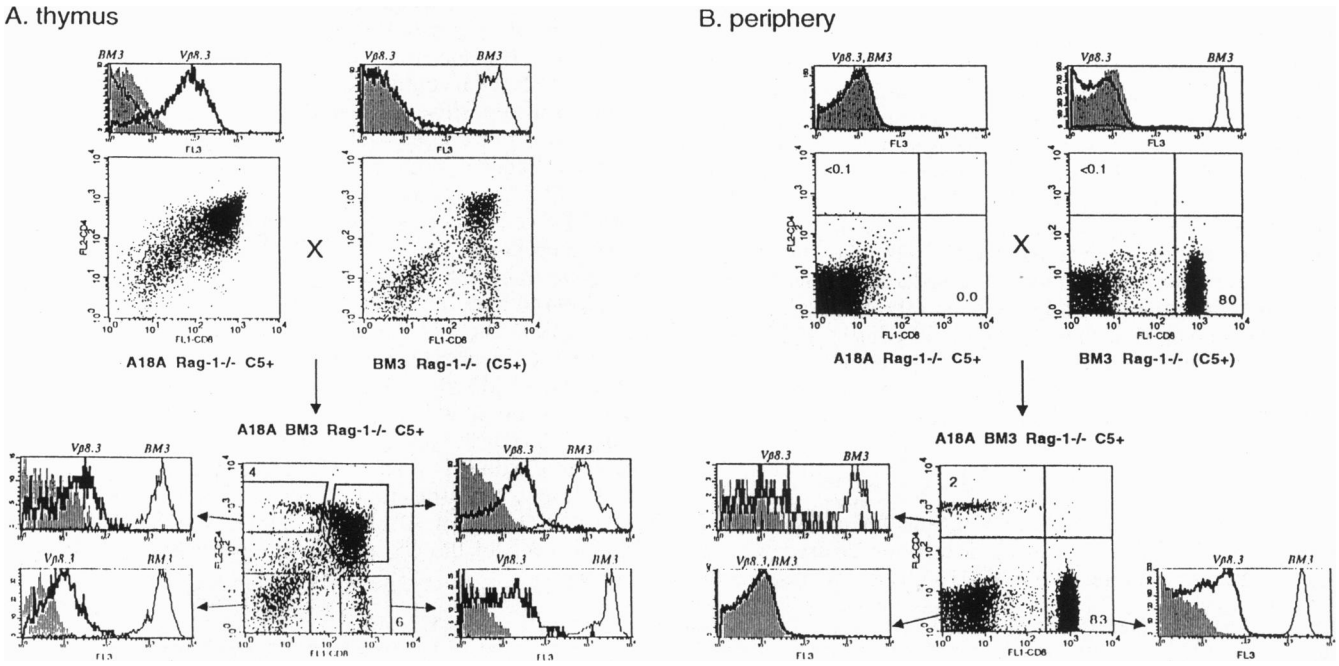

FIG. 2. Flow cytometric analysis of TCR Rag-$1^{-/-}$ transgenic mice crossed onto a $C5^+$ background (deleting for the single A18 TCR). Three color staining as described for Fig. 1 is shown for thymocytes (*A*) and peripheral lymphocytes (*B*) from single TCR Rag$1^{-/-}$ $C5^+$ mice and their dual TCR offspring. The numbers of CD4 T cells in the periphery of dual TCR $C5^+$ mice ranged from 1 to 5% of total cells in different experiments. Total thymocyte numbers were 0.8×10^8 for single TCR $C5^+$, 2×10^8 for single BM3, and 1.5×10^8 for dual TCR $C5^+$ transgenic mice.

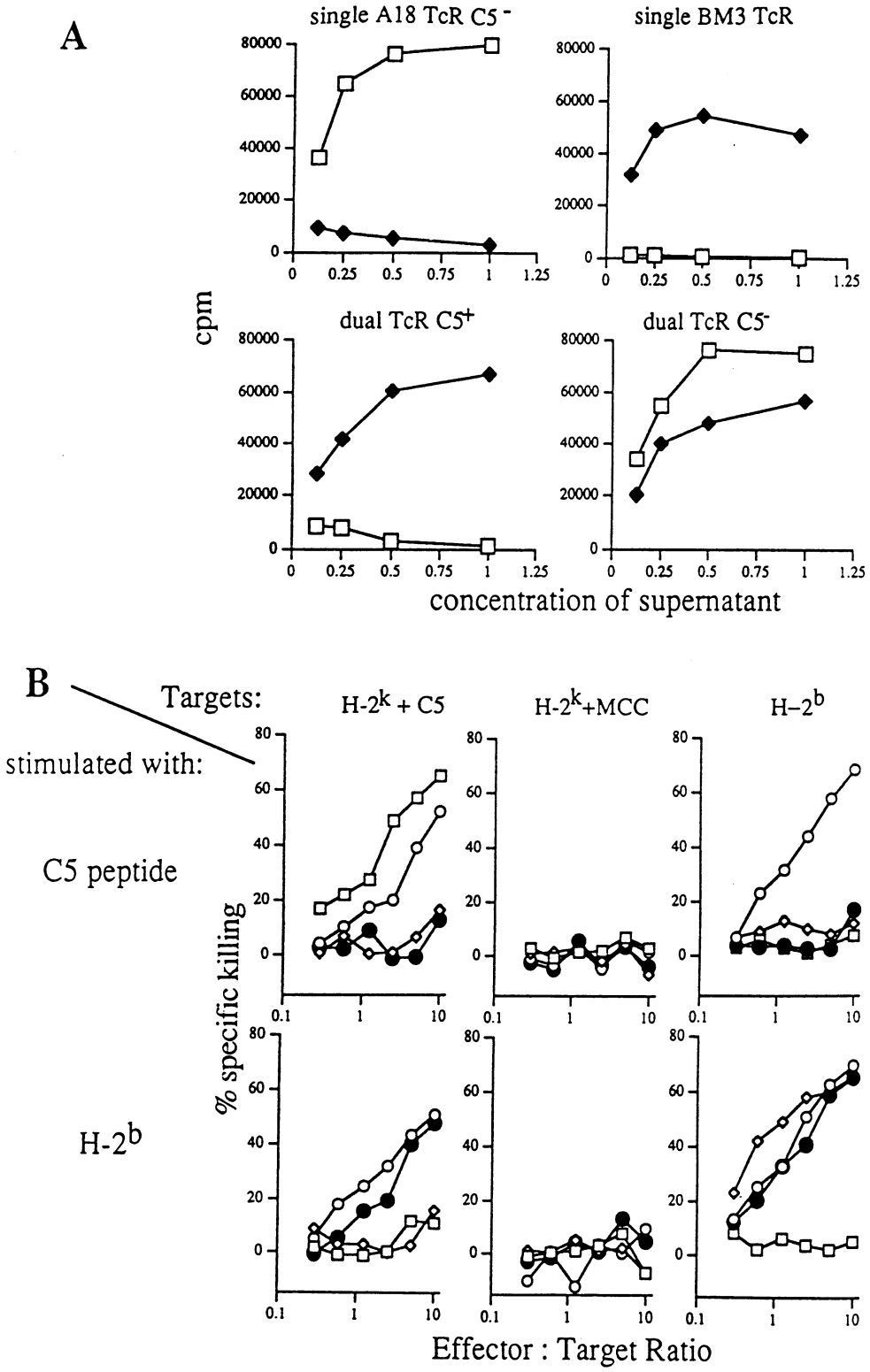

FIG. 3. Functional activity of cells from single and dual TCR transgenic mice. (*A*) Activation for IL-2 production. Spleen cells from single and dual TCR transgenic mice as indicated on top of the panels were tested for IL-2 production in response to 1 μM C5 peptide (□) or allo-H-2^b cells (◆). (*B*) The same cultures were tested for cytotoxicity in the JAM test. A18 single TCR (□), BM3 single TCR (◇), dual TCR C5$^-$ (○), and dual TCR C5$^+$(●), cultured with either C5 peptide (upper panels) or H-2^b APC (lower panels), were tested for cytotoxic activity against three targets: H-2E^k expressing tumor cells (LK35, ATCC HB98) pulsed with 1 μM C5 peptide or moth cytochrome *c* peptide 88–103 (MCC) and H-2^b expressing EL-4 tumor cells.

cells respond to stimulation with H-2^b by IL-2 production (Fig. 3*A*). Activation of CD4 cells through the class I-restricted BM3 receptor is possible because of its reported CD8 independence (15).

The crucial question was whether autoreactive effector function directed by the C5 specific receptor of rescued CD4 cells mice could be evoked after activation of such cells through their second T-cell receptor.

To test this, we took advantage of the fact that CD4 cells from A18 TCR transgenic mice develop into Th1 cells upon stimulation (23) and mediate antigen-specific killing activity in a cytotoxicity assay measuring apoptosis of target cells. As targets we used either H-2^k tumor cells pulsed with C5 peptide or a control peptide, or H-2^b tumor cells. This test demonstrated clearly that CD4 cells from dual TCR transgenic $C5^+$ mice possess autoreactive potential when activated via the BM3 receptor. They were able to kill not only allo-H-2^b targets, but also target cells loaded with C5 peptide, but they were not able to kill targets loaded with control peptide (Fig. 3*B*). In contrast, T cells carrying a single TCR could only kill those targets that expressed determinants corresponding to their receptor specificity; A18 killed H-2^k+C5, but not H-2^b, and BM3 killed H-2^b, but not H-2^k+C5. CD4 cells from dual TCR transgenic $C5^-$ mice could be activated to kill both targets by stimulation through either the C5 or the BM3 receptor (Fig. 3*B*). The functional results showing the A18 TCR specificity for H-2^k + C5 make it highly unlikely that extensive reshuffling of TCR chains between BM3 and A18 took place in dual TCR $C5^+$ transgenic mice.

DISCUSSION

The existence of T cells with two α chains is a natural consequence of gene rearrangement at the α chain locus, which only terminates when an $\alpha\beta$ TCR complex is expressed on the surface that results in positive selection of the cell (24). Although the initial description of dual receptor T cells in the periphery of mice and humans immediately prompted the suggestion that such cells might be involved in autoimmunity, subsequent argumentation disputed the likelihood of such an event (25). First, the demonstration that α chain gene rearrangements only proceed until a functional $\alpha\beta$ receptor is generated implies that there is only a small chance for T cells to express two functional, self major histocompatibility class-restricted receptors, and it was unclear whether an $\alpha\beta$ T-cell receptor, which had failed positive selection, could be functionally active in the periphery (26). Furthermore, it was assumed that thymocytes with receptors, which are specific for an ubiquitous self antigen, would inevitably be eliminated in the thymus whether they express one or two receptors (25)

Our experiments show that the latter assumption is incorrect. Thymocytes that express two T-cell receptors can avoid negative selection if the cell surface expression of auto-specific receptors is decreased, which could happen, for instance, as a consequence of competition for CD3 components. Thus, rearrangement and expression of additional receptors are not confined to situations in which positive selection fails but also take place under the pressure of negative selection. It is therefore conceivable that the peripheral repertoire of dual receptor T cells contains cells with auto-specific receptors, which would have been deleted in the thymus had they expressed only this receptor.

In our experimental system, negative selection has a strong impact on the number and functional capacity of C5-specific CD4 cells; only those dual receptor T cells leave the thymus which express the C5-specific receptor at levels too low to reach the triggering threshold for activation. Those cells are functionally inert as far as recognition of self antigen is concerned, be it as a consequence of failed positive selection through the C5 receptor or because of the impact of negative selection. In other situations, where self-specific T cells escaped from deletion through down-regulation of TCR, nonresponsiveness could be reversed *in vitro* by exposure to antigen or cytokines (6, 7) indicating differential sensitivities for triggering in different T-cell receptors.

The crucial point is that autoreactive effector function, such as antigen-specific killing, can be mediated through self-specific receptors despite their low expression levels, provided that the cells are activated via a second T-cell receptor. This emphasizes that, given one receptor succeeds in transmitting activation signals to the cell, a second receptor, which on its own would be functionally inert, can mediate effector function corresponding to its specificity. So far these results have been obtained *in vitro*; we have not been able to observe autoimmunity *in vivo* following priming of dual TCR mice with dendritic cells expressing cognate antigen for the BM3 receptor. However, we suspect that this may be a reflection of the experimental system rather than a general impossibility, since in the presence of high levels of systemically distributed self antigen, autoimmune effector function may not be detectable.

While autoreactive T cells may escape from thymic deletion through expression of a second T-cell receptor, their numbers are likely to be small if the self antigen has access to the thymus and their impact in autoimmune disease is uncertain. However, the periphery may in addition contain potentially autoreactive T cells that were never exposed to self antigen during their thymic differentiation and therefore express auto-specific receptors at higher levels. Although expression of two T-cell receptors is not a prerequisite for development of autoimmunity (27, 28), the presence of even just a few dual receptor T cells could contribute significantly to the initiation of the disease process. Animal models of autoimmune diseases provide evidence that even in genetically susceptible individuals and in the presence of large numbers of potentially autoreactive T cells onset of overt disease can be delayed (29). Alternatively, additional contributing factors such as exposure to pathogens in the animal facility can trigger disease (30). Autoimmune disease is frequently associated with preceding viral or bacterial infections (31). The basis for these correlations is poorly understood. It is possible that many self antigens do not normally gain access to professional antigen presenting cells, which are required for initial activation of naive T cells. Cross-reactive recognition of shared peptide sequences between infectious agents and self molecules (32) or bystander activation of autoreactive T cells as a consequence of local inflammatory reactions (33–35) have been postulated as a mechanism for activation of autoreactive T cells. Our results add the possibility that conventional recognition of infectious agents by an additional T-cell receptor on a potentially autoreactive cells could trigger autoimmune disease.

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