

Characterization of murine V γ 1, V δ 6 T cells

by

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ABSTRACT

The work described in this thesis began with an attempt to determine whether murine $\gamma\delta$ T cells respond to mycobacterial antigens. To this end we generated a panel of $\gamma\delta$ T cell hybridomas following *in vitro* stimulation of purified $\gamma\delta$ cells with mycobacterial purified protein derivative (PPD). 21 hybridomas that secreted IL-2 upon mAb mediated TCR crosslinking were chosen for further study. The hybridomas were primarily of the V γ 1,V δ 6 class, as all hybridomas contained an in frame V γ 1-J γ 4 rearrangement, and the majority contained an in frame V δ 6-J δ 1 rearrangement. Junctional sequences of the γ and δ chain genes were diverse. The hybridomas were found to secrete IL-2 spontaneously, and only modest increases in IL-2 secretion were observed upon addition of PPD. The high background level of IL-2 secretion complicates investigation of the antigenic specificity of the hybridomas.

The constitutive IL-2 secretion could be blocked by mAbs that bind to the CD3/ $\gamma\delta$ TCR complex, suggesting that IL-2 secretion depends on TCR-mediated activation. To identify additional molecules, and potential cellular TCR ligands, involved in the hybridoma activation, we generated mAbs that block hybridoma IL-2 secretion following immunization of hamsters with hybridoma cells. All mAbs that specifically blocked IL-2 secretion of the V γ 1,V δ 6 hybridomas either recognized an epitope of the $\gamma\delta$ TCR, or recognized the $\alpha\upsilon\beta$ 3 vitronectin receptor. Chapters 3 and 4 of this thesis describe characterization and expression analysis for anti-V γ 1 and anti- $\alpha\upsilon\beta$ 3 mAbs respectively.

To characterize primary V γ 1,V δ 6 T cells, particularly with regard to activation status and response to mycobacterial antigens, we generated mice transgenic for the γ and δ genes that encode the V γ 1,V δ 6 $\gamma\delta$ TCR of one of the hybridomas. These transgenic mice (A-2^m) contained increased numbers of V γ 1 cells in the thymus and peripheral lymphoid organs. A-2^m transgenic cells appeared to be in a resting state *in vivo* as determined by analysis of expression of surface activation markers and proliferative status, however, the cells could be activated by mAb mediated TCR crosslinking in the presence of modest amounts of recombinant IL-2. A-2^m transgenic cells did not display a convincing response to mycobacterial antigens, including PPD, Hsp 65 and isopentenyl pyrophosphate. Other notable features of the transgenic mice include the ability of the transgenes to rescue development of CD4⁺CD8⁺ thymocytes when crossed into the Rag1 deficient background, and the constitutive expression of the IL-2 receptor β chain by the A-2^m transgenic cells.

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Chapter 1: Introduction

Foreword

During the search for genes encoding the $\alpha\beta$ T cell receptor, a third rearranging gene designated γ was discovered (1, 2). Antibodies made against peptides derived from the γ sequence allowed identification of a distinct TCR designated $\gamma\delta$ (3). The δ gene was later cloned based on its position within the TCR α locus (4). Subsequent studies identified a distinct, new class of T cells that express the $\gamma\delta$ receptor and are designated $\gamma\delta$ T cells (5-7).

Unlike $\alpha\beta$ T cells whose function in cellular immunity was outlined long before the cloning of the $\alpha\beta$ TCR, $\gamma\delta$ T cells were identified based on the serendipitous isolation of the gene encoding the TCR γ chain, and therefore, their role in the immune system was unknown from the outset. Since their initial description, a vast effort has been directed towards understanding the function of $\gamma\delta$ cells. Despite the accumulation of a large body of literature describing various features of $\gamma\delta$ cells, the function of this class of T cells remains unknown. This introduction will highlight some of the fundamental information that is known about murine and human $\gamma\delta$ cells. The first section will describe some general features of $\gamma\delta$ T cells. The second section will discuss the specificity of $\gamma\delta$ T cells, and the last section will address the function of $\gamma\delta$ T cells. A small amount of material described in the body of the thesis has been incorporated into the introduction for the sake of completeness.

General features of $\gamma\delta$ T cells

Nomenclature of γ and δ gene segments

The genes encoding γ and δ TCR chains in both mice and man were identified by a number of groups at different times, and therefore, various different nomenclatures for γ and δ TCR genes are present in the literature. This can cause considerable confusion as the same gene segments are often referred to by different names in different publications. In the case of the

murine γ locus, it is particularly confusing as there are four different nomenclatures based on 1) order of identification, and publications by 2) Garman et al., (8) 3) Iwamoto et al., (9) and 4) Pelkonen et al., (10) These nomenclatures are listed in a table at the end of this introduction. The nomenclature based on order of identification is used in this thesis. More recently, a new WHO-IUIS nomenclature for all TCR segments has been devised(11).

$\gamma\delta$ T cell subsets

In mice, there are 5 major subsets of $\gamma\delta$ T cells that can be distinguished by such parameters as V γ gene usage, junctional diversity, time of appearance in ontogeny, tissue localization and thymus dependence. The earliest T cells to develop in the thymus are the V γ 5V δ 1 subset (12), which appear as early as embryonic day 15. The TCR of this class of cells is entirely homogeneous, encoded by γ and δ chains with canonical sequences (13). By one week after birth, no V γ 5 cells remain in the thymus, and in adult mice these cells specifically colonize the epidermis of the skin (12, 13). The second wave of T cells to develop in the thymus are the V γ 6V δ 1 (14) cells which also have a single canonical TCR. Upon leaving the thymus, these cells specifically colonize the epithelium of uterus, vagina and tongue (15). The next two $\gamma\delta$ subsets to appear in the thymus are the V γ 4 and V γ 1 populations(16, 17). V γ 4 cells have diverse TCRs based on the utilization of different V δ chains and junctional diversity of both the γ and δ chains. These cells are present in the peripheral immune system, and can be found in blood, lymph nodes and spleen. V γ 1 cells also have diverse TCRs that utilize different δ chains and exhibit junctional sequence diversity. Along with V γ 4 cells, V γ 1 cells constitute the major class of $\gamma\delta$ cells in the peripheral immune system of mice (18). Unlike V γ 4 cells, a large population of V γ 1 cells is present in the intestinal epithelium, and these V γ 1 intestinal intraepithelial lymphocytes can develop extrathymically (18). The final class of murine $\gamma\delta$ T cells are the V γ 7 cells which have diverse TCRs, specifically colonize the intestinal epithelium and for the most part, develop extrathymically (19-22).

In humans there are two major subsets of $\gamma\delta$ T cells defined by their use of two different δ chains. Rearrangements of V δ 2 to D δ 3 and V γ 1.8 or V γ 9 to

the J γ 1 cluster can be found in the thymus early in ontogeny (23, 24). Cells utilizing these chains are designated the V δ 2 subset. Predominant rearrangements that occur later in thymic ontogeny include rearrangements of V δ 1 to D δ 1 or D δ 2 and rearrangements of V γ gene segments from the V γ 2 family to the J γ 2 cluster (23, 24). Cells that use these TCR chains are referred to as the V δ 1 subset. After birth, the V δ 2 cells comprise a minority of $\gamma\delta$ cells in the thymus and are predominant in the blood. The percentage of V δ 2 cells in the blood increases with age. In contrast, after birth, the V δ 1 cells are predominant in the thymus and are a minority of $\gamma\delta$ cells in the blood, with their percentage in blood decreasing with age (25-29).

Determination of murine $\alpha\beta$ and $\gamma\delta$ T cell lineages

Several models have been proposed to explain the segregation of $\alpha\beta$ and $\gamma\delta$ T cell lineages. Rearrangements of γ and δ chain genes and surface expression of γ and δ chains precede those of α and β chains during thymic ontogeny (30, 31). In addition, non-productive rearrangements of γ chain genes are frequently observed in $\alpha\beta$ T cells. These facts led to the proposal that $\alpha\beta$ T cells develop from T cell precursors that failed to express a functional $\gamma\delta$ TCR (31, 32). In this model, complete γ and δ TCR gene rearrangements initially occur in common precursors at a time when only partial (D-J) rearrangements occur at the β locus. Generation of a functional $\gamma\delta$ TCR would inactivate the recombination machinery and commit the cell to the $\gamma\delta$ lineage. Failure to productively rearrange both γ and δ chain genes would allow the cell to proceed to further rearrangement of β and α chain genes and potential commitment to the $\alpha\beta$ lineage. Studies of transgenic mice that express rearranged γ and δ transgenes undermined this model that would predict that expression of γ and δ transgenes should disrupt $\alpha\beta$ T cell development. In contrast to this prediction, a first set of $\gamma\delta$ transgenics to be generated displayed normal development of $\alpha\beta$ cells (33). Transcripts of the transgenes were not present in the $\alpha\beta$ cells of these mice, leading to the notion that γ and/or δ chain genes have a silencer element that blocks their expression in cells of $\alpha\beta$ lineage. A second set of mice which had shorter transgenes displayed disrupted $\alpha\beta$ T cell development. It was concluded that these transgenes lacked the silencer element (34). These findings led to the

notion that transcriptional silencing of γ and δ genes is involved in commitment of precursor cells to the $\alpha\beta$ lineage. An α silencer was also identified that could function in commitment to the $\gamma\delta$ lineage (35).

Another model proposed that the δ locus cannot rearrange in $\alpha\beta$ lineage cells. This model was based on the finding that the δ locus present on circular DNAs generated from $V\alpha$ - $J\alpha$ rearrangement was almost exclusively in the germline configuration (36). Yet another model proposed that commitment to the $\alpha\beta$ lineage involves site-specific deletion of the δ locus by recombination between two elements (δ rec and $\psi J\alpha$) (37, 38). Two recent reports that describe the occurrence and characterization of δ chain gene rearrangements in $\alpha\beta$ lineage cells are incompatible with these two models (39, 40).

Several approaches including the expression of γ and δ transgenes in the SCID mouse background (41) and injections of bone marrow cells (42) or purified $\gamma\delta$ cells (43) into SCID mice have suggested the involvement of $\gamma\delta$ cells in development of $\alpha\beta$ lineage cells to the $CD4^+CD8^+$ double positive stage. However, characterization of δ gene deficient mice has shown that presence of functional δ chains is not required for development of $\alpha\beta$ T cells (44). Similarly, $\gamma\delta$ T cells can develop normally in mice deficient for the TCR β gene (45).

Based collectively on the above cited reports, it is currently believed that $\gamma\delta$ and $\alpha\beta$ cells emerge from common precursors and that lineage commitment is not controlled at the level of rearrangement of the TCR genes. Rather, it is believed that lineage commitment is either based on gene silencing or competition between the different TCRs or pre-TCRs. The potential role of $\gamma\delta$ cells in $\alpha\beta$ development remains unclear.

Thymic selection of murine $\gamma\delta$ T cells

In order to create a functional repertoire of $\alpha\beta$ T cells that can recognize foreign antigens in the context of self MHC and ignore self antigens, $\alpha\beta$ thymocytes undergo processes of positive and negative selection. Although in most cases the ligands for murine $\gamma\delta$ cells are unknown, several studies have addressed the issue of thymic selection of $\gamma\delta$ T cells. Studies of the sequences of rearranged $V\gamma 5$, $V\gamma 6$ and $V\delta 1$ genes from PCR amplified genomic DNA

from fetal thymocytes and epithelial T cells showed that there is greater junctional diversity among non-productive than among productive rearrangements. This observation suggested the existence of cellular selection for cells expressing the canonical TCRs (46). However, analysis of V γ -J γ and V δ -D δ -J δ rearrangements in mice deficient for the C δ region did not support this conclusion. In these mice, which cannot assemble functional $\gamma\delta$ TCRs, the relevant junctional sequences were comparably homogeneous to those described in normal mice (44). This finding favored the existence of a molecular mechanism (molecular constraint model) to limit the diversity of these junctional sequences. It is likely that rearrangements of these segments are greatly limited by molecular constraint, and that in addition, some cellular selection for canonical TCRs occurs.

Stronger evidence for thymic selection of $\gamma\delta$ T cells came from studies of $\gamma\delta$ transgenic mice. In one study, mice transgenic for genes encoding the V γ 4 $\gamma\delta$ TCR expressed by the KN6 hybridoma that recognizes the TL gene, T22 (47, 48), which is expressed in H-2^b mice but not in H-2^d, mice provided evidence for negative selection of the transgenic $\gamma\delta$ T cells (49). The KN6 transgenes were crossed into the H-2^b and H-2^d backgrounds, and it was found that these mice had similar numbers of transgenic thymocytes, but the number of transgenic cells was 10-fold lower in the spleens of the H-2^b mice than spleens of H-2^d mice. The transgenic cells derived from the H-2^b mice were also shown to be larger in size and lower in TCR density than those from H-2^d mice. In addition, $\gamma\delta$ T cells from H-2^b mice did not proliferate in response to H-2^b stimulator cells, although they could proliferate in the presence of exogenous IL-2, indicating that KN6 transgenic cells are rendered anergic in the H-2^b background (49). A second study of mice transgenic for the genes encoding the V γ 4 $\gamma\delta$ TCR from the clone G8, which also recognizes a TL gene product also provided evidence for negative selection of V γ 4 $\gamma\delta$ T cells (50).

Studies of mice derived from crossing the transgenics described above into the β 2m-deficient background provided evidence for positive selection of $\gamma\delta$ T cells (51, 52). Transgenic cells with high TCR density were present in the thymus of transgenic β 2m-deficient H-2^b and H-2^d mice, but they did not populate peripheral lymphoid tissues. The transgenic cells did not proliferate strongly in response to H-2^b stimulator cells, even in the presence of exogenous IL-2. In addition, transgenic cells in the β 2m-deficient background

were essentially all positive for J11d expression, while only half of the transgenic thymocytes were J11d positive in the normal background. As J11d is a marker for immature thymocytes, it appeared that expression of $\beta 2m$ and thus MHC class I type molecules is essential for maturation of the transgenic thymocytes. Thus at least some $\gamma\delta$ T cells undergo processes of thymic selection reminiscent of those described for $\alpha\beta$ T cells.

Extrathymic selection of murine $\gamma\delta$ T cells

Extrathymic selection of $\gamma\delta$ T cells has been suggested for two different classes of cells, the $\gamma\delta$ pulmonary resident lymphocytes and the $\gamma\delta$ intestinal intraepithelial lymphocytes (i-IELs). In one series of experiments, it was shown that two $\gamma\delta$ TCR sequences termed BID and GxYS are expressed by a large fraction of pulmonary resident lymphocytes from BALB/c mice and BALB.B mice but not from C57BL/6 mice (53-55). The BID and GxYS TCRs were found in (BALB/c \times C57BL/6) F1 hybrids and also in athymic BALB/c mice. Productively rearranged genes capable of encoding BID and GxYS TCRs were found among thymocytes of all strains tested, including C57BL/6 (54). Collectively, these studies concluded that expression of the BID and GxYS TCRs in the lungs requires extrathymic positive selection of cells expressing these receptors mediated by a molecule(s) that is present in BALB/c and absent in C57BL/6 and is not a classical MHC molecule.

The $\gamma\delta$ i-IELs are a major population of $\gamma\delta$ cells in mice. Unlike the case of peripheral lymphoid organs in which $\gamma\delta$ cells are a small minority of total T cells, among i-IELs, cells expressing the $\gamma\delta$ TCR are equally abundant as $\alpha\beta$ T cells. The extrathymic development of $\gamma\delta$ i-IELs has been suggested by their presence in nude mice and their Thy1 negative phenotype. Studies of i-IELs revealed that the percentage of i-IELs that express the V δ 4 chain varies from 20% to 50% among different strains of mice (56). In addition, F1 hybrids between the high and low expressors retained the high expression phenotype. Analysis of normal and thymectomized chimeras generated by injection of bone marrow from F1 mice into mice of the parent backgrounds indicated that the V δ 4 cells were positively selected by host cells in the high backgrounds and that this selection does not require a thymus. Analysis of V δ 4 expression among i-IELs from recombinant inbred strains, and strains

with recombinant H-2 loci, revealed that the selection process that leads to the V δ 4-high phenotype is dependent on a gene linked to the MHC class II region and requires I-E expression.

More recently, the issue of whether development of the i-IEL $\gamma\delta$ subset is truly extrathymic has been questioned. Analysis of euthymic and thymectomized Rag2 deficient mice that were reconstituted with bone marrow from H-2-matched, Rag2⁺ donors showed that while $\gamma\delta$ i-IELs can develop in the thymectomized recipients, they are present in significantly greater numbers in the euthymic mice (57). Furthermore, recent studies found a dramatic decrease in the number of $\gamma\delta$ i-IEL in mice thymectomized 24 to 72 hrs before birth compared to euthymic controls (58, 59). These studies suggest that presence of a thymus or thymically derived T cells could be important in $\gamma\delta$ i-IEL development.

Specificity of $\gamma\delta$ T cells

Classical MHC proteins

The majority of $\alpha\beta$ T cells recognize peptide antigens presented by MHC class I and class II molecules. $\alpha\beta$ cells generally express one of the two co-receptor molecules, CD4 and CD8, which are involved in recognition of class II and class I molecules, respectively. One of the hallmarks of most $\gamma\delta$ cells is their CD4⁻CD8⁻, double negative phenotype. The fact that $\gamma\delta$ cells do not express the co-receptors for class I and class II molecules suggests that similar recognition of class I or class II molecules is not a feature of $\gamma\delta$ T cells. Indeed the high degree of alloreactivity between individuals with different MHC class I and class II alleles that is a striking feature of $\alpha\beta$ T cells is not observed for $\gamma\delta$ T cells, again suggesting that recognition of class I and class II proteins is not a general feature of $\gamma\delta$ T cells. Nevertheless, some murine and human $\gamma\delta$ T cells have been shown to respond to MHC class I and class II molecules. In mice, a $\gamma\delta$ T cell line was isolated from draining lymph node cells one week after footpad immunization with allogeneic spleen cells in complete Freund's adjuvant. This cell line was shown to respond to H-2D^k (60). In addition, a $\gamma\delta$ T cell clone was generated following repeated stimulation of lymph node cells from nude mice with allogeneic spleen cells. This clone was shown to

respond to I-E^k (61) and several lines of evidence suggested that the recognition did not require peptide presentation (62).

In humans, T cell clones that respond to the class I molecules, HLA-A2 (63) or HLA-A24 (64), were generated following culture of purified CD4⁺CD8⁻ healthy donor-derived peripheral blood lymphocytes in the presence of allogeneic stimulator cells. The recognition of the HLA-A2 molecules appeared to be peptide-dependent, because mutations in the A2 molecule that affect the response of A2 restricted $\alpha\beta$ cells also affected the response of the $\gamma\delta$ cells (63). Several human $\gamma\delta$ T cell clones have been isolated that respond to various class II alleles. In one study, a panel of alloreactive $\gamma\delta$ T cell clones that recognize different HLA-DR alleles was generated (65). Other reports describe the isolation of human $\gamma\delta$ T cell clones specific for peptides from tetanus toxoid in the context of HLA-DRw53 (66) or HLA-DR4 (67). These studies suggest that peptide antigens can be recognized in the context of MHC molecules by $\gamma\delta$ cells, however the nature of peptide presentation could differ between $\alpha\beta$ and $\gamma\delta$ cells.

Despite these examples, it appears that in general, $\gamma\delta$ cells do not recognize classical MHC proteins. Surveys of large numbers of murine $\gamma\delta$ hybridomas (68) and human $\gamma\delta$ T cell clones activated in limiting dilution culture (69) showed that the vast majority fail to respond to classical MHC proteins. In addition, $\gamma\delta$ T cell responses can not generally be inhibited by antibodies against classical MHC proteins, again suggesting that recognition of classical MHC proteins is not typical of $\gamma\delta$ T cells (70).

Non classical MHC proteins

In addition to the classical MHC class I and class II proteins, the MHC locus encodes a variety of MHC-like proteins. In mice, as described above, Two $\gamma\delta$ T cell hybridomas were shown to respond to TL gene products (47, 48, 60, 71). TL gene products resemble class I molecules and were initially identified on leukemic T cells. Various TL gene products are also expressed on normal thymocytes and on epithelial cells in the intestine (72, 73). The specific expression of several TL-encoded proteins on intestinal epithelial cells that are in close proximity to the $\gamma\delta$ i-IELs raises the interesting possibility that $\gamma\delta$ i-IELs recognize antigens presented by TL-encoded proteins (73). It has

been reported that one TL-encoded protein, T23^b, can bind peptides. Furthermore, a murine $\gamma\delta$ T cell line was identified that responds to a synthetic Glu:Tyr copolymer presented by the T23^b protein (74). The generality of murine $\gamma\delta$ recognition of antigens presented by TL-encoded products has yet to be established.

The human MHC locus also encodes a number of class I-like molecules, some of which may be related to the TL-encoded proteins in mice. So far direct evidence for recognition of TL-like proteins by human $\gamma\delta$ T cells has not been obtained. CD1 molecules are another subset of class I-like glycoproteins that require $\beta 2m$ for stable cell surface expression (75, 76). In humans, some $\alpha\beta$ cells respond to CD1b while $\gamma\delta$ clones have been identified that respond to CD1c (77-79). $\alpha\beta$ cells have been shown to respond to the mycobacterial lipid, mycolic acid, presented by the CD1b molecule (79). Therefore, it is possible that some human $\gamma\delta$ T cells also respond to lipid antigens in the context of CD1c molecules. However, the frequency of $\alpha\beta$ cells and $\gamma\delta$ cells that recognize CD1 appears to be very low.

So far, antigen presenting molecules that activate a large fraction of $\gamma\delta$ T cells have not been identified. It does not appear that $\gamma\delta$ cells generally respond to classical MHC proteins, and examples of $\gamma\delta$ reactivity to MHC-like proteins also seem to be exceptions. Since, alloreactivity is not a feature of $\gamma\delta$ cells, it is likely that antigen presenting molecules for $\gamma\delta$ cells are non-polymorphic. It is also possible that most $\gamma\delta$ cells do not respond to presented antigens. Instead they could directly associate with foreign antigens or cell surface proteins expressed on stressed or damaged cells.

Mycobacterial antigens

The reactivity of human $\gamma\delta$ T cells to mycobacterial antigens is well established. Several $\gamma\delta$ T cell clones derived from a BCG immune donor (80), patients with tuberculoid leprosy (81), or patients with rheumatoid arthritis (66) were initially shown to respond to recombinant mycobacterial heat shock proteins. Later, it was shown that $\gamma\delta$ T cells from healthy subjects with no discernible prior exposure to mycobacterial antigens also display a strong response to heat killed mycobacteria or mycobacterial extracts in the presence of antigen presenting cells. It was also realized that mycobacterial heat shock

proteins were not the major antigenic components for the $\gamma\delta$ cells (82-84). In one study, half of the peripheral blood $\gamma\delta$ cells from some donors were shown to respond to killed mycobacteria, while only a minority of resulting $\gamma\delta$ lines also responded to PPD or mycobacterial Hsp 65 (82). The major human V γ 2V δ 2 $\gamma\delta$ T cell antigens present in mycobacterial extracts were initially found to reside in a low molecular weight fraction (2-10 kD) and were shown to be protease resistant and phosphatase sensitive (83, 84). The chemical nature of some of these novel antigens has been recently described. In one case, a γ derivative of thymidine triphosphate was shown to stimulate V γ 2V δ 2 $\gamma\delta$ T cells (85), however, the chemical nature of this compound was not determined. Another study showed that V γ 2V δ 2 $\gamma\delta$ cells respond to synthetic monoalkyl phosphates such as monoethyl phosphate (86). Subsequently, isopentenyl pyrophosphate and related prenyl phosphate derivatives were purified from *Mycobacterium tuberculosis*, and shown to be potent antigens for V γ 2V δ 2 $\gamma\delta$ T cells (87). Recently the requirement for presentation of these antigens was analyzed, and it was found that a non-conventional extracellular presentation pathway that does not require antigen uptake, antigen processing or expression of classical MHC molecules is necessary for the $\gamma\delta$ response (88).

In mice, the reactivity of $\gamma\delta$ T cells to mycobacterial antigens is not as well established. Initially it was reported that CD4⁻CD8⁻ T cells accumulate in the draining lymph nodes of mice immunized with killed mycobacteria (89). These cells were not directly shown to be $\gamma\delta$ T cells, and since that report, even with the availability of anti- $\gamma\delta$ TCR mAbs, it has not been possible to directly show accumulation of $\gamma\delta$ cells in mice following exposure to mycobacterial antigens. In another study, a panel of hybridomas obtained by fusing thymocytes from newborn mice or splenocytes from adult mice with BW5147 cells were shown to produce IL-2 in response to mycobacterial purified protein derivative (PPD) (68, 90). Many of these hybridomas also responded to spleen cells incubated with recombinant mycobacterial Hsp 65 or peptide 180-196 from mycobacterial Hsp 65 (68, 91). A lesser response to the corresponding peptide from murine Hsp 63 was also observed (91). These hybridomas were primarily of the V γ 1V δ 6 class and exhibited extensive junctional diversity (92). One problem with these studies was the high background of constitutive IL-2 secretion by the hybridomas. Specific responses to antigens could only be

observed under certain conditions, rendering interpretation of these experiments questionable.

In one study, $\gamma\delta$ resident pulmonary lymphocytes were shown to be activated by aerosolized PPD (93, 94), and another report showed that V γ 5V δ 1 dendritic epidermal cells respond to heat stressed keratinocytes (95), indicating possible recognition of heat shock proteins. In general, several lines of evidence have suggested recognition of mycobacterial and endogenous Hsps by murine $\gamma\delta$ cells, however this response to mycobacterial antigens is not as clearly delineated as that of the human V γ 2V δ 2 cells to low molecular weight antigens.

Superantigens

Superantigens were first identified for $\alpha\beta$ cells as antigens that stimulate all $\alpha\beta$ cells expressing particular V β chains regardless of α chain use and junctional sequence. Such antigens are presented by MHC class II proteins and bind to non-diverse elements of certain V β chains and to a non-polymorphic region of the class II molecule. Staphylococcal enterotoxin A (SEA) is a superantigen for $\alpha\beta$ cells that has also been shown to be a superantigen for V γ 2 cells (96, 97). In this case, the SEA is also presented by class II molecules and binds to a non-diverse region of the V γ 2 chain.

Many observed $\gamma\delta$ responses to such antigens as microbial extracts, heat shock proteins and tumor cells, involve $\gamma\delta$ subsets with particular δ and /or γ chains and extensive TCR junctional diversity, suggesting that these responses may be determined by superantigens (70). Hopefully, with the identification of presenting molecules and the further determination of the structure of antigenic components and their interactions with the $\gamma\delta$ TCR and presenting molecules, this issue will be resolved.

Interaction with antigen

In addition to the above examples, many instances of $\gamma\delta$ stimulation by viral, bacterial, parasitic and tumor cell antigens have been described. Certainly $\gamma\delta$ cells have the capacity to recognize a broad spectrum of antigens which can differ dramatically in chemical nature, including peptides, low

molecular weight phosphorylated compounds and superantigens. It is quite likely that antigen recognition by $\gamma\delta$ cells is fundamentally different from that of $\alpha\beta$ cells. Indeed recent comparison of the CDR3 structures of the known antigen receptors concluded that the $\gamma\delta$ receptor is more similar in this respect to immunoglobulins than to the $\alpha\beta$ TCR (98). This raises the possibility that in general $\gamma\delta$ cells recognize unprocessed, conformationally dependent antigens without the requirement for conventional presentation. $\gamma\delta$ cells could therefore represent a class of cells whose antigen recognition is similar to B cells and whose response is more aligned with a cellular immune response.

$\gamma\delta$ T cell function

Potential immune functions

$\gamma\delta$ T cells share a number of features with $\alpha\beta$ T cells that imply various potential roles for $\gamma\delta$ cells in immune responses. First, $\gamma\delta$ cells have been shown to have cytolytic activity (99-101), and can lyse target cells using the same cytolytic molecules, perforin and serine esterases 1 and 2, as other cytolytic cells (102-104). Indeed, $\gamma\delta$ i-IELs from some strains have been shown to have constitutive cytolytic activity (105-107). In addition, some $\gamma\delta$ cells have been shown to express the Fc receptors expressed by NK cells that mediate antibody based cellular cytotoxicity (108, 109). Thus $\gamma\delta$ cells may have the capacity to kill infected or stressed host cells, and to kill invading pathogens.

Second, $\gamma\delta$ T cells have been shown to secrete a variety of lymphokines (reviewed in (70)), and recently one report described a parallel Th1/Th2 dichotomy in $CD4^+$ $\alpha\beta$ cells and $\gamma\delta$ cells. In this study, the response of $\gamma\delta$ cells to the two pathogens, *L. monocytogenes* and *Nippostrongylus brasiliensis* was analyzed. *L. monocytogenes* has previously been shown to elicit an $\alpha\beta$ Th1 response typified by secretion of IL-2 and IFN- γ , while *N. brasiliensis* has been shown to induce an $\alpha\beta$ Th2 type response characterized by secretion of IL-4, IL-5 and IL-10. Priming with *L. monocytogenes* produced $\gamma\delta$ T cells that secreted the Th1 lymphokine, IFN- γ , while priming with *N. brasiliensis* induced $\gamma\delta$ cells that secrete the Th2 cytokine, IL-4. The peak response of the $\gamma\delta$ cells occurred prior to the $\alpha\beta$ response, suggesting that the $\gamma\delta$ cells are

involved in the primary immune reaction and could have an incipient role in determining the Th1/Th2 outcome of the response (110).

Two other studies suggested a regulatory role for $\gamma\delta$ cells in determining the immune response. In one study, T cells from TCR $\gamma\delta$ knock out mice were shown to be deficient for antigen induced IFN- γ production following infection with *M. bovis* BCG (111). It was concluded that interactions between $\gamma\delta$ and $\alpha\beta$ T cells are essential for IFN- γ production in response to that antigen. In the other study, regulation of IgE responses following exposure to inhaled antigen was shown to depend on antigen dependent CD8⁺ $\gamma\delta$ T cells (112). In this case suppression of the IgE component of the anti-OVA response following inhalation of OVA could be induced in naive recipients by transfer of as few as 5×10^2 $\gamma\delta$ cells. These "suppressor cells" were shown to produce INF- γ , and it was suggested that they function by regulating the response of CD4⁺ $\alpha\beta$ T cells. Since $\gamma\delta$ cells are present in such low numbers in the peripheral lymphoid organs of mice and man, it is an attractive idea that they have a regulatory role in the immune response, rather than functioning directly as effector cells.

$\gamma\delta$ cells and infectious disease

The finding that $\gamma\delta$ cells are present in a variety of epithelial tissues prompted the theory that $\gamma\delta$ cells act as a first line of defense against invading pathogens. One indication that $\gamma\delta$ T cells function in the immune response to pathogens is the increased numbers of $\gamma\delta$ cells that are frequently observed following infection (reviewed in (70, 113, 114)). In one early study, accumulation of $\gamma\delta$ T cells was found in the lesions of leprosy and leishmaniasis patients (81). Subsequently, increases in $\gamma\delta$ T cell numbers have been observed in rodents and humans following exposure to a variety of infectious agents including bacteria, viruses and parasites. One report described expansion of V γ 2 cells in the blood of humans infected with *Salmonella* (115). Similar increases in the numbers of $\gamma\delta$ T cells have been observed in patients infected with Epstein Barr virus (116), human immunodeficiency virus (117, 118), and in patients with *Plasmodium falciparum* (119) and Schistosomiasis (120) parasitic infections. Increases in $\gamma\delta$ T cell numbers at the site of infection have been observed in rodents

following i.p. infection with *Salmonella* (121) and *L. monocytogenes* (122, 123) or following intranasal infection with influenza virus (124). Increases in $\gamma\delta$ T cell numbers have also been observed in mice infected with the parasites *Trypanosoma cruzi* and *Plasmodium chabaudi*.

More direct evidence for the function of $\gamma\delta$ cells in response to infectious agents was achieved through experiments with mice depleted of $\gamma\delta$ T cells. Mice depleted of $\gamma\delta$ T cells following administration of anti- $\gamma\delta$ TCR mAb cleared *Listeria* from spleen and liver more slowly than untreated controls (122, 125, 126). In two additional studies, $\gamma\delta$ depleted mice infected with *Leishmania major* were shown to have larger lesions than non-depleted controls (127) and $\gamma\delta$ depleted mice were shown to be more susceptible to lethal *Salmonella* infection than $\gamma\delta^+$ controls (113). Experiments with $\gamma\delta$ knock out mice also provided evidence for the role of $\gamma\delta$ cells in the immune response against pathogens. In one study, $\gamma\delta$ deficient mice were shown to be unable to form granulomas in the liver in response to infection with *Listeria*. (128) Instead, these mice tended to form liver abscesses. Granuloma formation is part of the normal response that controls *Listeria* infection, therefore, this study suggested that $\gamma\delta$ cells are essential for the generation of certain immune responses. Although the precise functional role of $\gamma\delta$ cells in the immune responses to pathogens has not been defined, collective evidence from infected patients and mice exposed to a variety of infectious agents suggests the involvement of $\gamma\delta$ cells in the immune response against infectious diseases.

$\gamma\delta$ cells and cancer

Various lines of evidence suggest that $\gamma\delta$ T cells may perform a surveillance function against tumors. In mice $\gamma\delta$ cells were shown to proliferate in response to B cell lymphomas (129). More recently, protection against T cell leukemias was observed in mice transgenic for a $V\gamma 1J\gamma 4C\gamma 4$ cDNA (130). In humans $\gamma\delta$ cells derived from children with Burkitt's lymphoma have been shown to lyse autologous tumor cells, and an interaction was observed between the $\gamma\delta$ TCR and the surface Ig of the tumor (131). In addition, there are numerous reports of $V\gamma 2V\delta 2$ T cells responding to Daudi Burkitt's lymphoma cells (132-134). $\gamma\delta$ cells with autologous tumor

cell lytic activity have been isolated from patients with Burkitt's lymphoma or acute lymphoblastic leukemia in complete remission (135). Furthermore, $\gamma\delta$ T cells have been found among tumor infiltrating lymphocyte populations in lung carcinoma, renal carcinoma, Wilm's tumor, melanoma and sarcoma (136-138). In the cases of the lung carcinoma and renal carcinoma, the infiltrating $\gamma\delta$ cells were shown to have autologous tumor cell lytic activity, however, not all tumor infiltrating $\gamma\delta$ cells have been found to have specific lytic activity. Further study is required to establish that $\gamma\delta$ cells play a protective role against tumors.

Development of intestinal epithelium

As discussed above, $\gamma\delta$ cells are present in a number of epithelial tissues, however their function at these sites has not been determined. One recent study addressed the role of $\gamma\delta$ i-IELs by comparing the intestinal epithelium in TCR δ deficient and TCR β deficient mice (139). The $\gamma\delta$ deficient mice were found to have reduced generation of crypt cells, a reduction in the ability of crypt cells to migrate to the ends of villi, and a downregulation of MHC class II expression on intestinal epithelial cells. These effects were not observed in $\alpha\beta$ deficient mice. This work suggests that $\gamma\delta$ i-IELs have a regulatory role in the generation and differentiation of intestinal epithelial cells.

Conclusion

Despite the large amount of knowledge about $\gamma\delta$ T cells that has been generated since the discovery of the γ gene, a clear understanding of their function has remained elusive. This may be partly due to the fact that, so far, many of the lines of investigation into the function of $\gamma\delta$ cells have been biased by already established notions of how the immune system works. While the inability to discover an essential function for $\gamma\delta$ cells, and the lack of a dramatic phenotype in mice deficient for the TCR δ gene may be viewed as discouraging, the evolutionary conservation of $\gamma\delta$ T cells argues for their function. Hopefully, such work as the recent characterization of the novel low molecular weight antigens for human V γ 2V δ 2 $\gamma\delta$ T cells will lead to identification of unique functions of this class of T cells.

Table of murine γ gene nomenclatures

1	C γ 1 locus				C γ 3 locus			C γ 2 locus		C γ 4 locus					
1	V γ 7	V γ 4	V γ 6	V γ 5	J γ 1	C γ 1	V γ 3	J γ 3	C γ 3	C γ 2	J γ 2	V γ 2	V γ 1	J γ 4	C γ 4
2		V γ 2	V γ 4	V γ 3	J γ 1	C γ 1	V γ 1.3	J γ 3	C γ 3	C γ 2	J γ 2	V γ 1.2	V γ 1.1		
3		V γ 6	V γ 5	V γ 4		C γ 2	V γ 3		C γ 3	C γ 1		V γ 2	V γ 1		C γ 4
4	V γ 4.4	V γ 4.3	V γ 4.2	V γ 4.1	J γ 4	C γ 4	V γ 3	J γ 3	C γ 3	C γ 1	J γ 1	V γ 1	V γ 2	J γ 2	C γ 2

1. Order of identification
2. Garman et al.
3. Iwamoto et al.
4. Pelkonen et al.

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Chapter 2: Generation and characterization of $\gamma\delta$ T cell hybridomas.

Abstract

In order to study the reactivity of murine $\gamma\delta$ T cells to mycobacterial antigens, we have generated a panel of $\gamma\delta$ T cell hybridomas following stimulation of primary cells with PPD *in vitro*. $\gamma\delta$ expressing hybridomas were screened for the ability to produce IL-2 upon non-specific crosslinking of the CD3/TCR complex, and 21 lines satisfying this requirement were further analyzed. The majority of these lines expressed TCRs composed of V γ 1 and V δ 6 chains. When these hybridomas were tested for reactivity to PPD, we observed that the cells secrete high background levels of IL-2 spontaneously. The constitutive activation of the hybridomas complicates analysis of their antigenic specificity.

Introduction

$\gamma\delta$ T cells comprise a minor subset of total T cells in mice and man. The low abundance of $\gamma\delta$ T cells has impeded attempts to understand their function, and the antigenic specificity of $\gamma\delta$ T cells remains largely unknown. A considerable effort has been applied to examine the reactivity of $\gamma\delta$ T cells to mycobacterial antigens. Initially, in the case of human $\gamma\delta$ T cells, several $\gamma\delta$ T cell lines were shown to respond to mycobacterial heat shock proteins (1-3). Subsequently, it became apparent that $\gamma\delta$ T cells isolated from healthy individuals respond to heat killed mycobacteria and mycobacterial extracts, and that mycobacterial heat shock proteins were not the primary stimulating components (4). The major human $\gamma\delta$ T cell stimulating components of mycobacterial extracts were initially shown to reside in a low molecular weight fraction (2-10 kD) and were found to be protease resistant (5, 6). More recently, the chemical nature of these antigens has been described (7, 8). The human $\gamma\delta$ cells that respond to these mycobacterial antigens are all of the V γ 2, V δ 2 subset.

In mice, the response of $\gamma\delta$ T cells to mycobacterial antigens is less clearly defined. Initially, CD4⁻CD8⁻ T cells were shown to accumulate in the draining lymph nodes of mice following immunization with killed mycobacteria (9), however these cells were not directly shown to be $\gamma\delta$ cells. In another study, a series of hybridomas obtained by fusing thymocytes from newborn mice or splenocytes from adult mice with BW5147 thymoma cells were shown to produce IL-2 in response to mycobacterial purified protein derivative (PPD) (10). Many of these hybridomas also responded to spleen cells incubated with recombinant mycobacterial Hsp 65 or peptide 180-196 from mycobacterial Hsp 65, and to a lesser degree, to spleen cells incubated with the corresponding peptide from murine Hsp 63 (11). These hybridomas were all of the V γ 1, V δ 6 class and exhibited extensive junctional diversity (12) (13). This study was complicated by the fact that these hybridomas secreted high background levels of IL-2 spontaneously, and specific response to mycobacterial antigens could only be observed under certain conditions. Therefore the interpretation of these results is questionable.

To more directly address the issue of whether murine $\gamma\delta$ T cells respond to mycobacterial antigens, we have generated a collection of $\gamma\delta$ T cell hybridomas following stimulation of purified $\gamma\delta$ T cells with PPD in vitro. A subset of these hybridomas that produced IL-2 upon non-specific CD3/TCR stimulation was characterized. The majority of these hybridomas were shown to be of the V γ 1, V δ 6 type. As in the previous study, the junctional sequences of these hybridomas were diverse and the hybridomas secreted high background levels of IL-2 spontaneously, rendering analysis of their antigenic specificity difficult.

Materials and Methods

Mice: C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME)

Antibodies: mAbs used were H57-597, anti-C β (14) ; GK1.5, anti-CD4 (15); 53-6.7, anti-CD8 (16); RA3.6B2, anti-CD45R/B220 (17); M1/70 and anti-Mac-1 (18). All of these mAbs were purified from culture supernatant either by ion-exchange chromatography on DEAE-cellulose or by affinity chromatography on protein A-sepharose (Pharmacia, Uppsala, Sweden), and biotinylated by standard procedures. Biotin labeled Goat anti-mouse IgM was purchased from Caltag Lab. (San Francisco, CA); PE-labeled anti-CD3 ϵ , FITC labeled anti- $\gamma\delta$ TCR, purified anti- $\gamma\delta$ TCR and purified anti-CD3 ϵ were purchased from Pharmingen (San Diego, CA).

Immunofluorescence staining and flow cytometric analysis: Cells (10^5 - 10^6) were incubated in staining buffer (PBS, 2%FCS, 0.1%NaN₃) with the indicated labeled Abs for 40 min at 4 $^{\circ}$ C. After two washes, 10,000 viable cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). Dead cells were gated out by their staining with propidium iodide.

Cell purifications and cultures: The preparation of $\gamma\delta$ T cell blasts used to produce $\gamma\delta$ T-cell hybridomas was as follows: Thymus and spleen cell suspensions were prepared according to standard procedures. Thymocytes were incubated with biotinylated anti-CD4 and anti-CD8 mAbs and splenocytes were incubated with biotinylated anti-TCR β , anti-CD4, anti-CD8, anti-B220 and anti-MAC-1 mAbs for 35 minutes on ice. After washing the unbound Abs, the cells were incubated with Tosyl-activated magnetic beads (Dynal Inc., Great Neck, NY) that had been previously coupled to streptavidin following the manufacturer's instructions. The incubation was performed for 60 min. at 4 $^{\circ}$ C with a slight rocking of the tubes. After separation of the beads, unbound cells were cultured in complete medium (DME supplemented with L-glutamine, sodium pyruvate, β -mercaptoethanol, non essential amino acids, penicillin and streptomycin, plus 10% fetal bovine serum) together with irradiated (1000 rads) B6 spleen cells (2×10^6 /ml) in the presence of 50 μ g/ml of PPD and 10U/ml of rIL-2.

Production of T-cell hybridomas: 3-5 days after initiation of the cultures described above, growing blasts were fused to the TCR $\alpha\beta^-$ variant of the BW5147 thymoma cell line (19) at a 1:1 ratio in 0.5 ml of 50% polyethylene glycol as described. The cells were then distributed in 96 well flat-bottomed plates with either HAT or AH medium. The hybridomas named T and S come from PPD stimulated thymocytes and splenocytes respectively.

PCR and sequencing analysis: The following primers were used.

V γ 1_{ext}: 5'-CCGGCAAAAAGCAAAAAGT-3'

J γ 4_{ext}: 5'-GCAAATATCTTGACCCATGA-3'

V γ 1_{int}: 5'-TCTACCTCAACCTTGAA-3'

J γ 4_{int}: 5'-GGCAAATATCTTGACCC-3'

V δ 6_{ext}: 5'-TCAAGTCCATCAGCCTTGTC-3'

J δ 1_{ext}: 5'-CCAAGTGACTGTGGAACCAA-3'

V δ 6_{int}: 5'ACAGCCAGAGGATTCAG-3'

J δ 6_{int}: 5'-TCGTCTTTGGACAGGA-3'

Genomic DNA from hybridoma cells (T and S) was prepared by the Proteinase K/Phenol extraction/ethanol precipitation method. Sequences spanning the V γ 1-J γ 4 and V δ 6-J δ 1 junctions were amplified by PCR using the V γ 1_{ext}, J γ 4_{ext} and V δ 6_{ext}, J δ 1_{ext} primer pairs respectively. For direct sequencing of the amplified hybridoma junctions, two PCRs were performed in parallel, each one containing either one of the primers kinased. To produce single-stranded DNA after PCR amplification, the double-stranded product was digested with λ exonuclease as originally described (20). Both strands were then sequenced by the dideoxy chain termination method using the Sequenase enzyme (USB, Cleveland, OH) and the appropriate internal primers. For hybridomas that contained more than one rearranged sequence for either of the loci, PCR products were band isolated, cloned into the pUC12 vector and then sequenced by the dideoxy chain termination method using the universal and reverse primers.

IL-2 assays: Hybridoma cells were cultured in flat-bottomed 96-well plates (100,000/well) either alone, with 50 μ g/ml PPD, with 2×10^5 mitomycin-C treated B6 spleen cells, with 50 μ g/ml PPD and 2×10^5 mitomycin-C treated B6 spleen cells or in wells coated with the anti- $\gamma\delta$ mAb, GL4. IL-2 production was assayed after 18-24 hrs by the growth of HT-2 cells, scored by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) assay as

originally described (21). Absolute values of IL-2 were determined by comparing sample values to a standard curve derived from parallel quantification of fixed amounts of recombinant IL-2 (Gibco BRL, Gaithersburg, MD). To assay for inhibition of IL-2 secretion, hybridoma cells were cultured for 18-24 hrs in the presence of the indicated amounts of soluble anti-CD3 ϵ (2C11) or anti- $\gamma\delta$ TCR (GL4), and then the culture supernatants were assayed for IL-2 content as described above.

Results

Generation of $\gamma\delta$ T cell hybridomas

$\gamma\delta$ T cells from spleen and thymus of adult B6 mice were purified by antibody mediated depletion of $\alpha\beta$ T cells, B cells and macrophages. The remaining cells were cultured for 4 days in the presence of 50 $\mu\text{g/ml}$ PPD, 10 U/ml recombinant human IL-2 and irradiated syngeneic spleen cells, and then fused with $\alpha\beta^-$ BW5147 cells. Resulting hybridomas were analyzed for surface expression of the $\gamma\delta$ TCR and then subcloned. The subcloned hybridomas expressing the $\gamma\delta$ TCR were screened for the ability to secrete IL-2 when activated via crosslinking of the CD3/TCR complex with the 2C11 antibody. A group of 21 $\gamma\delta$ hybridomas satisfying this requirement was selected for further study. Staining analysis of fifteen of these hybridomas with monoclonal antibodies (mAbs) against the CD3 ϵ chain and the TCR δ chain is shown in Fig. 1. Some of the hybridomas exhibit unstable expression of the CD3/TCR complex as indicated by the presence of CD3 ϵ^- , TCR δ^- subpopulations in some lines, however, with repeated subcloning it was possible to maintain each hybridoma with the majority of cells positive for $\gamma\delta$ TCR surface expression.

PCR and sequencing analysis

Since the hybridomas were generated following stimulation with PPD, it was of interest to determine whether exposure to this antigen preparation prior to fusion yielded hybridomas that express specific γ and δ chains. Genomic DNA samples from the hybridomas were first screened by PCR for rearrangements of the V γ 1-J γ 4 and V δ 6-J δ 1 loci, as previous reports indicated that V γ 1, V δ 6 hybridomas respond to PPD and mycobacterial hsp 65 (10, 11, 13). 21 of 21 hybridomas yielded bands corresponding to V γ 1-J γ 4 rearrangement, and 17 of the 21 hybridomas yielded bands corresponding to V δ 6-J δ 1 rearrangement (data not shown). Since V δ 6 alleles comprise a gene family, and it is not certain that the particular V δ 6 primer utilized amplifies all members of the family, it is possible that the remaining 4 hybridomas could contain undetected V δ 6 rearrangements.

To determine whether the rearranged V γ 1-J γ 4 and V δ 6-J δ 1 alleles detected by PCR could encode functional TCR chains, the PCR products were sequenced and the junctional sequences were analyzed. Each of the 21 hybridomas contained an in frame rearranged V γ 1-J γ 4 sequence, and each of the 17 hybridomas that were positive for V δ 6-J δ 1 rearrangement contained an in frame rearranged V δ 6-J δ 1 sequence, as shown in tables 1 and 2 respectively. The V γ 1-J γ 4 and V δ 6-J δ 1 sequences exhibited extensive junctional diversity, resulting from both exonucleolytic cleavage and addition of N nucleotides. This extensive diversity is maintained upon translation of the nucleotide sequences into amino acid sequences for both the V γ 1-J γ 4 and V δ 6-J δ 6 junctions. Thus the hybridomas generated following stimulation in vitro with PPD display considerable homogeneity in their expression of V γ 1 and V δ 6 chains, and extensive diversity in the junctional sequences of both the γ and δ chains.

Spontaneous reactivity of V γ 1, V δ 6 hybridomas

To analyze potential reactivity to PPD, the hybridomas were cultured under several conditions and analyzed for IL-2 secretion. Fig. 2 shows the results of one experiment in which 12 of the hybridomas were cultured alone (1), with 50 μ g/ml PPD (2), with mitomycin C-treated B6 spleen cells (3), with 50 μ g/ml PPD and mitomycin C-treated B6 spleen cells (4), and in wells coated with the anti- $\gamma\delta$ TCR mAb, GL4 (5). In general, hybridomas that produced IL-2 upon non-specific stimulation also produced IL-2 spontaneously. This background level of IL-2 production constituted a significant proportion of the maximal level of IL-2 production induced by non-specific crosslinking of the TCR with mAbs. In some cases, modest increases in IL-2 production were observed upon addition of PPD, however these increased values were generally within two-fold of the background values. Absolute amounts of IL-2 secretion varied among experiments depending on the state of the cells, and possibly on the batch of PPD. However, the general trends of the experiment, including the high background of spontaneous IL-2 secretion, and the lack of a significant increase in IL-2 secretion in response to addition of PPD were consistently observed. The high degree of spontaneous IL-2 production by the hybridomas renders it difficult to study their response to antigens.

Inhibition of spontaneous IL-2 secretion by anti CD3/TCR mAbs

To determine whether the spontaneous IL-2 secretion is dependent on an interaction involving the $\gamma\delta$ TCR, hybridoma cells were cultured in the presence of soluble anti-CD3 and anti- $\gamma\delta$ mAbs, and culture supernatants were analyzed for IL-2 concentration. Fig. 3A shows the results of an experiment in which four of the hybridomas were cultured in the presence of a series of 3-fold dilutions of the anti-CD3 ϵ mAb, 2C11. IL-2 secretion was effectively blocked by addition of 2C11 in a dose-dependent manner. This inhibition by soluble 2C11 was observed for all hybridomas tested. IL-2 secretion was also inhibited by addition of anti- $\gamma\delta$ TCR mAbs in a dose-dependent manner as shown in Fig. 3B. However, anti- $\gamma\delta$ mAbs were not as effective as 2C11 in blocking IL-2 secretion, as some hybridomas still produced significant amounts of IL-2 even in the presence of high concentrations of anti $\gamma\delta$ -mAb, for example, S3.61.2, in Fig. 3B. The inhibition of IL-2 secretion by the anti-CD3/TCR mAbs suggests that this spontaneous response involves activation via the $\gamma\delta$ TCR complex, and that it could depend on an interaction between the $\gamma\delta$ TCR and other molecules expressed on the cell surface and/or soluble antigens present in the cultures.

Discussion

We have generated a panel of $\gamma\delta$ T cell hybridomas following stimulation of purified murine $\gamma\delta$ T cells in vitro with PPD. The hybridomas were screened for the ability to secrete IL-2 upon non-specific stimulation of the CD3/TCR complex, and a group of 21 hybridomas fulfilling this requirement was chosen for further study.

21/21 hybridomas contained an in frame V γ 1-J γ 4 rearrangement and at least 17/21 contained an in frame V δ 6-J δ 1 rearrangement. The junctional sequences of the V γ 1 and V δ 6 genes exhibited extensive diversity, and this diversity is maintained upon translation of the nucleotide sequence into amino acid sequence.

When the $\gamma\delta$ hybridomas were tested for reactivity to PPD, we observed that the cells secrete significant amounts of IL-2 when cultured alone, however, only modest increases in IL-2 production were detected in the presence of PPD. Spontaneous IL-2 secretion could be inhibited by the addition of antibodies against the CD3/TCR complex to the cultures, suggesting that IL-2 secretion is dependent on an interaction involving the $\gamma\delta$ TCR. The high background level of IL-2 secretion by these cells renders it difficult to study their potential reactivity to mycobacterial antigens.

It is interesting that the procedure utilized to select these hybridomas generated a group of hybridomas with such uniform expression of the V γ 1, V δ 6 TCR. The only two restrictions applied in producing these cell lines were the stimulation with PPD prior to fusion and the stipulation that the hybridomas be capable of secreting IL-2. As V γ 4 cells comprise roughly 50% of the $\gamma\delta$ cells in the adult spleen and thymus (22, 23), and V γ 4 cells have previously been shown to secrete IL-2 (24), it is likely that the high proportion of V γ 1, V δ 6 cells among the hybridomas reflects the specific activation of cells of this type prior to fusion. In this regard it is noteworthy that previous $\gamma\delta$ hybridomas generated from newborn thymocytes and adult splenocytes, that were reported to be reactive to PPD and mycobacterial Hsp 65, were all of the V γ 1, V δ 6 class (10-13).

Due to the high background of IL-2 secretion by the hybridomas, we could not directly test whether these cells respond to mycobacterial antigens, therefore the nature of the stimulation prior to fusion is open to speculation.

It is possible that the specific generation of V γ 1, V δ 6 hybridomas was independent of the addition of PPD prior to fusion and was merely a consequence of constitutive activation of primary V γ 1, V δ 6 cells in culture. It is also possible that the specific generation of V γ 1, V δ 6 hybridomas reflects direct or indirect stimulation of V γ 1, V δ 6 cells by antigens present in the PPD. The predominant expression of the V γ 1, V δ 6 TCR among the hybridomas is compatible with the presence of a mycobacterial superantigen for V γ 1, V δ 6 cells. However, the extensive junctional diversity exhibited by the hybridoma TCRs is also compatible with their recognizing diverse antigens.

The constitutive IL-2 secretion of the V γ 1, V δ 6 hybridomas raises several interesting issues. It is possible that this spontaneous reactivity is an innate feature of V γ 1, V δ 6 cells, and that even primary cells of this type are autoreactive. It is also possible that the spontaneous hybridoma reactivity is a consequence of the fusion. The hybridomas were generated by fusing $\gamma\delta$ cells from B6 mice with BW5147 cells derived from AKR mice. Thus the hybridoma cells may express allo-antigens from the AKR background or tumor antigens that could be recognized by the V γ 1, V δ 6 TCR. In this regard, it is interesting that human V γ 2, V δ 2 cells which respond to mycobacterial antigens have also been shown to respond to antigens expressed on tumor cells (25).

We have taken two approaches to further examine the reactivity of V γ 1, V δ 6 T-cells. The first was to generate monoclonal antibodies that block spontaneous IL-2 secretion by the hybridomas. By this means we hoped to identify cell surface molecules involved in the response. The second was to generate mice transgenic for the rearranged V γ 1 and V δ 6 genes from one of the hybridomas. Such mice should have increased numbers of V γ 1, V δ 6 cells that would allow examination of primary cells of this type, particularly with regard to spontaneous and mycobacterial reactivity. Chapters 3 and 4 of this thesis present the characterization of anti -V γ 1 and anti -integrin mAbs respectively, that were isolated by their ability to block IL-2 secretion of the V γ 1, V δ 6 hybridomas, and chapter 5 presents the characterization of mice transgenic for rearranged V γ 1 and V δ 6 genes.

Figure 1. Expression of the $\gamma\delta$ TCR on subcloned hybridomas.

Fifteen of the hybridomas were labeled with anti-CD3 ϵ PE (y-axis) and anti- $\gamma\delta$ TCR FITC (x-axis) and analyzed by flow cytometry. The names of the hybridomas are listed above the dot plots.

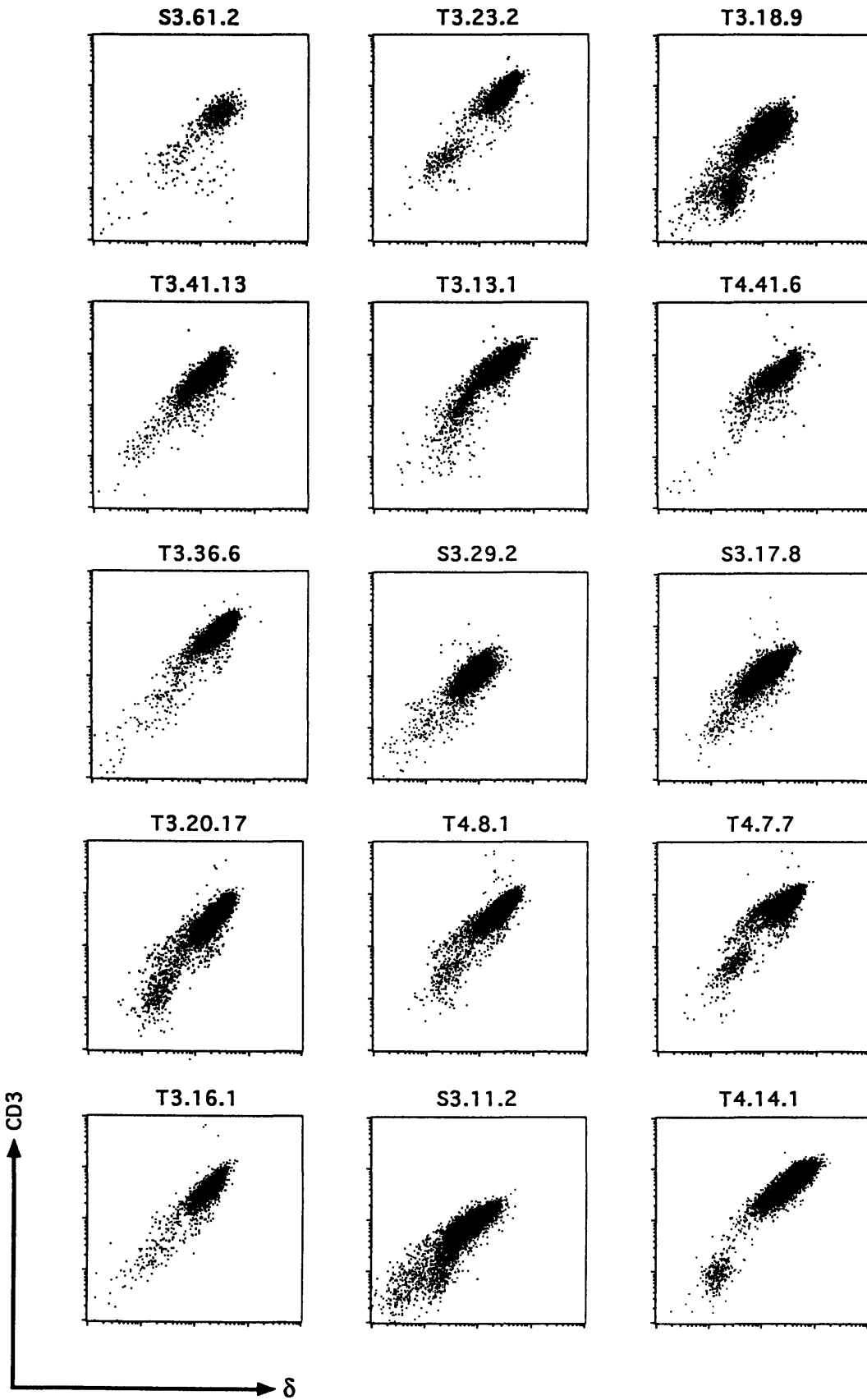


Table 1. Hybridoma V γ 1-J γ 4 junctional sequences.

Genomic DNA from the hybridomas was amplified with V γ 1 and J γ 4 specific primers and sequenced as described in Materials and Methods. The V γ 1 and J γ 4 germline sequences are listed above the hybridoma sequences. P denotes palindromic nucleotides and N denotes nucleotides not present in the germline.

Hybridoma V γ 1-J γ 4 junctional sequences

	V	P	N	P	J
Germline	GTC TGG ATA AA	TT		GA	TCA GGC
T3.13.1	GTC TGG				TCA GGC
T3.23.2	GTC TGG ATA AA		AAGGGG	A	TCA GGC
T3.41.13	GTC TGG ATA		GGA		GCC
T3.16.1	GTC TGG AT		CCG	A	TCA GGC
T3.18.9	GTC TGG AT		CAG		A GGC
T3.20.17	GTC TGG		GGG		GGC
T3.40.3	GTC TGG A		G	A	TCA GGC
T4.7.7	GTC TGG ATA AA		GTCT		GGC
T4.14.1	GTC TGG		CCCC	GA	TCA GGC
T4.36.6	GTC TGG A		CCCCCTTC	A	TCA GGC
T4.41.6	GTC TGG		CCCC	GA	TCA GGC
T4.8.1	GTC TGG		CCG	A	TCA GGC
T4.3.2	GTC TGG		AT		A GGC
T4.2.1	GTC TGG		GGAGGGC		CA GGC
T4.15.3	GTC TGG AT		CAGG		TCA GGC
T4.7.6	GTC TGG AT		TTCGTGG		GGC
S3.11.2	GTC TGG ATA		C		CA GGC
S3.17.8	GTC TGG AT		GCA	A	TCA GGC
S3.29.2	GTC TGG A			GA	TCA GGC
S3.29.3	GTC TGG A			GA	TCA GGC
S3.22.9	GTC TGG				GGC

Table 2. Hybridoma V δ 6-J δ 1 junctional sequences.

Genomic DNA from the hybridomas was amplified with V δ 6 and J δ 1 specific primers and sequenced as described in Materials and Methods. The V δ 6 and J δ 1 germline sequences are listed above the hybridoma sequences. P denotes palindromic nucleotides and N denotes nucleotides not present in the germline.

Hybridoma V86-J81 junctional sequences

V

D AND N

J

Germiline CTC TCG GAA CTG

CT ACC GAC AAA CTC

T3.13.1	CTC TCG GAA CTG	ATAGGAIATGGCCCTATCGGAGGGATACGGG	ACTC
T3.23.2	CTC TCG GAA	CCATGGCTCCGGAGGGATACGAGTT	CC GAC AAA CTC
T3.16.1	CTC TCG GAA CTG	ACTGCCGGGGGCTTACGAGCTCGGG	CC GAC AAA CTC
T3.20.17	CTC TCG GAA CT	TTGGCAIATCGGAGGATCG	CT ACC GAC AAA CTC
T3.40.3	CTC TCG GAA	GGGTGGCATAITCGACGGAGGGATACGAG	AA CTC
T4.7.7	CTC TCG GAA	CCCTATGGCAIATATCGGAGGGATACGAGGGG	C GAC AAA CTC
T4.36.6	CTC TCG G	TATGGGGGGCATAIATAGGCCCCCGGAGGGATACGAG	CT ACC GAC AAA CTC
T4.8.1	CTC TCG GAA CTG	ACTGCCGGGGGCTTACGAGCTCGGG	CC GAC AAA CTC

Germiline CTC TGG GAG CTG

CT ACC GAC AAA CTC

T3.41.13	CTC TGG GAG CT	GATCGGAGGGATAGGCG	CT ACC GAC AAA CTC
T3.18.9	CTC TGG GAG CT	CGATAITCGGAGGGATACGATCCCCAG	CT ACC GAC AAA CTC
T4.14.1	CTC TGG GAG CTG	GGCCGGAGGGATACGAGCT	CC GAC AAA CTC
T4.41.6	CTC TGG GAG CTG	GGCCGGAGGGATACGAGCT	CC GAC AAA CTC
T4.2.1	CTC TGG GAG C	CCCAITATGGCCCAATACGAG	CT ACC GAC AAA CTC
S3.11.2	CTC TGG GAG CTG	GATCGGAGGGATAGGCG	A CTC
S3.17.8	CTC TGG GAG CTG	GCGGGGATATGGCAITATGAAGGGAAATCGGAGGCCATACGAAAGCAT	CC GAC AAA CTC
S3.29.2	CTC TGG GAG CTG	GAGGGAGGGATACGAAAG	C AAA CTC
S3.29.3	CTC TGG GAG CTG	GAGGGAGGGATACGAAAG	C AAA CTC

T4.3.2 -----

T4.15.3 -----

T4.7.6 -----

S3.22.9 -----

Figure 2. Assay of hybridoma reactivity.

12 representative hybridomas were cultured alone (1), with 50 µg/ml PPD (2), with mitomycin-C treated B6 spleen cells (3), with 50µg/ml PPD and mitomycin-C treated B6 spleen cells (4) and in wells coated with anti-γδ TCR mAb (5) and analyzed for IL-2 production as described in Materials and Methods. All hybridomas produced IL-2 spontaneously. In some cases, modest increases in IL-2 production were observed upon addition of PPD, however the high background reactivity impedes analysis and interpretation of antigen response.

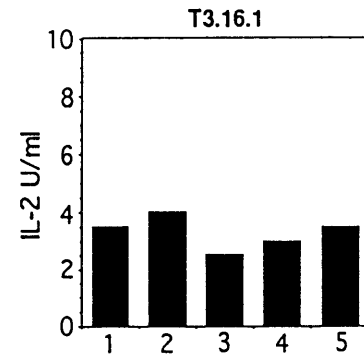
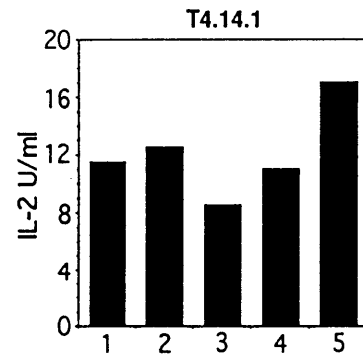
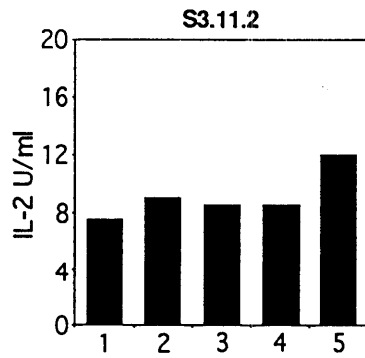
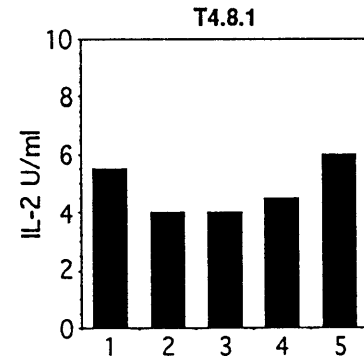
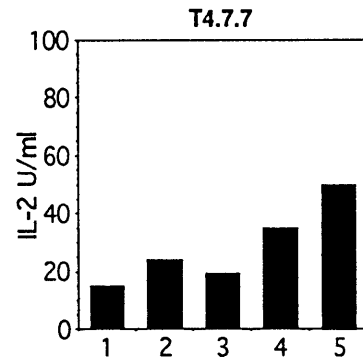
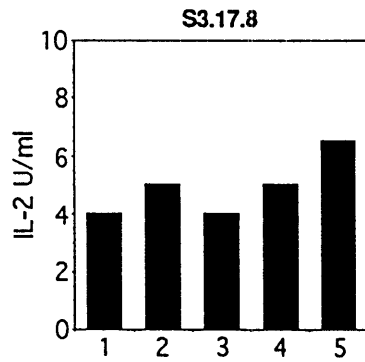
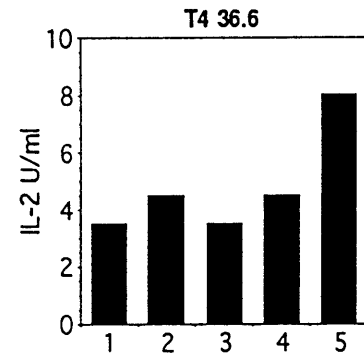
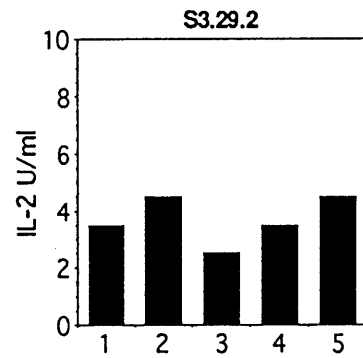
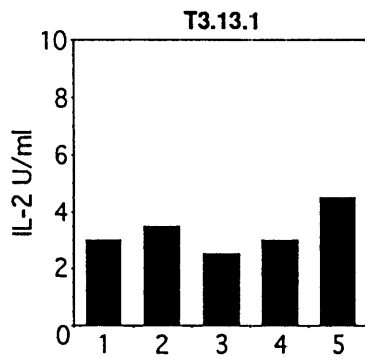
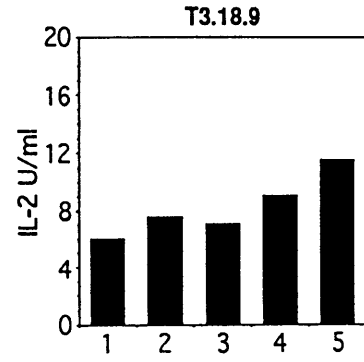
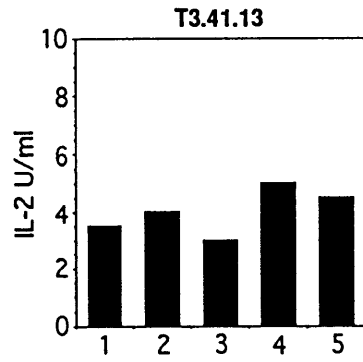
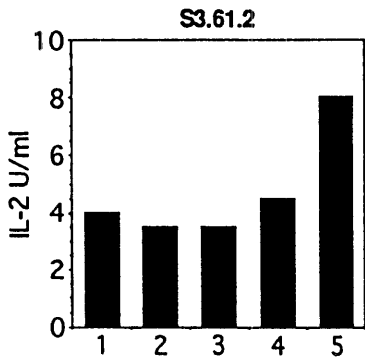
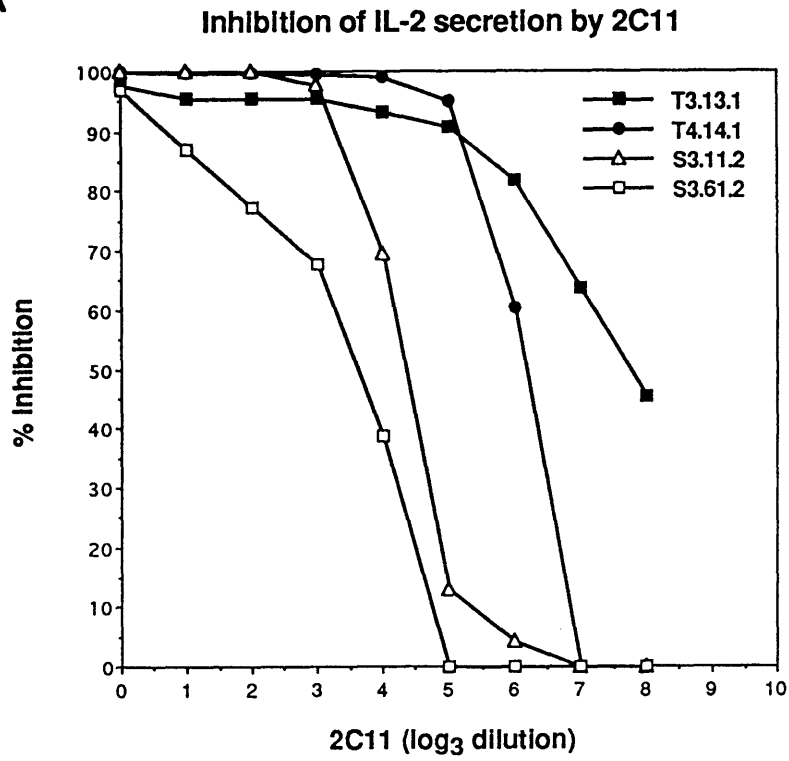
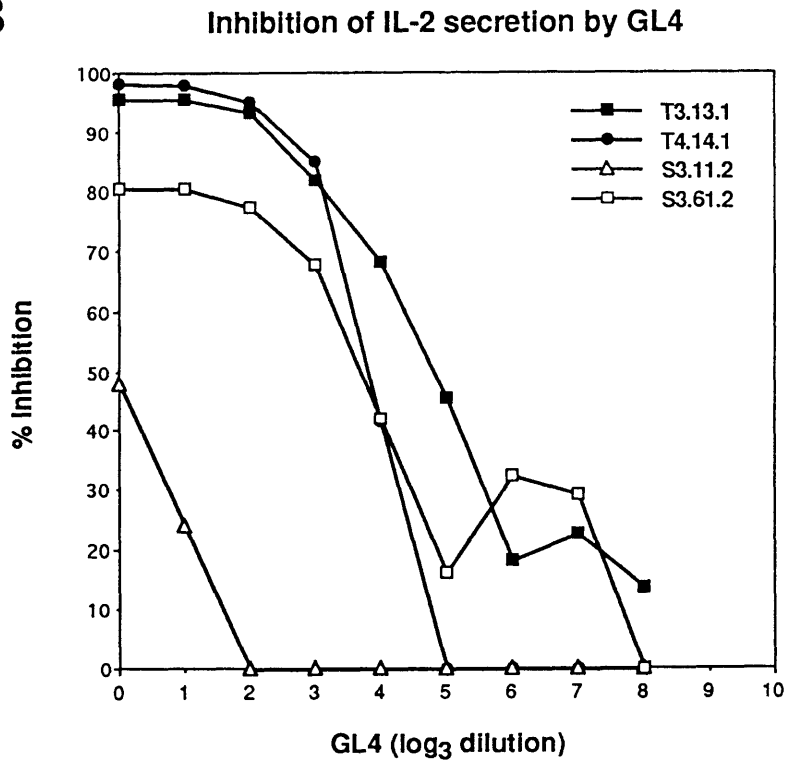


Figure 3. Inhibition of spontaneous IL-2 secretion by addition of anti-CD3/TCR complex mAbs.

Four hybridomas were cultured in the presence of 3-fold dilution series of the anti-CD3 ϵ (A) and anti- $\gamma\delta$ TCR (B) mAbs and analyzed for IL-2 production as described in Materials and Methods. The maximum concentration of each mAb was 5 μ g/ml. IL-2 secretion was blocked by both mAbs in a dose dependent manner. Blocking of IL-2 secretion was a general feature of all hybridomas tested.

A**B**

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Chapter 3: Ontogenic development and tissue distribution of V γ 1-expressing $\gamma\delta$ T lymphocytes in normal mice.

Abstract

A hamster monoclonal antibody (mAb) recognizing an epitope in the V γ 1-J γ 4-C γ 4 chain of the $\gamma\delta$ T cell receptor (TCR) has been generated. Using this mAb we have quantitated the occurrence of V γ 1-bearing $\gamma\delta$ T cells in the developing thymus and in the lymphoid organs and several epithelia of adult mice. The V γ 1 expressing cells constitute a minor $\gamma\delta$ T cell subpopulation during fetal and early post-natal life, but they constitute a major population of $\gamma\delta$ T cells in the thymus and in the peripheral lymphoid organs in adult mice. In addition, we found that V γ 1-bearing cells comprise a large proportion (15-60%) of the $\gamma\delta$ T cells present in the intestinal epithelium (i-IEL) in all strains of mice tested. V γ 1-positive i-IEL are present in athymic (nude) mice and in antigen-free mice, demonstrating that they can develop extrathymically and that their presence in the intestinal epithelium is independent of the antigenic load of the gut. Our results show that V γ 1-bearing lymphocytes account for the largest population of $\gamma\delta$ T cells in the mouse. This population includes a thymus-dependent component that homes to the secondary lymphoid organs and a thymus-independent component that constitutes a major fraction of the $\gamma\delta$ i-IELs.

Introduction

T lymphocytes bearing the $\gamma\delta$ T cell receptor (TCR) constitute a minor T cell subpopulation in the thymus and in the peripheral lymphoid organs, but are often the major T cell type in epithelial tissues (1-9). $\gamma\delta$ T cells comprise several different subsets as defined by such distinctive parameters as TCR repertoire, time of appearance during ontogeny, thymus dependence and anatomical localization (reviewed in (10)). The physiological role of $\gamma\delta$ T cells is still poorly understood, partially due to the lack of knowledge about $\gamma\delta$ T cell ligands.

A large fraction of $\gamma\delta$ T cell hybridomas expressing a TCR composed of $V\gamma 1$ - $J\gamma 4$ - $C\gamma 4$ and $V\delta 6$ - $C\delta$ chains is constitutively activated to secrete interleukin-2 (IL-2) (11). The role of a specific interaction involving the TCR in this activation has been suggested by the ability of mAbs against the TCR/CD3 complex to inhibit the spontaneous IL-2 production and by the fact that transfection of the $V\gamma 1$ / $V\delta 6$ cDNAs into a TCR⁻ murine hybridoma confers the phenotype of constitutive activation onto the recipient cells (11, 12). In some cases, the reactivity of $V\gamma 1$ / $V\delta 6$ TCR expressing hybridomas to purified protein derivative (PPD) and to the 180-196 amino acid region of the mycobacterial heat shock protein HSP 60 has been demonstrated (13), although failures to detect such reactivities have also been reported (14, 15). Constitutively activated hybridomas expressing the $V\gamma 1$ / $V\delta 6$ TCR have been isolated from newborn and adult thymus (11, 14, 16), adult spleen (16), liver (17), skin epidermis (18) and from the intestinal epithelium of weanling mice (15), suggesting that $\gamma\delta$ T cells expressing the $V\gamma 1$ gene product are normally present in different lymphoid organs and epithelia. Nevertheless, neither quantification of $V\gamma 1$ -bearing $\gamma\delta$ T cells in different tissues, nor studies on the

origin of these cells has been performed, mainly due to the lack of mAbs specifically recognizing this population. Here we describe a hamster mAb (named 2.11) specific for the mouse V γ 1-C γ 4 protein and the studies performed to analyze these issues. Our results show that V γ 1-bearing lymphocytes account for the largest population of $\gamma\delta$ T cells in the mouse, including a thymus-dependent component that localizes to the secondary lymphoid organs and a thymus-independent component that comprises a significant fraction of the $\gamma\delta$ intestinal intraepithelial lymphocytes (i-IEL).

Materials and Methods

Animals: C57BL/6 (B6), BALB/c, DBA/2, and C3H/HeJ (C3H) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or from Iffa-Credo (France). BALB.B10 and BALB.C3H mice were purchased from Bomholtgard (Denmark) and bred at the MIT facilities. BALB/c SPF (specific pathogen free) and antigen-free (AgF) were a kind gift of Dr. C. Heusser (Ciba-Geigy, Basel, CH). Athymic (nu/nu) and (nu/+) littermates were purchased from The Jackson Laboratory, Charles River Laboratories Inc. (Wilmington, MA), and Iffa-Credo. All other mouse strains were purchased from The Jackson Laboratory. Adult Armenian hamsters were obtained from Cytogen Research (West Roxbury, MA).

Antibodies: For complement mediated killing we used anti-CD4 (RL.174, ref. (19)) and anti-CD8 (HO 2.2, ref. (20)). Both mAbs were used as culture supernatants. Other mAbs used were H57-597, anti-C β (21); GK1.5, anti-CD4 (22), 53-6.7, anti-CD8 (23), RA3.6B2, anti-CD45R/B220 (24); M1/70 anti-Mac-1 (25) 3A10, anti pan- δ (2) and 536 anti-V γ 5 (26). All of these mAbs were purified from culture supernatant either by ion-exchange chromatography on DEAE-cellulose or by affinity chromatography on protein A-sepharose (Pharmacia, Uppsala, Sweden), and biotinylated by standard procedures. Biotin labeled Goat anti mouse IgM was purchased from Caltag Lab. (San Francisco, CA); biotin-labeled anti-CD5 was from Becton-Dickinson (Mountain View, CA); and FITC-labeled anti-CD8 β , FITC-labeled anti-Thy1, biotin-labeled anti-V δ 4 and biotin-labeled anti-V γ 4 were from PharMingen (San Diego, CA.).

Immunofluorescence staining and flow cytometric analysis: Cells (10^5 - 10^6) were incubated in staining buffer (PBS, 3%FCS, 0.1%NaN₃) with the indicated labeled Abs for 30 min on ice. After two washes the cells were incubated with

streptavidin-PE (Southern Biotechnology, Birmingham, AL) for 15 min on ice. After another two washes, 10,000 viable cells were analyzed using a FACScan flow cytometer (Becton-Dickinson). Dead cells were gated out by their staining with propidium iodide.

Cell purifications and cultures: The preparation of dendritic epidermal cells (DEC) and i-IELs has been previously described in detail (27, 28). The preparation of $\gamma\delta$ T cell blasts used to produce $\gamma\delta$ T-cell hybridomas was as follows: Thymus and spleen cell suspensions were prepared according to standard procedures. Thymocytes were incubated with biotinylated anti-CD4 and anti-CD8 mAbs and splenocytes were incubated with biotinylated anti-TCR β , anti-CD4, anti-CD8, anti-B220 and anti-MAC-1 mAbs for 35 min on ice. After washing the unbound Abs, the cells were incubated with Tosyl-activated magnetic beads (Dynal Inc., Great Neck, NY) that had been previously coupled to streptavidin following the manufacturer's instructions. The incubation was performed for 60 min at 4°C with a slight rocking of the tubes. After separation of the beads, unbound cells were cultured in complete medium (DME supplemented with L-glutamine, sodium pyruvate, β -mercaptoethanol, nonessential amino acids, penicillin and streptomycin, plus 10% fetal bovine serum) together with irradiated (1000 rads) B6 spleen cells (2×10^6 /ml) in the presence of 50 μ g/ml of PPD and 10U/ml of rIL-2.

Alternatively, thymocytes were treated with anti-CD4 and anti-CD8 mAbs and complement for 45 min at 37°C. CD4⁻CD8⁻ alive cells were purified by density gradient centrifugation using Lympholyte M (Cederland Lab. Hornby, ON, Canada) and cultured (5×10^5 /ml) in complete medium plus 10U/ml of rIL-2, in plates previously coated with the 2.11 mAb (20 μ g/ml).

Production of T-cell hybridomas: 3-5 days after initiation of the cultures described above, growing blasts were fused to the TCR $\alpha\beta$ ⁻ variant of the

BW5147 thymoma cell line (29) at a 1:1 ratio in 0.5 ml of 50% polyethylene glycol as described (30). The cells were then distributed in 96 well flat-bottomed plates with either HAT or AH medium. The hybridomas named T and S come from PPD stimulated thymocytes and splenocytes respectively. The hybridomas named BTC come from thymocytes activated with the 2.11 mAb.

Production of the 2.11 mAb: Armenian hamsters were immunized intraperitoneally three times at three week intervals with 2×10^6 - 10^7 irradiated, TCR $\gamma\delta^+$ T3.13.1 hybridoma cells resuspended in saline. The T3.13.1 hybridoma expresses a TCR composed of V γ 1 and V δ 6.3 chains and constitutively secretes IL-2 when cultured in vitro. Three days after the last injection, spleen cells were fused with the murine myeloma SP2/0 at a ratio of 10:1 (spleen cell/myeloma) in 1 ml of 50% PEG as described (2). The cells were then distributed in 96 well flat-bottomed plates with HAT medium. Culture supernatants from growth-positive wells were tested for their ability to inhibit the constitutive IL-2 production of the immunizing hybridoma. IL-2 production was assayed by the growth of HT-2 cells, scored by the MTT assay as originally described (31). Cells from wells inhibiting >75% of the IL-2 secretion were subcloned several times by limiting dilution until stable Ab secreting hybrids were obtained.

Cell surface labeling and immunoprecipitation: 8×10^6 V γ 1-expressing hybridoma cells were harvested by centrifugation, washed four times with PBS and resuspended in four mls of PBS. Cells were split into nine glass test tubes coated with Iodogen (Pierce, Rockford, IL), and 200 μ Ci Na¹²⁵I was added to each tube. After 15 min incubation on ice with occasional mixing, cells were pooled and washed four times with PBS. The cell pellet was lysed in 1.5 ml ice cold lysis buffer (10mM Tris-HCl pH 7.6, 150mM NaCl, 1% Triton X-

100, 1mM PMSF), vortexed for 20s, and incubated on ice for 30 min. The lysate was clarified by centrifugation at 13,000 rpm at 4° in a microfuge. One-tenth volume of normal hamster serum was added to the supernatant followed by two rounds of pre-clearing with one-tenth volume protein-A sepharose beads (Pharmacia). One-quarter of the precleared lysate was immunoprecipitated with 10µl of either 2.11 or 3A10 coated protein A sepharose beads.

Immunoprecipitates were washed, resuspended in reducing sample buffer, and subjected to SDS-PAGE on a 10% gel, according to standard procedures. Radioactive bands were visualized using a Fugi BAS 2000 Bioimage Analyzer.

Polymerase chain reaction (PCR) and sequencing analysis:

The following primers were used:

V γ 1: 5'-CCGGCAAAAAGCAAAAAGT-3';

J γ 4: 5'-GCAAATATCTTGACCCATGA-3' and

pan C γ : 5'-CTTATGGAGATTTGTTTCAGC-3'.

Genomic DNA from hybridoma cells (T and S) was prepared by the Proteinase K/Phenol extraction/ethanol precipitation method. Sequences spanning the V-J junction were amplified by PCR using primers specific for the V γ 1 and J γ 4 genes. For each sample, two PCRs were performed in parallel, each one containing either one of the primers kinased. To produce single-stranded DNA after PCR amplification, the double-stranded product was digested with λ exonuclease as originally described (32). Both strands were then sequenced by the dideoxy chain termination method using the Sequenase enzyme (USB).

Total cellular RNA from hybridoma cells (BTC) was prepared by the guanidine isothiocyanate/Acid phenol extraction method. cDNA was synthesized with Oligo-dT using superscript reverse transcriptase (Gibco-BRL)

according to the manufacturer's instructions. Sequences spanning the V-J junction were amplified by PCR using primers specific for the V γ 1 and all four C γ genes. After amplification, four μ l of each PCR reaction mixture was incubated with Shrimp Alkaline Phosphatase and Exonuclease I to remove the excess of primers and dNTPs and sequenced by the dideoxy chain termination method using the Sequenase enzyme as described in the Sequenase Version 2.0 DNA Polymerase for sequencing PCR products kit (Pharmacia-USB).

RESULTS

Characterization of the 2.11 mAb

The mAb 2.11 was selected by its ability to block spontaneous IL-2 production by the V γ 1/V δ 6 expressing $\gamma\delta$ T cell hybridoma that had been used for immunization. Initial studies showed that this mAb binds to, and inhibits spontaneous IL-2 production by 10 of 10 V γ 1/V δ 6 expressing T cell hybridomas, but does not inhibit IL-2 production by $\alpha\beta$ -bearing T cell hybridomas (not shown). Furthermore, the 2.11 mAb binds to the immunizing hybridoma cells but not to variants that have lost expression of the $\gamma\delta$ TCR (not shown), suggesting that the 2.11 mAb recognizes some component of the $\gamma\delta$ TCR. SDS PAGE analysis of immunoprecipitates from lysate of ^{125}I surface-labeled V γ 1 hybridoma cells revealed an apparently identical pattern of 2 bands for the 2.11 mAb and the pan- $\gamma\delta$ specific mAb, 3A10 (Fig 1). This confirms that the 2.11 mAb is specific for TCR $\gamma\delta$.

To characterize further the specificity of the 2.11 mAb, we stained several $\gamma\delta$ T cell hybridomas known to express different V γ and V δ gene products. The mAb 2.11 did not bind to previously characterized $\gamma\delta$ T cell hybridomas that are known to express TCRs containing V γ 4, V γ 5, V γ 6 or V γ 7 chains (30, 33). In contrast, this mAb recognized 14 of 14 $\gamma\delta$ T cell hybridomas that produce IL-2 spontaneously (not shown). Sequence analysis of rearranged γ genes in those 14 hybridomas showed that all contain a functionally rearranged V γ 1 gene (Fig. 2A). These data demonstrate that the 2.11 mAb recognizes the V γ 1-C γ 4 protein but do not allow us to exclude the possibility that it also recognizes the V γ 2-C γ 2 protein. However, three additional lines of evidence reinforce the conclusion that the 2.11 mAb is specific for the V γ 1-C γ 4

protein. (1) 2.11⁺ hybridomas obtained by fusion of BW5147 cells with CD4⁻ CD8⁻ thymocytes that had been activated in vitro with the 2.11 mAb all expressed a functionally rearranged V γ 1-C γ 4 mRNA (Fig. 2B), while they each expressed one of five different V δ genes (not shown). 2) Immunoprecipitation of the $\gamma\delta$ TCR by the 2.11 mAb performed with four different $\gamma\delta$ T cell hybridomas showed a 43kD γ chain compatible with the V γ 1 chain but incompatible with the V γ 2 chain (1). 3) Expression of functionally rearranged V γ 1-C γ 4 mRNA, but not of V γ 2-C γ 2 mRNA, correlates with the 2.11⁺ phenotype in sorted 2.11⁺ and 2.11⁻ $\gamma\delta$ T cell populations (see appendix 2). Taken together, these data strongly suggest that the 2.11 mAb recognizes only the product of the V γ 1-C γ 4 gene .

Ontogeny of $\gamma\delta$ T cells expressing the V γ 1-C γ 4 gene product in the thymus

Using the 2.11 mAb, we quantitated the number of V γ 1-bearing $\gamma\delta$ thymocytes in B6 mice as a function of age. For comparison, we also determined the number of $\gamma\delta$ thymocytes expressing the V γ 4 or the V γ 5 chains using the respective antibodies that had previously been produced (1, 26). The results are summarized in Fig 3. In agreement with previous results (26), V γ 5-expressing thymocytes constituted the major $\gamma\delta$ T cell population at day 15 of gestation, but their number decreased thereafter until the first week after birth, when they became nearly undetectable. V γ 4-expressing thymocytes also appeared in fetal life and their number increased until one week of age, when they represented around 60% of all $\gamma\delta$ thymocytes. Thereafter the number of V γ 4⁺ thymocytes decreased to the adult levels (around 45% of all $\gamma\delta$ thymocytes). V γ 1-expressing cells constituted a very minor population during fetal and early post-natal life. Their number increased rapidly 1-3

weeks after birth, concomitant with the decrease of V γ 4-expressing thymocytes, and quickly reached the adult levels of around one third of all $\gamma\delta$ thymocytes (Fig 3A).

In spite of the very low numbers of V γ 1-expressing cells in the fetal thymus, these cells are detectable in the spleen at birth, constituting around 20% of all $\gamma\delta$ T cells in that organ. Here again, their numbers increased very rapidly, and by one week of age they reached the adult level which is around 40% of all $\gamma\delta$ splenic T cells in B6 mice (Fig 3B).

Tissue distribution of V γ 1-C γ 4 expressing $\gamma\delta$ T cells

We then quantitated the proportion of V γ 1-bearing $\gamma\delta$ T cells in the peripheral lymphoid organs and in different epithelia in adult B6, BALB/c and C3H mice. The results are summarized in Table 1. As can be seen, V γ 1-bearing cells represent 30-50% of all $\gamma\delta$ T cells in the spleen and lymph nodes of the three strains studied. Given that V γ 4-bearing cells also represent around half of the $\gamma\delta$ T cells in the peripheral lymphoid organs in these mouse strains (1, 34), these data show that V γ 1 and V γ 4-bearing cells comprise most of the spleen and lymph node $\gamma\delta$ T cells.

Although $\gamma\delta$ T cell clones expressing a TCR composed of V γ 1 and V δ 6 chains have been isolated from the skin epithelium (18), we found that no more than 2% of the $\gamma\delta$ -bearing DEC cells express the V γ 1-C γ 4 gene product in any of the three strains examined (B6, BALB/c and C3H).

We also found that close to 30% of the $\gamma\delta$ i-IELs in B6 mice were recognized by the 2.11 mAb and were thus likely to express the V γ 1 gene product. This observation was surprising because previous reports analyzing

mRNA expression of V γ genes in i-IELs, had led to the belief that V γ 1-bearing cells are at most a small minority of $\gamma\delta$ i-IELs. Stainings of B6 i-IELs with the anti-pan $\gamma\delta$ mAb 3A10 together with either the 2.11 mAb or an anti-V δ 4 mAb are shown in fig 4. The anti-V δ 4 mAb recognizes a fraction of the $\gamma\delta$ i-IEL that is mainly included in the V γ 7⁺ population (35). In this particular experiment, the V γ 1 bearing cells represent 28% of the $\gamma\delta$ i-IEL population. It is worth noting that the level of TCR expression of V γ 1 i-IELs is significantly lower than that of other $\gamma\delta$ -TCR⁺ i-IELs (compare gate A in both stainings). This has been consistently observed in many individual mice and in all three strains. We conclude that V γ 1⁺ cells comprise a significant population of $\gamma\delta$ i-IELs.

V γ 1-C γ 4 expressing i-IELs originate outside the thymus

$\gamma\delta$ i-IELs, as is the case for many $\alpha\beta$ i-IELs, have been shown to have a thymus-independent origin, as evidenced by their presence in normal numbers in athymic nude mice and in thymectomized, lethally irradiated, bone marrow-reconstituted chimeras (36-38). Furthermore, thymus independent $\gamma\delta$ cells are phenotypically different from thymus dependent, peripheral $\gamma\delta$ T cells. Thus, while most peripheral $\gamma\delta$ T cells do not express CD4 or CD8 molecules, most of the $\gamma\delta$ i-IEL express the α but not the β chain of the CD8 molecule (39). Furthermore, while all peripheral $\gamma\delta$ T cells express the Thy1 and CD5 molecules, only 30-50% of the $\gamma\delta$ i-IELs express Thy1 and all of them are negative for expression of the CD5 antigen (40-42). Given the possibility that the V γ 1-bearing i-IEL population could arise from thymus-dependent peripheral V γ 1 cells through migration to the intestinal epithelium, we quantitated the V γ 1-expressing cells in i-IELs isolated from

athymic nude mice and analyzed the phenotype of V γ 1-bearing cells in i-IEL preparations isolated from normal animals. The percentage of $\gamma\delta$ -i-IELs marked by the 2.11 mAb in normal and nude B6, BALB/c and C3H mouse strains is shown in Table II. As can be seen, the proportion of V γ 1-bearing cells among $\gamma\delta$ i-IELs was very similar in normal and nude mice of all strains examined.

The expression of Thy1, CD5, CD8 α and Cd8 β among total $\gamma\delta$ TCR⁺ or 2.11⁺ i-IELs in B6 mice is shown in Table III. In concordance with previous observations (39, 41, 42), virtually all the cells stained with the anti- δ mAb were negative for the expression of CD5 and CD8 β , while 80-90% of them expressed the α chain of the CD8 molecule, and close to 40% of them expressed the Thy1 antigen. The 2.11⁺ population displayed a very similar phenotype with regard to these markers, with most of the cells being CD5⁻CD8 α ⁺ β ⁻. The only significant difference found between total $\gamma\delta$ ⁺ and 2.11⁺ i-IELs was the proportion of Thy1-positive cells (38% and 50%, respectively). As V γ 1⁺ cells represented around 25% of the total $\gamma\delta$ i-IELs in these experiments, these data imply that the Thy1 antigen is expressed in 50% of the V γ 1⁺ cells and in 20% of the V γ 1⁻ $\gamma\delta$ T cell population.

Normal i-IEL are constitutively cytolytic in a redirected lysis assay (6, 43). This assay uses an Fc receptor-expressing target cell and relies on the crosslinking of TCR on effector cells by mAbs against the TCR/CD3 complex that also bind to the Fc receptor to trigger lysis. B6 i-IELs lyse P815 target cells in the presence of the 2.11 mAb (data not shown) indicating that the V γ 1⁺ i-IEL population is constitutively cytolytic.

From these experiments we conclude that, similar to the $V\gamma 7^+$ i-IEL population, $V\gamma 1^+$ i-IEL also mature extrathymically, and that these $\gamma\delta$ T cell populations are phenotypically and functionally similar.

The proportion of $V\gamma 1^+$ cells among $\gamma\delta$ i-IELs is independent of the antigenic load of the intestine.

The presence of $V\gamma 1$ mRNA in the i-IEL population of some individual mice or in mice infected with the parasite *Eimeria* has been reported (44, 45), leading to the suggestion that colonization of the gut could result in antigen-driven accumulation or expansion of few incipient $V\gamma 1^+$ cells. To study the effect of microbial colonization and/or food antigens on the extent of $V\gamma 1$ -bearing cells among $\gamma\delta$ i-IELs, we quantitated the proportion of $V\gamma 1^+$ cells in i-IELs isolated from specific pathogen free (SPF) and antigen-free (AgF) BALB/c mice. The results of one such experiment are shown in Table IV. As can be seen, the proportion of $V\gamma 1$ -bearing i-IELs was virtually identical in SPF and AgF BALB/c mice. These experiments demonstrate that the presence of the $V\gamma 1$ -bearing population in the epithelium of the small intestine is independent of the antigenic load of the gut epithelium.

Expression of $V\gamma 1$ -C $\gamma 4$ i-IELs in different mouse strains

We then quantitated the proportion of $V\gamma 1$ -bearing lymphocytes in the i-IEL population of 12 different strains of mice, including some MHC congenic strains in different backgrounds. The results are summarized in Fig 5. Several conclusions can be drawn from this analysis. First, all strains tested

contained a sizable proportion of V γ 1-bearing $\gamma\delta$ i-IELs that ranged from 15-60% of all $\gamma\delta$ -bearing i-IELs. Second, the representation of V γ 1-bearing lymphocytes among $\gamma\delta$ i-IELs seems to be strain specific. It should be pointed out that some of the data presented were obtained using identical strains of mice that were acquired from different sources and housed in different places. The low standard deviation observed within a single strain suggests that genetic rather than environmental factors are responsible for the observed strain-to-strain differences in the proportion of V γ 1-bearing lymphocytes. Third, although differences in the percentage of $\gamma\delta$ i-IELs expressing the V γ 1 gene product are found between MHC congenic strains, these differences are relatively small when compared with those found between strains that differ genetically outside of the MHC locus. These data suggest that genes other than MHC genes are involved in the regulation of the number of V γ 1-bearing cells.

DISCUSSION

In this chapter we describe a new mAb specific for the V γ 1-C γ 4 chain of the $\gamma\delta$ TCR. The use of this mAb allowed us to quantitate and characterize V γ 1-expressing $\gamma\delta$ T cells in the thymus of fetal and adult animals, as well as in the peripheral lymphoid organs and in several epithelia. Our data, while confirming some previously drawn conclusions, revealed a few new facts about the composition of various $\gamma\delta$ T cell subsets in developing and adult mice.

V γ 1-bearing cells constitute a small minority of the $\gamma\delta$ thymocytes throughout fetal life. Their number increases substantially during the first weeks of post-natal life, concomitant with a decrease in the number of V γ 4-bearing thymocytes. By three weeks of age the V γ 1 and V γ 4 subpopulations reach their adult values of around 30 and 50% of the total $\gamma\delta$ thymocytes, respectively. At that time, V γ 5-bearing cells are virtually absent and V γ 7-bearing thymocytes represent no more than 5% of total adult $\gamma\delta$ thymocytes ((46) and our unpublished observations). Furthermore, although functional rearrangements of V γ 6 and V γ 2 genes have been described in the adult thymus (47), it is believed that $\gamma\delta$ thymocytes expressing these γ chains constitute a small minority among the total $\gamma\delta$ thymocytes. Although no other V γ genes have been identified, a possibility that some minor populations of $\gamma\delta$ T cells express hitherto unidentified TCR γ chains cannot be excluded. Taken together, these data are consistent with the possibility that, at least in B6 mice, other V γ genes could be expressed in the adult thymus.

In the spleen and lymph nodes, V γ 1- and V γ 4-bearing cells are also the major subpopulations of the $\gamma\delta$ T cell population, constituting close to 90% of all $\gamma\delta$ lymphocytes in these peripheral lymphoid organs. The actual

proportion of V γ 1- and V γ 4-expressing cells varies in different strains of mice. Nevertheless, they constitute the large majority of the splenic and lymph node $\gamma\delta$ T cells in most of the common laboratory strains ((34) and unpublished results). Thus, it appears that the proportions of the two $\gamma\delta$ T cell subsets are coordinated; a decrease in the frequency of cells expressing one of these V γ chains is accompanied by a corresponding increase in the frequency of cells expressing the other V γ chain. A similar situation can be observed concerning the expression of V γ 1 and V γ 7 gene products within the $\gamma\delta$ i-IEL population. Together, V γ 1 and V γ 7-bearing cells represent close to 90% of the $\gamma\delta$ i-IEL population, and their relative frequencies in different strains of mice are also coordinated (Fig 5 and unpublished results). This is particularly important to consider in the context of positive and/or negative selection for $\gamma\delta$ T cells. It has been proposed that the expansion or overrepresentation of T cells utilizing a particular $\gamma\delta$ receptor could be used as an indication that positive selection has occurred (34, 38). Although such increased representation of a specific $\gamma\delta$ T cell type may suggest that cellular selection has occurred, the fact that the overrepresentation of T cells utilizing a defined V γ chain is concomitant to a similar underrepresentation of T cells using another V γ chain precludes definition of the selection as either positive or negative or both.

Although V γ 1-bearing T cells have been isolated from the skin of C3H mice (18), our data show that V γ 1-bearing cells constitute a minor population (0.5-2%) of total $\gamma\delta$ DEC in that strain as well as in the other strains tested. On the other hand, it has been shown that the skin of nude mice is colonized by thymus-independent V γ 1-bearing $\gamma\delta$ T cells (48), demonstrating that these cells can home to the epithelium of the skin. Furthermore, recent analysis of

IL-7 transgenic mice that spontaneously develop dermatitis (49) has shown a massive infiltration or expansion of $\gamma\delta$ T cells other than V γ 5-bearing cells in the skin. It is possible, therefore, that the representation of V γ 1-bearing cells in the skin epithelium is increased upon infection and could, therefore, vary in different mouse colonies.

The most striking finding concerning the tissue localization of $\gamma\delta$ T cells expressing the V γ 1-C γ 4 chain is that they constitute a substantial proportion of $\gamma\delta$ i-IELs in normal mice. V γ 1⁺ cells represent 15-60% of all $\gamma\delta$ i-IELs in different strains of mice and their presence is independent of the thymus and of the antigenic load of the gut. The fact that this population has been overlooked is not readily understood, although some plausible explanations can be put forward. To date, four different groups have analyzed TCR γ rearrangement or TCR γ mRNA expression in i-IELs, reaching the conclusion that the V γ 1-bearing population is minor, if not absent, in the normal $\gamma\delta$ i-IEL population. The first group (50) did not analyze V γ 1 rearrangements in i-IELs because their previous work (7) had shown that the major γ protein expressed in i-IELs had a relative molecular mass of 34-35kD, which is clearly different from the V γ 1-C γ 4 protein (Mr=41-42kD (1)). Besides the fact that the immunoprecipitation technique is not very sensitive and is certainly not quantitative (because of the differential labeling rates of different proteins and the specificity of the antisera used), it should also be noted that the size of the V γ 1-C γ 4 chain is very similar to the size of most of the V δ -C δ proteins. Thus, it is possible that the δ chain band in the gels masked the V γ 1-C γ 4 protein.

Two other groups (51, 52) analyzed TCR γ rearrangements in i-IELs by PCR techniques using V γ and J γ specific primers. To analyze V γ 1 and V γ 2

rearrangements they used an identical set of primers. The $V\gamma$ -specific primer cross-hybridizes with $V\gamma 1$ and $V\gamma 2$ genes, while the $J\gamma$ -specific primer cross-hybridizes with all four $J\gamma$ sequences. A closer look at the primers used reveals that the $V\gamma$ primer has a sequence identical to the $V\gamma 2$ gene but contains a single mismatch with respect to the $V\gamma 1$ sequence in the second base at its 3' end. Similarly, the $J\gamma$ primer has a sequence identical to the $J\gamma 1$ and $J\gamma 2$ segments but has two mismatches with respect to the $J\gamma 4$ segment, one of which is located in the fourth base of its 3' end. The $V\gamma 2$ gene appears to be rearranged in almost all $\gamma\delta$ T cells and in many $\alpha\beta$ T cells (see appendix 2), while with the exception of a small number of other $\gamma\delta$ T cells, the $V\gamma 1$ gene is almost exclusively rearranged in cells expressing the $V\gamma 1$ - $C\gamma 4$ protein. Thus, it seems very likely that, in the PCR conditions used by these authors, the $V\gamma 2$ - $J\gamma 2$ rearrangements would be preferentially amplified over the $V\gamma 1$ - $J\gamma 4$ rearrangements. Finally, the fourth group analyzed the expression of $V\gamma$ mRNA in i-IELs by in situ hybridization using anti-sense probes, but failed to detect any cells expressing either $V\gamma 1$ or $V\gamma 2$ mRNA (53). Further experiments conducted by these authors were limited by the preparation of cDNAs with a $C\gamma 1$ specific primer and subsequent amplification of the cDNA with primers specific for the $V\gamma 4$, $V\gamma 6$ and $V\gamma 7$ genes, thereby excluding $V\gamma 1$ - $C\gamma 4$ and $V\gamma 2$ - $C\gamma 2$ from the analysis. As this report shows no positive control for the hybridization of any of the $V\gamma$ specific probes, it is possible that their failure to detect $V\gamma 1$ and $V\gamma 2$ mRNA simply reflects the low sensitivity of the in situ hybridization technique.

We, thus, believe that the normal $\gamma\delta$ i-IEL population contains a relatively high frequency of cells expressing the $V\gamma 1$ - $C\gamma 4$ gene product. This frequency is comparable to the frequency of cells expressing the $V\gamma 7$ - $C\gamma 1$ chain.

The frequency of the V γ 1⁺ i-IEL varies in different strains of mice, but appears to be quite constant among different individuals of the same strain, even when mice are housed in different colonies. This suggests that genetic factors, rather than environmental factors, are primarily responsible for the determination of V γ 1⁺ i-IEL frequency.

A striking characteristic of $\gamma\delta$ T cell subsets is the rather strict correlation among their onset of appearance in development, the V γ (and V δ) genes utilized to encode the TCR, and their homing to distinct peripheral sites. Thus, V γ 5- and V γ 6-expressing cells are primarily, if not exclusively, produced early in the fetal thymus, and home to the epithelium of skin and of uterus, vagina and tongue respectively (9, 26, 30, 54-57). V γ 4-bearing cells appear later in the fetal thymus, constituting the major $\gamma\delta$ thymocyte population at birth, and preferentially home to the peripheral lymphoid organs (1, 2, 33). V γ 7-bearing cells, on the other hand, develop from precursors that do not require a thymus for maturation and home to the intestinal epithelium (36-38, 50, 51, 53). Until this study only a few exceptions to these rules were observed. Thus, around 2% of the $\gamma\delta$ thymocytes bear the V γ 7 protein (46), and roughly 5% of $\gamma\delta$ i-IELs express the V γ 4 chain in all mouse strains examined, including athymic mice (our unpublished observations). In clear contrast to the other $\gamma\delta$ T cell subsets, the V γ 1-bearing $\gamma\delta$ T cells seem to develop and home in a more diversified fashion. Thus, they can develop in the thymus and migrate to the peripheral lymphoid organs where, together with the V γ 4 subset, they represent the vast majority of the $\gamma\delta$ T cells. On the other hand, V γ 1-bearing cells can also develop extrathymically and constitute, together with the V γ 7 subset, the large majority of $\gamma\delta$ i-IELs. Thus, the ability to rearrange and express the V γ 1 gene is

not limited to a particular $\gamma\delta$ T cell precursor, nor is the differentiation of V γ 1-bearing $\gamma\delta$ T cells restricted to a defined milieu. Thymus-independent V γ 1-bearing cells have also been found in the skin of nude mice (48) and CD8 $\alpha^+\beta^-$ $\gamma\delta$ T cells, which are believed to have a thymus independent origin, have been found in the liver of normal mice (58).

The presence of V γ 1-bearing cells in lymphoid organs and epithelia might be related to their specificity. A large fraction of V γ 1 cells appears to recognize an endogenous antigen expressed by lymphocytes (11). These cells can be further stimulated in the presence of PPD and Hsp proteins or peptides (11, 13), which led to the suggestion that they might recognize autologous stress proteins (57, 59). In some instances, proliferation or accumulation of V γ 1 bearing cells has been shown to occur *in vivo* after infection of mice with different bacteria or parasites, suggesting a physiological role for those cells in the defense against pathogens (45, 60, 61). A similar reactivity has also been described for a subset of human $\gamma\delta$ T cells that express the V γ 9 gene product. Cells of this type respond to mycobacterial extracts (and in some cases to mycobacterial and endogenous Hsps) and recognize an undefined antigen on the surface of some tumor cell lines of lymphoid origin (62-70). Cells with these specificities are restricted in the use of V γ and V δ genes, but there is considerable diversity in the junctional sequences of their TCR genes (66, 71-74). As in the case of V γ 1 cells in the mouse, V γ 9 cells in humans appear late in ontogeny, but compose a major $\gamma\delta$ T cell population in adult individuals (75, 76). This parallelism, suggests a precise and important function of these cells. A precise definition of the ligands recognized by these cells will certainly help to understand their physiological role.

Table I - Expression of V γ 1-C γ 4+ $\gamma\delta$ T cells in different tissues

TISSUE	2.11 ⁺ cells (% of total $\gamma\delta$ T cells)*+	
	STRAIN	
	C57BL/6	C3H/HeJ
Spleen	34.3 \pm 5.3	55.4 \pm 5.2
LN	38.9 \pm 4.4	54 \pm 5.4
i-IEL	24 \pm 3.1	36 \pm 8.3
DEC	0.7	1.6
		1.4

* Cells were stained and analyzed as in Fig. 2
+ At least three determinations were performed except for DEC where the average of two experiments is given. A pool of 2-5 mice was used for each determination.

Table II - Expression of V γ 1+ i-IELs in euthymic and athymic (nu/nu) mice

	2.11 ⁺ cells (% of total $\gamma\delta$ T cells)*+
BALB/c (nu/+)	26.1
BALB/c (nu/nu)	23.4
C57BL/6 (nu/+)	24.5
C57BL/6 (nu/nu)	29.2
C3H (nu/+)	46.1
C3H (nu/nu)	39.8

* Cells were stained and analyzed as in Fig. 3

+ The average of two determinations is given. A pool of 2-3 mice was used for each determination.

Table III - Phenotype of V γ 1+ i-IELs

i-IEL population	% of cells expressing a)			
	Thy1	CD8 α	CD8 β	CD5
$\gamma\delta^+$ (3A10 $^+$)	38.6;38.0	89.9;86.6	1.6;1.8	<1
V γ 1 $^+$ (2.11 $^+$)	50.8;50.0	79.0;81.9	1.0;1.4	<1

a) C57BL/6 IEL were stained with mAbs against the indicated antigen and counterstained with either anti- δ (3A10) or anti-V γ 1 (2.11) mAbs. Numbers represent the percentage of cells expressing the defined antigen among total $\gamma\delta$ cells (upper) or 2.11 $^+$ cells (lower). Results from two independent experiments are shown.

Table IV - Expression of V γ 1+ i-IELs in Specific Pathogen Free (SPF) and Antigen Free (AgF) Balb/c mice

	2.11 ⁺ cells (% of total $\gamma\delta$ T cells)*+
BALB/c SPF	18.1
BALB/c AgF	19.2

* Cells were stained and analyzed as in Fig. 3

+ The average of two determinations is given. Three mice were used for each determination.

Figure 1: 2.11 and the pan $\gamma\delta$ specific mAb, 3A10, immunoprecipitate an identical complex from surface-labeled V γ 1-expressing hybridoma cells. Lysate from ^{125}I -labeled cells was immunoprecipitated with 2.11 or 3A10 mAbs and subjected to SDS-PAGE under reducing conditions. 2.11 immunoprecipitate (lane 1), 3A10 immunoprecipitate (lane 2). Molecular weight markers are indicated in kilodaltons.

1

2

— 200

— 97

— 68

— 43

— 29

2.11

3A10



Figure 2: V γ 1-J γ 4 junctional sequences of 2.11⁺ T cell hybridomas. DNA (A) or cDNA (B) was amplified by PCR with V γ 1-J γ 4 or V γ 1-C γ primers respectively. The products obtained were directly sequenced as described in Materials and Methods. Hybridomas in A) were derived from adult thymocytes (T) and splenocytes (S) activated in vitro with PPD. Hybridomas in B) were derived from $\gamma\delta$ thymocytes activated in vitro with the 2.11 mAb. P, palindromic nucleotides (54); N, nucleotides not present in the germline sequence.

germline Vy1 germline Jy4

	Vy1	P	N	P	Jy4
germline Vy1	TGT GCA GTC TGG ATA AA	TT			
germline Jy4		GA			TCA GGC ACA
A)					
T3.13.1	TGT GCA GTC TGG				TCA GGC ACA
T4.7.7	TGT GCA GTC TGG ATA AA		GCTC		GGC ACA
T3.23.2	TGT GCA GTC TGG ATA AA		AAGGG		TCA GGC ACA
S3.11.2	TGT GCA GTC TGG ATA		C		CA GGC ACA
T3.41.13	TGT GCA GTC TGG ATA		GGA		GGC ACA
T4.14.1	TGT GCA GTC TGG		CCCC		TCA GGC ACA
S3.17.8	TGT GCA GTC TGG AT		GCA		TCA GGC ACA
T4.36.6	TGT GCA GTC TGG A		CCCCCTTC		TCA GGC ACA
T3.16.1	TGT GCA GTC TGG AT		CC		TCA GGC ACA
S3.29.2	TGT GCA GTC TGG A				TCA GGC ACA
T3.18.9	TGT GCA GTC TGG AT		CAG		A GGC ACA
T3.20.17	TGT GCA GTC TGG		GGG		GGC ACA
T4.41.6	TGT GCA GTC TGG		CCCC		TCA GGC ACA
T4.8.1	TGT GCA GTC TGG		CC		TCA GGC ACA
B)					
BTC 1	TGT GCA GTC TGG		GC		TCA GGC ACA
BTC 7	TGT GCA GTC TGG AT		T		GGC ACA
BTC 54	TGT GCA GTC TGG AT		TA		GC ACA
BTC 57	TGT GCA GTC TGG		G		TCA GGC ACA
BTC 58	TGT GCA GTC TGG		GTC		TCA GGC ACA
BTC 59	TGT GCA GTC TGG AT		T		TCA GGC ACA
BTC 60	TGT GCA GTC TGG AT		GG		CA GGC ACA
BTC 63	TGT GCA GTC TGG A		AGGGG		GGC ACA
BTC 66	TGT GCA GTC TGG ATA AA				TCA GGC ACA
BTC 78	TGT GCA GTC TGG		GT		TCA GGC ACA

Figure 3: Differential expression of $\gamma\delta$ chains on thymocytes and splenocytes of C57BL/6 mice of different ages. Cells were stained with FITC-labeled anti-C δ mAb (3A10) and biotin-labeled anti-V γ 1 (2.11), anti-V γ 5/V δ 1 (536) and anti-V γ 4 (UC3-10A6) mAbs followed by streptavidin PE. 2000-5000 $\gamma\delta$ -positive cells per sample were analyzed with a FACScan. The percentages of 2.11 (o), 536 () and UC3-10A6 (◇) -positive cells among 3A10-positive cells is shown. Cell pools were always used for the analysis and each point represents the mean of at least two independent determinations

Ontogeny of V γ 1 cells in thymus and spleen

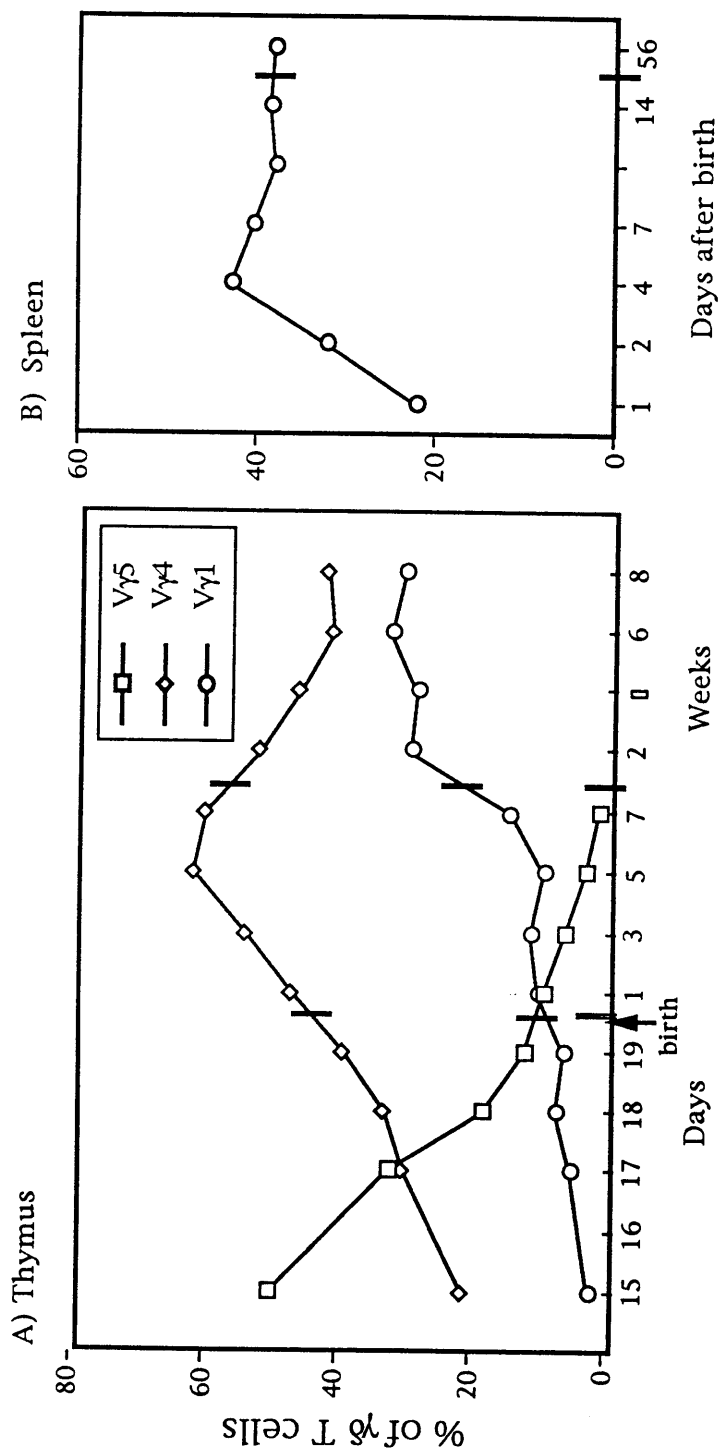


Figure 4: Expression of V γ 1 and V δ 4 in i-IELs from C57BL/6 mice. i-IEL were labeled with 3A10 FITC and either 2.11- or GL2-biotin and analyzed as described in Materials and Methods. Gates A and B denote TCR low and TCR high $\gamma\delta$ T cell populations. The fractions of 2.11⁺ cells and GL-2⁺ cells among 3A10⁺ i-IELs were 28% and 24% respectively.

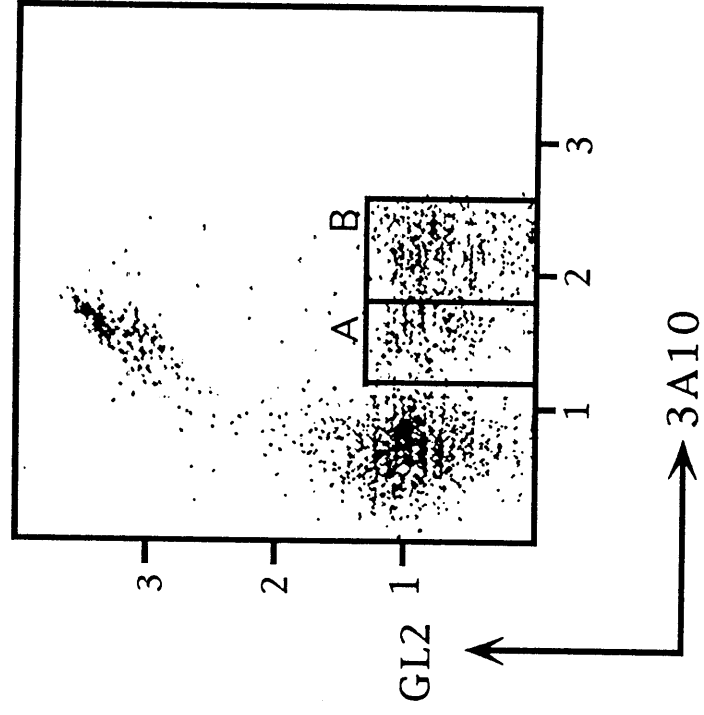
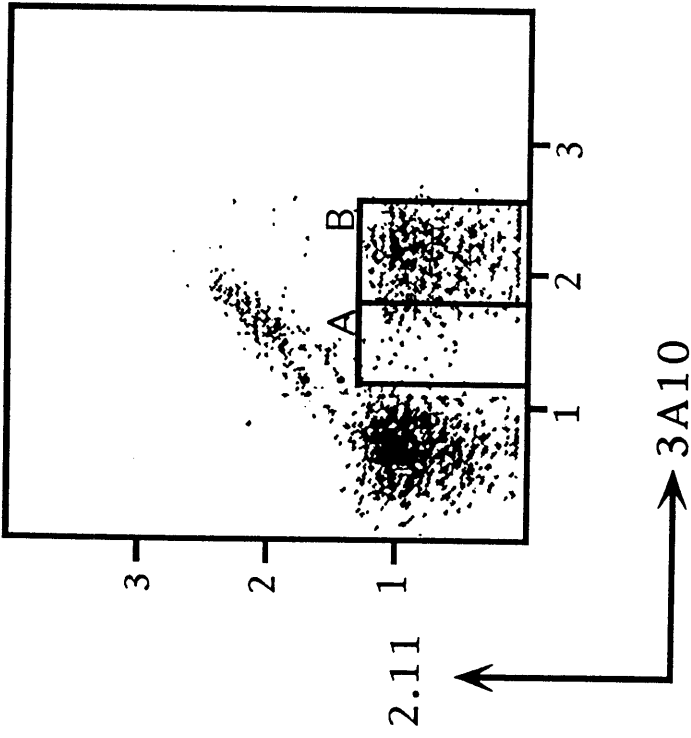
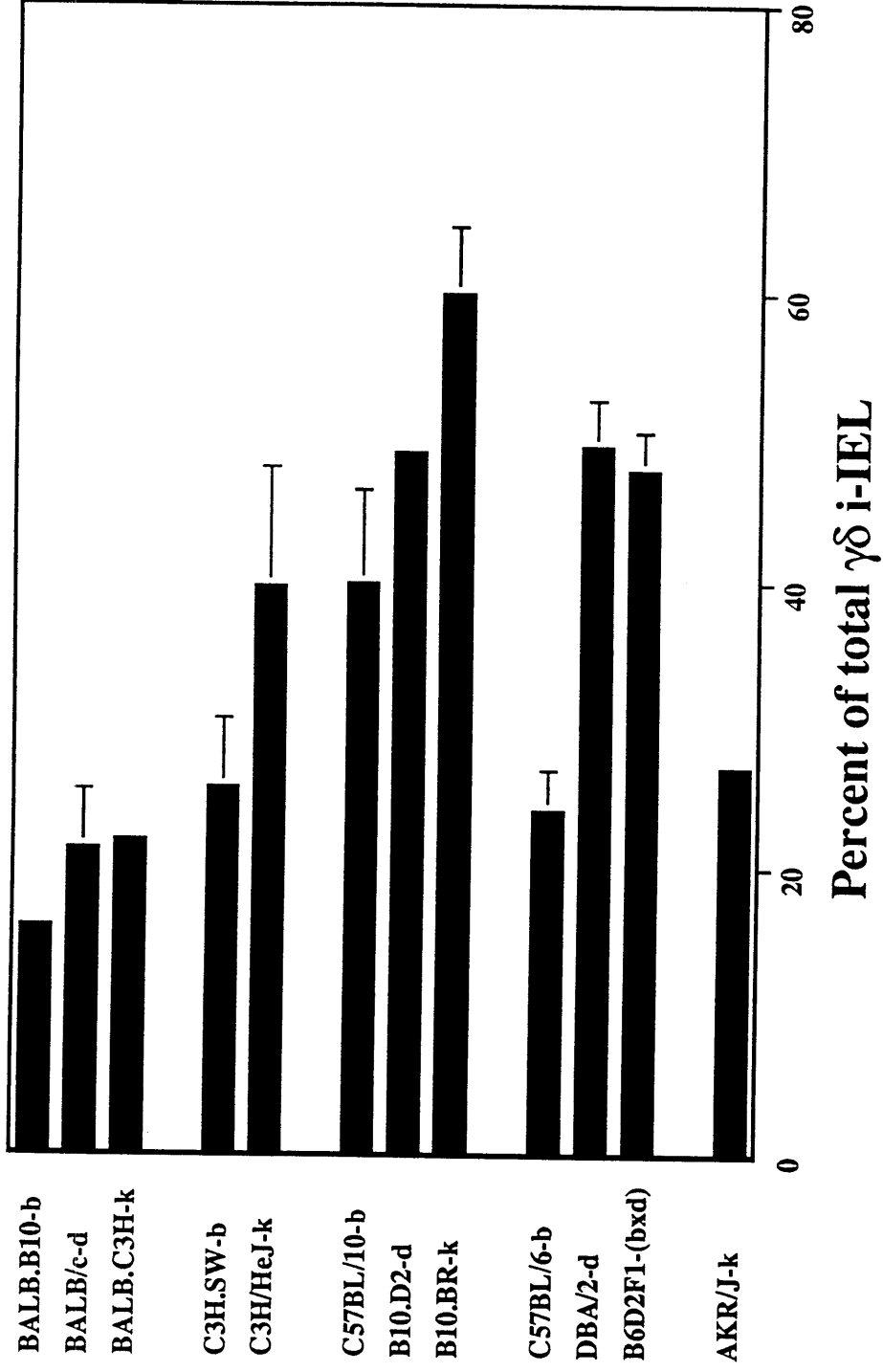


Figure 5: Analysis of V γ 1 usage in inbred mice. i-IEL from the indicated mouse strains were analyzed for 2.11 and 3A10 expression by fluorescence flow cytometry. Data are shown as the percentage of 3A10⁺ cells that are 2.11⁺. When standard deviations are shown, at least three determinations were performed; otherwise the mean of two experiments is given. Two to five mice were used for each determination. In one experiment, five B6 mice were analyzed individually. The small letter after each strain denotes the MHC haplotype.

V γ 1 i-IELs in different strains of mice



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Chapter 4: Expression of αv and $\beta 3$ integrin chains on murine lymphocytes.

Abstract

The vitronectin receptor is a member of the integrin family of adhesion protein receptors and binds a broad spectrum of ligands, including fibronectin and fibrinogen in addition to vitronectin. We have generated four monoclonal antibodies that recognize the murine $\alpha v\beta 3$ vitronectin receptor. Biochemical and expression analysis showed that two of the mAbs are specific for the αv chain, and two are specific for the $\beta 3$ chain. The mAbs are effective blocking reagents and inhibited cell adhesion to vitronectin, fibrinogen and fibronectin. Staining analysis revealed expression of αv and $\beta 3$ on certain populations of murine thymocytes, splenocytes and bone marrow cells. The expression of αv and $\beta 3$ appeared to be modulated at specific stages of thymocyte development, suggesting a possible function for the $\alpha v\beta 3$ vitronectin receptor in T cell development.

Introduction

Integrins comprise a large family of heterodimeric cell surface proteins composed of α and β chains that mediate cell-cell interactions and interactions between cells and the extracellular matrix (ECM) (1-3). Integrins are expressed on all cells and are involved in a number of fundamental cell processes including adhesion, migration, activation and differentiation. The expression and functions of integrins have been particularly well studied with respect to lymphocytes, which require a variety of cell-cell and cell-ECM interactions to perform their complex programs of immune surveillance and antigen response (4, 5). The functions of integrins depend on binding to specific adhesion proteins, such as fibronectin, often through recognition of the tripeptide, arginine-glycine-aspartic acid (RGD), binding motif (6).

The human $\alpha v \beta 3$ vitronectin receptor was originally identified as a heterodimeric molecule with vitronectin binding activity (7) and later shown to be related to other members of the integrin family (8). Subsequent studies demonstrated that this receptor has a broad binding specificity and can mediate binding to fibronectin, fibrinogen, von Willebrand factor and thrombospondin in addition to vitronectin (9-12). More recently, a mAb specific for the murine $\alpha v \beta 3$ vitronectin receptor ($\alpha v \beta 3$) was isolated (13, 14), and used to identify $\alpha v \beta 3$ as a costimulatory molecule required for spontaneous activation of $\gamma \delta$ T cell hybridomas (15).

In the process of identifying cell surface molecules involved in the constitutive IL-2 secretion of murine V γ 1, V δ 6 T cell hybridomas, we generated a series of monoclonal antibodies (mAbs) that recognize murine $\alpha v \beta 3$. Here, we present the characterization of four of these mAbs, including the determination of their chain specificities and staining analysis of several

lymphoid populations. Contrary to previous reports (13, 16), we observed detectable levels of αv and $\beta 3$ expression on certain populations of thymocytes and splenocytes in addition to bone marrow cells. Interestingly, we observed differential expression of the αv and $\beta 3$ chains on discrete populations of thymocytes, suggesting a possible role for this receptor in T cell development.

Materials and Methods

Animals: C57BL/6 (B6) mice were obtained from Taconic (Germantown, NY) or from Iffa-Credo (L'Arbresle, France). Rag-1 deficient mice have been previously described (17). Adult Armenian Hamsters were obtained from Cytogen Research (West Roxbury, MA).

Antibodies and antisera: The mAbs used were 145-2C11, anti-CD3, RM4-5, anti-CD4, 53-6.7, anti-CD8 α , RA3-6B2, anti-B220 and 2C9.G2, anti-CD61. These mAbs were purchased as FITC or biotin conjugates from Pharmingen (San Diego, CA). The anti- β 1 antiserum was previously described (18). The anti- β 3 antiserum was a gift from the Hynes lab. The anti- α v antiserum was purchased from Chemicon (Temecula, CA).

Production and purification of the anti-vitronectin receptor mAbs: The production of mAbs capable of inhibiting the constitutive IL-2 production by V γ 1, V δ 6 expressing $\gamma\delta$ T cell hybridomas has been previously described in detail (19). 8-B11 is a hamster IgG and was purified from tissue culture supernatant by protein G affinity chromatography. The other three mAbs (8-2D, 8-B3 and 4-10D) are of the IgM class, and were partially purified from tissue culture supernatant. Briefly, cells were adapted to grow in either Hybriboma-SFM (Gibco-BRL, Gaithersburg, MD) or Optimem (Gibco-BRL) in the absence of serum, and the collected supernatants were extensively filtered through a 100kD pore-size membrane (YM100, Amicon Inc, Beverly, MA). Purified mAbs were conjugated with FITC or biotin according to standard procedures.

Cell Surface Labeling and Immunoprecipitation: Fig1 A and B: 10^7 cells were harvested by centrifugation, washed three times with PBS, resuspended in 200 μ l of PBS and labeled with 1 mCi of 125 I Na by the lactoperoxidase method.

After three washes, the cell pellet was lysed in 1 ml of ice cold lysis buffer (10mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% BSA, 1mM PMSF), vortexed for 20s, and incubated on ice for 30 min. The lysate was clarified by centrifugation at 13,000 rpm at 4°C in a microfuge. 100µl of normal hamster serum was added to the supernatant followed by two rounds of pre-clearing with one-tenth volume Protein A - Sepharose beads (Pharmacia, Uppsala, Sweden). One quarter of the precleared lysate was incubated with 10µg of biotinylated mAb followed by incubation with one-tenth volume of Streptavidin-Sepharose beads. Immunoprecipitates were washed, resuspended in sample buffer, and subjected to SDS-PAGE on a 7-10% mini-gel according to standard procedures. Radioactive bands were visualized using X-Ray film. Fig 1C: The derivation and culture of embryonic day 9 (E9) fibroblasts was previously described (20). One 6 cm plate of ~60% confluent E9 fibroblasts was labeled as a monolayer with 1mCi ¹²⁵I Na, and roughly seventy-million hybridoma cells were labeled in suspension with 2 mCi ¹²⁵I Na by the lactoperoxidase-glucose oxidase method (21). Labeled E9 and hybridoma cells were washed extensively and lysed in 1ml and 2 ml respectively of ice cold lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.5 mM CaCl₂, 0.5% NP-40, 1mM PMSF, 0.02 mg/ml aprotinin and 0.0125 mg/ml leupeptin). The lysates were clarified by centrifugation at 13,000 rpm at 4°C in a microfuge. 135 µl of each clarified lysate was separated for precipitation with the anti-αv serum and the remaining 1ml of the E9 lysate was mixed with 1 ml of the hybridoma lysate. The mixed lysate was split into a 800µl fraction for precipitation with the antisera and a 1200 µl fraction for precipitation with the mAbs. Samples to be precipitated with the antisera were pre-cleared with 25% volume protein A-Sepharose, and samples to be precipitated with the biotinylated mAbs were incubated with biotinylated RM4-5, anti-CD4

(Pharmingen) and pre-cleared with 25% volume Ultralink immobilized streptavidin (Pierce, Rockford IL). The pre-cleared 135 μ l portions of the original lysates and 270 μ l portions of the mixed lysate were mixed with 100 μ l of 10 mg/ml BSA followed by addition of either 2-5 μ l of α v, β 1 or β 3 antiserum, or 5-10 μ g of the biotinylated anti- α v β 3 mAbs as indicated. After 2hrs incubation at 4 $^{\circ}$ C, 50 μ l protein A-Sepharose beads (50% slurry pre-coated with 10 mg/ml BSA in lysis buffer) was added for the antiserum precipitations and 50 μ l Ultralink immobilized streptavidin (50% gel pre-coated with 10 mg/ml BSA in lysis buffer) was added for the biotin-mAb precipitations. After 2 hrs rotation at 4 $^{\circ}$ C, the beads were sedimented and washed 4x with cold lysis buffer. Samples were then boiled in 50 μ l of non-reducing SDS-PAGE sample buffer, and subjected to SDS-PAGE on a 6% gel.

Immunofluorescence staining and flow cytometric analysis: Cells (5×10^5) were incubated for 1 hr at 4 $^{\circ}$ C in 25 μ l staining buffer (PBS, 2%FCS, 0.1% NaN₃) with 20 μ g/ml Fc Block (Pharmingen), followed by addition of 25 μ l of 40% normal hamster serum (NHS) in staining buffer. After 30 min, the cells were spun down and resuspended in staining buffer with the indicated labeled Abs for 40 min. at 4 $^{\circ}$ C. After two washes, the cells were incubated with streptavidin-PE (Pharmingen, or Southern Biotechnology, Birmingham, AL) for 15 min at 4 $^{\circ}$ C. After two additional washes, viable cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Dead cells were gated out by staining with propidium iodide. Normal mouse serum was substituted for NHS in the staining of Rag1 deficient thymocytes.

For competition experiments, the blocking with Fc block and NHS was omitted. Cells were first incubated with a 10-fold excess of the indicated unlabeled antibodies for 15 min, before the addition of the biotinylated mAbs. The rest of the staining and analysis was performed as indicated above.

Adhesion assay: Preparations of mouse vitronectin (Telios Pharmaceuticals, San Francisco, CA), mouse fibronectin (Gibco-BRL), mouse fibrinogen (Sigma Chemicals, St-Louis, MO), rat collagen type I, mouse collagen type IV (Sigma) or mouse laminin (ICN biomedical, Aurora, OH) were diluted in PBS and distributed in 96-well flat bottom tissue culture plates (Costar, Cambridge, MA). After an overnight incubation at 4°C, the coated wells were washed twice with PBS and incubated for two hours at 37°C with 100 µl of adhesion medium (RPMI-0.2% BSA). Cells were incubated for 5 min with PBS-EDTA (1mM), washed three times with adhesion medium and resuspended at a concentration of 5×10^5 cells/ml. 100µl of the cell suspension was added to the saturated wells, and after a 10 sec centrifugation at 200g, the plates were incubated at 37°C for 2 hrs. For the inhibition assays, the cells were preincubated with the indicated concentrations of Ab or peptide (GRGDSP or GRGESP, Sigma) for 45 min at 4°C in 96 well plates in a final volume of 100µl before being transferred to the coated plates. Non-adherent cells were removed from the wells by the careful addition and removal of 200 µl of adhesion medium repeated three times. Finally, 200µl of RPMI containing 5% FCS and 10µg MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) was added to each well and the plates were incubated at 37°C for 3 hrs. The plates were centrifuged for 3 min at 200g and the supernatants were flicked out from the plates. 200µl of DMSO was then added to the wells and pipetted up and down until the precipitated salt was dissolved. The O.D. was then read at 540nm wavelength. All conditions were performed in duplicate. Percent adherence is defined as the O.D. of the coated wells divided by the O.D. of wells that had the same number of input cells and were not washed. The linear correlation between the number of cells in the wells and the O.D. was checked in every experiment.

Results

Characterization of anti-vitronectin receptor monoclonal antibodies

The monoclonal antibodies, 8-2D, 8-B3, 4-10D, and 8-B11, were identified based on their ability to block spontaneous IL-2 secretion of murine V γ 1, V δ 6 expressing $\gamma\delta$ T-cell hybridomas. SDS-PAGE analysis of immunoprecipitations with each of the four antibodies from lysates of ^{125}I surface-labeled hybridoma cells under non-reduced (Fig. 1A) and reduced (Fig. 1B) conditions revealed the characteristic pattern of bands for $\alpha\nu\beta$ 3. Under non-reduced conditions, the band of approximately 140kD corresponds to the $\alpha\nu$ chain and the band of approximately 90 kD corresponds to the β 3 chain. Under reduced conditions, the bands of approximately 120 kD and 25 kD correspond to the two subunits of the $\alpha\nu$ chain, and the band of approximately 100 kD corresponds to the β 3 chain. The size of the β 3 chain appears smaller under non-reduced conditions due to the presence of intrachain disulphide bonds (22). To further define the specificity of the four mAbs, immunoprecipitations were performed with the four mAbs and anti- $\alpha\nu$ and anti- β 3 antisera on mixed lysates from ^{125}I surface labeled embryonic fibroblast cells and hybridoma cells. As shown in Fig. 1C, the embryonic cells express the $\alpha\nu$ chain primarily in combination with β 1, and to a considerably lesser extent, in combination with β 3 and β 5, while the hybridoma cells express the $\alpha\nu$ chain primarily in combination with the β 3 chain. The mAbs, 8-B3 and 4-10D, precipitated $\alpha\nu$ in combination with β 1, β 3 and β 5 from mixed lysate, while the mAbs, 8-2D and 8-B11, precipitated $\alpha\nu$ only in combination with β 3 (Fig. 1C). This result suggests that 8-B3 and 4-10D bind

specifically to the αv chain and 8-2D and 8-B11 bind to epitopes of the $\alpha v\beta 3$ heterodimer.

To confirm the epitope specificities suggested by the immunoprecipitation analysis, binding competition assays were performed with the four mAbs and a previously characterized anti- $\beta 3$ mAb, 2C9.G2. As summarized in table 1, the five antibodies could be divided into two groups based on their ability to compete with each other for binding. 8-B3 and 4-10D effectively competed with each other for binding and failed to compete with 8-2D, 8-B11 and 2C9.G2 for binding. Accordingly, 8-2D, 8-B11 and 2C9.G2 competed with each other for binding and failed to compete with 8-B3 and 4-10D for binding. These results are in agreement with the specificities assigned based on the immunoprecipitation data.

To further delineate the specificities of the four mAbs, resting platelets were stained and analyzed by flow cytometry. Resting platelets are known to express the $\beta 3$ chain as part of the fibrinogen receptor and to express much lower levels of the αv chain (23, 24). As shown in Fig. 2, resting platelets were negative for staining with the 8-B3 and 4-10D mAbs, and were positive for staining with the 8-2D and 8-B11 mAbs. Taken together, the immunoprecipitation results, binding competition data and platelet staining analysis strongly suggest that 8-B3 and 4-10D specifically bind to the αv chain and that 8-2D and 8-B11 specifically bind to the $\beta 3$ chain.

Inhibition of substrate binding

The anti-vitronectin receptor antibodies were selected by a functional assay and therefore it was of interest to determine which receptor-ligand interactions are inhibited by antibody binding. As shown in Fig. 3A, BW5147

cells adhere strongly to vitronectin and to a lesser degree, to fibronectin and fibrinogen. Cell adhesion to vitronectin was specifically inhibited by addition of 8-B3, 4-10D, 8-2D or 8-B11 antibodies and by addition of RGD peptide (Fig. 3B and C). Similar results were obtained for fibronectin and fibrinogen binding (data not shown), indicating that the spectrum of ligand binding that is inhibited by the αv and $\beta 3$ antibodies matches the previously characterized broad binding specificity of the $\alpha v\beta 3$ vitronectin receptor.

Expression of αv and $\beta 3$ chains on primary lymphocytes

To examine the expression of the αv and $\beta 3$ chains on lymphocytes *in vivo*, cell preparations from thymus, spleen and bone marrow of 8 week old C57BL/6 mice were stained with the 8-B3 and 8-B11 mAbs in combination with a variety of other mAbs and analyzed by flow cytometry. In thymus, CD3⁺ cells were positive for staining with both 8-B3 and 8-B11, while the majority of CD3⁻ cells exhibited significantly lower staining with both mAbs (Fig. 4A), suggesting that in the thymus, $\alpha v\beta 3$ is primarily expressed on mature thymocytes. In spleen, CD3⁺ T cells were stained by both 8-B3 and 8-B11, and there appeared to be differential expression of $\alpha v\beta 3$ on CD4⁺ and CD8⁺ cells as CD4⁺ cells stained more brightly than CD8⁺ cells with both mAbs (Fig. 4 B, C and D).

In bone marrow, B220⁻ cells could be divided into two populations based on staining with 8-B3 and 8-B11 (Fig. 4F). The 8-B3 and 8-B11 positive population appear to be precursors of granulocyte/monocyte lineage and the 8-B3 and 8-B11 negative population appear to be erythroid precursors based on forward and side scatter properties of the cells (data not shown). The

B220^{low} pre B cells and the B220^{high} B cells in the bone marrow were positive for staining with 8-B3 and exhibited detectable staining on some cells with 8B11 compared to negative control mAbs (Fig. 4F). A similar staining profile was observed for B220⁺ B cells in the spleen (Fig. 4E).

Stainings of each of the above cell populations were performed in parallel with 4-10D, 8-2D and 2C9.G2 and consistent results were obtained (data not shown).

Expression of $\alpha\upsilon$ and $\beta 3$ chains on CD4⁻CD8⁻ thymocytes

In the stainings of the thymus, there appeared to be a small population of CD3⁻ cells that were positive for staining with 8-B3 and 8-B11. Since CD3⁺ thymocytes were positive for $\alpha\upsilon$ and $\beta 3$ expression, and CD3⁻ thymocytes, which consist largely of the CD4⁺CD8⁺ double positive population, were low for $\alpha\upsilon$ and $\beta 3$ expression, it was of interest to determine whether this small population consists of the CD4⁻CD8⁻ thymocytes, which could express $\alpha\upsilon$ and $\beta 3$ prior to their development into CD4⁺CD8⁺ cells. For this purpose, thymocytes from Rag1 deficient mice, in which T cell development is blocked at the CD4⁻CD8⁻ stage (17), were stained with 8-B3 and 8-B11. As shown in Fig. 5, immature thymocytes from Rag1⁻ mice were positive for staining with both 8-B3 and 8-B11, indicating that $\alpha\upsilon\beta 3$ is expressed at the CD4⁻CD8⁻ stage of T cell development. Thus a modulation of expression of $\alpha\upsilon\beta 3$ appears to occur in T cell development, with CD4⁻CD8⁻ cells expressing high levels of $\alpha\upsilon$ and $\beta 3$, CD4⁺CD8⁺ cells expressing much reduced levels of $\alpha\upsilon$ and $\beta 3$, and mature CD3⁺ cells expressing significant levels of $\alpha\upsilon$ and $\beta 3$.

Discussion

We have generated four mAbs, 8-2D, 8-B3, 4-10D and 8-B11, specific for the murine $\alpha v\beta 3$ vitronectin receptor. Immunoprecipitation analysis, binding competition assays and platelet staining analysis indicate that 8-B3 and 4-10D are specific for the αv chain, and 8-2D and 8-B11 are specific for the $\beta 3$ chain. These mAbs were identified based on their ability to block constitutive IL-2 secretion of V $\gamma 1$, V $\delta 6$ $\gamma \delta$ T cell hybridomas, confirming previous work that demonstrated the involvement of the vitronectin receptor in this spontaneous IL-2 production (15). Studies in which RGD peptides were shown to inhibit such spontaneous IL-2 secretion (15), and transfection experiments in which a V $\gamma 1$, V $\delta 6$ TCR was expressed in cells negative for expression of the vitronectin receptor (25), have led to the notion that engagement of the vitronectin receptor is a required coreceptor interaction for this spontaneous response. More recently, it was proposed that engagement of the vitronectin receptor leads to activation directly through the CD3 ζ chain and that the V $\gamma 1$ -C $\gamma 4$ chain merely facilitates the interaction between $\alpha v\beta 3$ and CD3 ζ (26).

The issue of whether the vitronectin receptor functions in V $\gamma 1$ cell activation *in vivo* awaits the development of a system to study responses of primary V $\gamma 1$ cells and identification of potential ligands that activate primary V $\gamma 1$ cells. However, we were able to detect surface expression of the $\alpha v\beta 3$ receptor on V $\gamma 1$ transgenic thymocytes and splenocytes, (unpublished observations) consistent with a possible function of this receptor on V $\gamma 1$ cells *in vivo*.

Since the mAbs were selected with a functional blocking assay, they were likely to block the binding interactions between $\alpha v\beta 3$ and its ligands.

We have shown that 8-B3, 4-10D, 8-2D and 8-B11, can each effectively block cell adhesion to vitronectin, fibronectin and fibrinogen. These mAbs should prove useful for delineating the involvement of the αv and $\beta 3$ chains in various cell adhesion interactions.

We have used the four mAbs to study the expression of the αv and $\beta 3$ chains on lymphocytes *in vivo*. Previous studies suggested that $\alpha v\beta 3$ is not expressed on thymocytes and splenocytes and that expression is only detectable following cell activation or long term *in vitro* culture (13, 16). In contrast to these findings, we observed expression of both αv and $\beta 3$ on certain populations of thymocytes and splenic B and T cells. We also observed expression of αv and $\beta 3$ on certain populations of bone marrow cells, consistent with previous reports (13, 16). Staining intensities of most of the positive cell populations, and particularly of B cells and pre B cells, were higher for the anti- αv mAbs than for the anti- $\beta 3$ mAbs, raising the possibility that on some lymphocyte populations, receptors composed of the αv chain complexed with β chains other than $\beta 3$ may be expressed. Such receptors as $\alpha v\beta 1$ and $\alpha v\beta 5$ have been previously described (27, 28).

Stainings of some of the lymphocyte populations with the anti- $\alpha v\beta 3$ mAbs, and particularly of pre B and B cells with the anti- $\beta 3$ mAbs were of low intensity, however we believe that they represent surface expression of αv and $\beta 3$ on these cells *in vivo*, because we observed similar staining patterns with two independent anti- αv and 3 independent anti- $\beta 3$ mAbs. We cannot formally exclude the possibilities that the stainings represent low level cross reactivity with different antigens or non-specific sticking of each of the mAbs to certain cell populations, however we find these interpretations unlikely. Therefore, it is likely that $\alpha v\beta 3$ is expressed on resting CD3⁺ T cells, B cells and pre B cells *in vivo*.

There are two possible explanations for the difference between these results and the previous studies. First, it is possible that the mAbs we selected have higher affinity for $\alpha v\beta 3$ than those used previously. Second, we used biotinylated mAbs followed by addition of streptavidin phycoerythrin to detect expression, which is generally more sensitive than direct detection with FITC- coupled mAbs, as was utilized in the previous reports. In support of these explanations, we were also unable to detect expression on primary lymphocytes with FITC- coupled mAbs, and we did observe higher expression of αv and $\beta 3$ on activated lymphocytes relative to primary resting cells (unpublished observations).

Previous reports have described developmentally regulated expression of the $\beta 4$ integrin and fibronectin receptors on mouse thymocytes (29, 30).

Interestingly, we found that expression of $\alpha v\beta 3$ also appears to be modulated during T cell development. Thus we observed high expression of $\alpha v\beta 3$ on $CD4^-CD8^-$ thymocytes, while $CD4^+CD8^+$ thymocytes displayed considerably lower $\alpha v\beta 3$ expression, and mature $CD3^+$ thymocytes and peripheral T cells were positive for $\alpha v\beta 3$ expression. The differential expression of $\alpha v\beta 3$ on discrete populations of thymocytes raises the possibility that this receptor is functionally involved in T cell development. The mAbs described here should prove useful in further studying this issue.

Table 1: Binding competition

	8-B3	4-10D	8-2D	8-B11	2C9.G2
8-B3	+	+	-	-	-
4-10D	+	+	-	-	-
8-2D	-	-	+	+	+
8-B11	-	-	+	+	+
2C9.G2	-	-	+	+	+

Binding competition analysis was performed as described in the methods section. Cells were preincubated with the mAbs listed in the far left column and were then stained with the mAbs listed in the top row. Competition for binding is indicated by "+" and lack of competition for binding is indicated by "-".

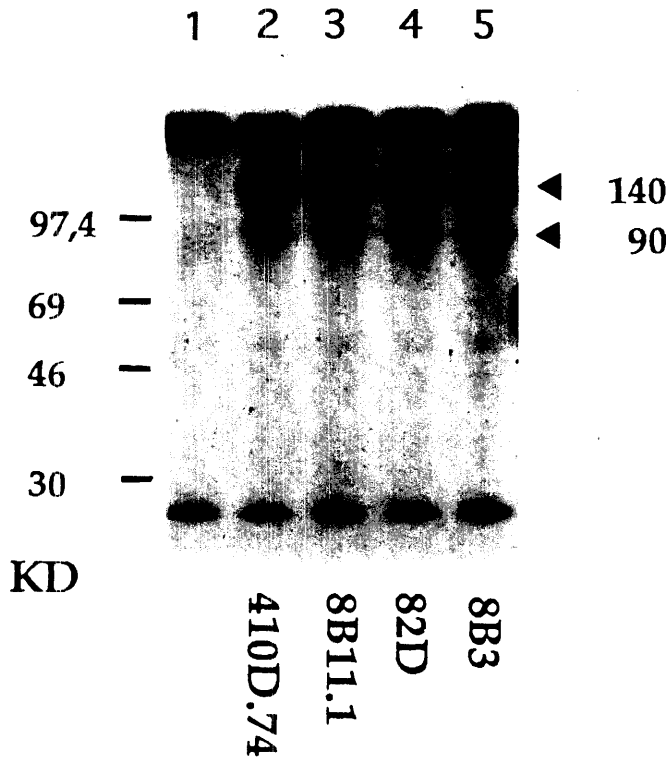
Figure 1:

A) SDS-PAGE analysis of immunoprecipitates from lysate of ^{125}I -labeled hybridoma cells run under non-reduced conditions. Lanes: 1, beads alone; 2, 4-10D; 3, 8-B11; 4, 8-2D; 5, 8-B3. Molecular weight standards are indicated in kilodaltons.

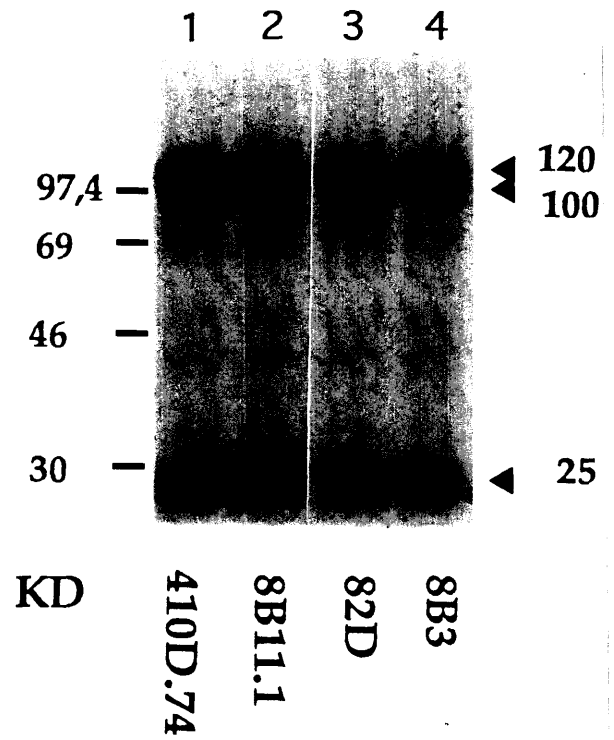
B) SDS-PAGE analysis of immunoprecipitates from lysate of ^{125}I -labeled hybridoma cells run under reduced conditions. Lanes: 1, 4-10D; 2, 8-B11; 3, 8-2D; 4, 8-B3. Molecular weight standard are indicated in kilodaltons

C) SDS-PAGE analysis of immunoprecipitates from lysates of ^{125}I labeled hybridoma cells and E9 fibroblasts and mixed lysate, run under non-reduced conditions. Lanes: 1, E9 fibroblast lysate, anti- αv serum; 2, hybridoma lysate, anti- αv serum; 3-9, mixed lysate precipitated with: 3, anti- αv serum; 4, anti- β1 serum; 5, anti- β3 serum; 6, 8-2D; 7, 8-B3; 8, 4-10D; 9, 8-B11. Lanes 1-4 were exposed for a shorter time than lanes 5-9, because the anti- αv and anti- β1 sera immunoprecipitate much more efficiently than the other antibodies. T3 = hybridoma cells; E9 = embryonic d9 fibroblasts; M = mixed lysate

A



B



C

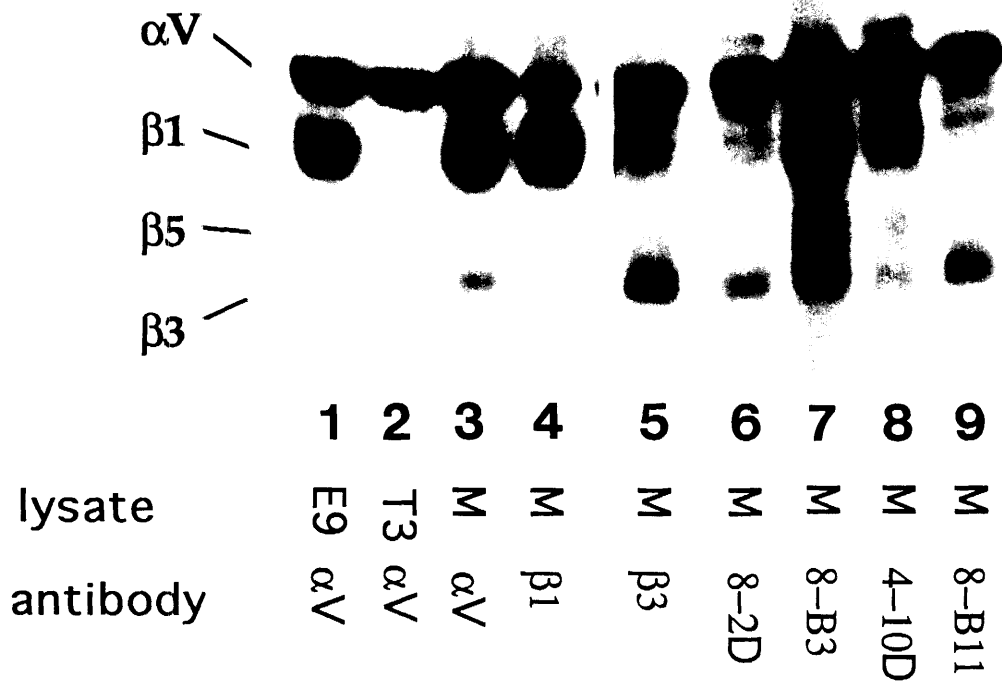


Figure 2:

FACS analysis of platelets with the anti- $\alpha v\beta 3$ mAbs. Platelets were diluted in Tyrode buffer (5mM hepes, 150 mM NaCl, 2.5 mM KCl, 5.5 mM glucose 0.1% BSA) and incubated with the indicated FITC-labeled antibodies for 30 min on ice. The cells were further diluted and analysed with a FACScan without washing to avoid aggregation. Shown is the fluorescence intensity profile of cells stained with the 8-B3 and 8-2D mAbs. The profile observed with the the 8-2D mAb was similar to that observed with 8-B11 and with that of an anti- δ TCR mAb used as a negative control (not shown). Similarly, the profiles obtained with the 8-B3 and 4-10D mAbs were almost indistinguishable.

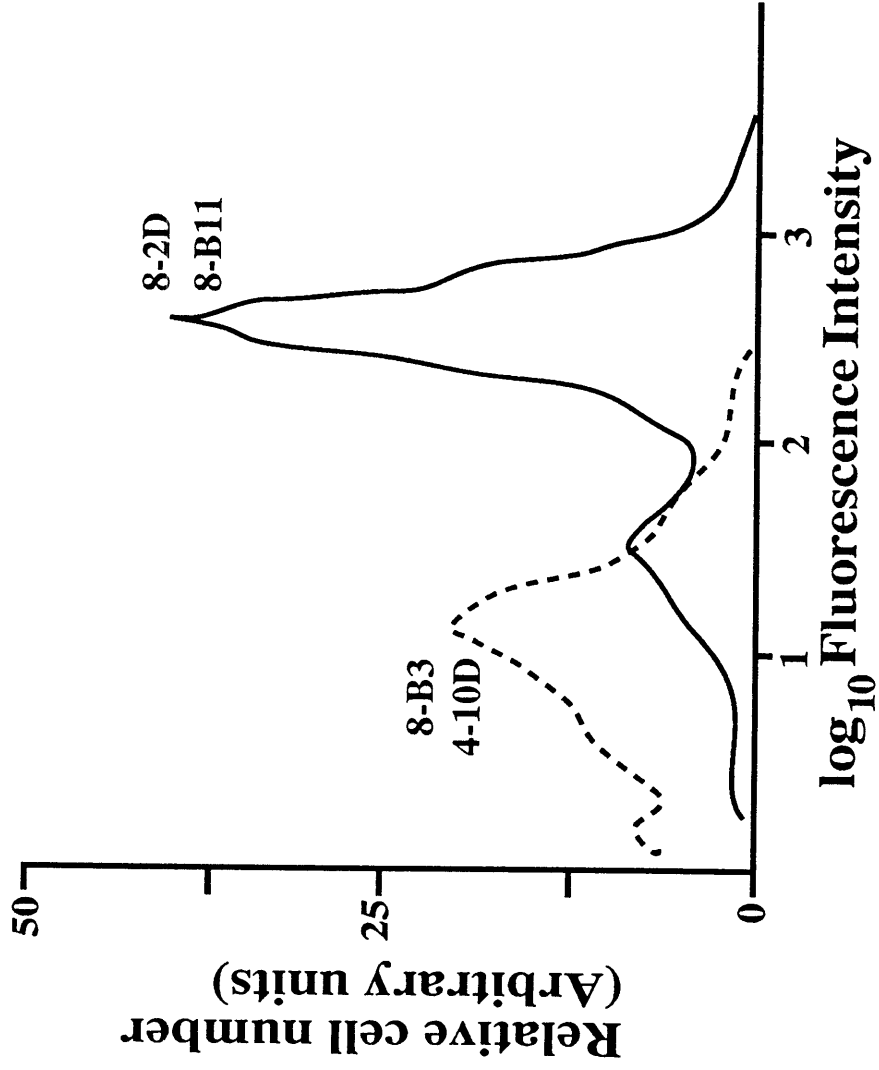


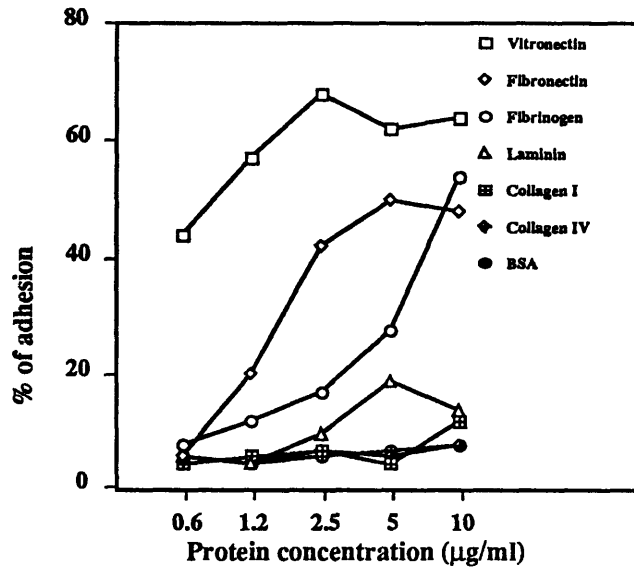
Figure 3:

A) Adhesion of BW5147 cells to ECM-proteins. BW5147 cells (5×10^4 /well) were incubated in wells previously coated with increasing concentrations of the indicated ECM-proteins. The wells were washed and the remaining cells incubated with MTT. Percent adherence was calculated as described in the Methods section.

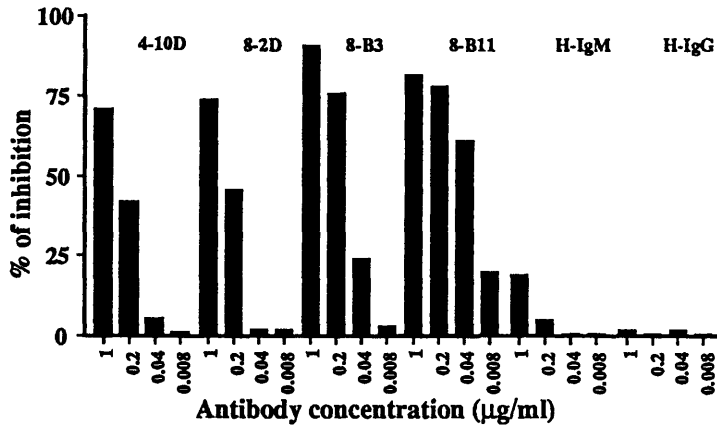
B-C) Inhibition of adhesion to vitronectin by anti- $\alpha v \beta 3$ mAbs (B) and GRGDSP and GRGESP peptides (C). BW5147 cells were assayed for adhesion to wells coated with vitronectin ($1 \mu\text{g}/\text{ml}$) as described for (A), after preincubation with and in the continuous presence of the indicated mAbs or peptides. H-IgM and H-IgG denote irrelevant hamster IgM and IgG mAbs respectively. One of three independent experiments is shown. Similar results were obtained with wells coated with fibronectin ($5 \mu\text{g}/\text{ml}$) or fibrinogen ($10 \mu\text{g}/\text{ml}$) and when a V γ 1-expressing hybridoma was used instead of the BW5147 cell line.

Cell adhesion properties and inhibition of adhesion with anti- α V β 3 mAbs and RGD peptide

A



B



C

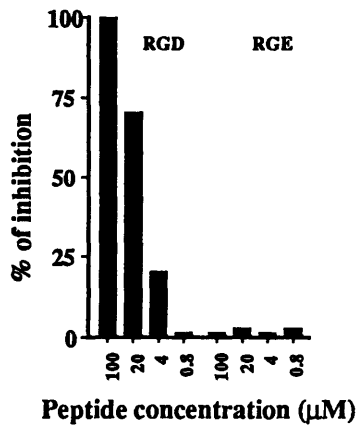


Figure 4:

Expression of $\alpha\beta3$ on different lymphoid populations. Thymus (A), Spleen (B,C,D and E) and bone marrow cells (F) were stained with the indicated antibodies as described in the Methods section and analyzed with a FACScan. The 8-B3, 8-B11 and 1.9 mAbs were used as biotin conjugates and their binding was detected with streptavidin-PE (vertical axes). The CD3 (A and B), CD4 (C), CD8 (D) and B220 mAbs (E and F) were used as FITC conjugates (horizontal axes). 1.9 is a hamster IgG anti- $\gamma\delta$ TCR clonotype mAb used as a negative control. Consistent results were obtained with the 4-10D, 8-B11 and 2C9.G2 mAbs., although staining levels of some populations such as the CD3⁺ thymocytes were higher with 4-10D than 8-B3.

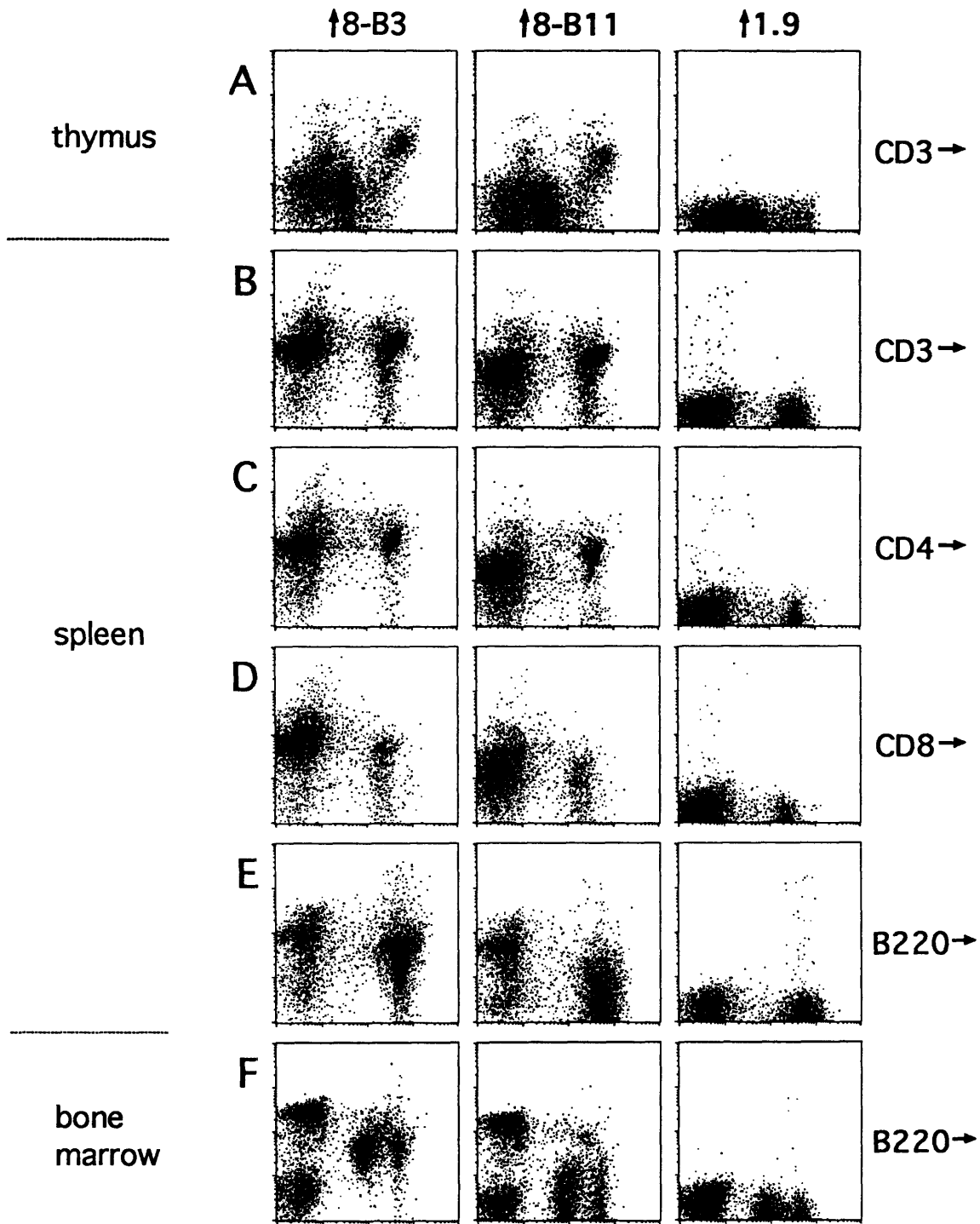
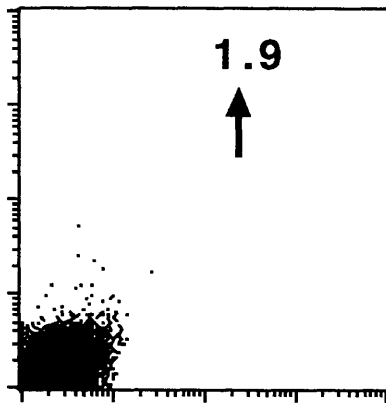
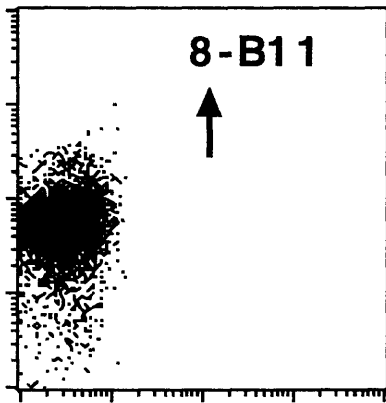
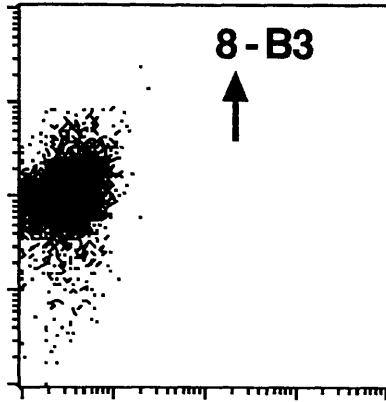
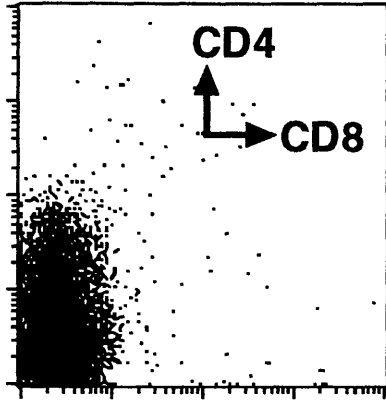


Figure 5:

Expression of $\alpha\gamma\beta 3$ on CD4⁻CD8⁻ thymocytes. Thymocytes from Rag1 deficient mice were stained with the indicated antibodies as described in the Methods section. The 8-B3, 8-B11 and 1.9 mAbs were used as biotin conjugates and their binding was detected with streptavidin-PE. 1.9 is a hamster IgG anti- $\gamma\delta$ clonotype mAb used as a negative control.



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Chapter 5: Generation and characterization of V γ 1, V δ 6 transgenic mice.

Abstract

In order to study the reactivity of primary V γ 1, V δ 6 $\gamma\delta$ T cells, we have generated mice transgenic for the rearranged γ and δ TCR chain genes from a V γ 1, V δ 6 $\gamma\delta$ T cell hybridoma, T3.13.1. The transgenic mice have increased numbers of V γ 1 expressing T cells in thymus and spleen, and most of these cells express the T3.13.1 $\gamma\delta$ TCR clonotype. The transgenes appear to be specifically expressed in the $\gamma\delta$ lineage, and transgene expression does not appear to disrupt development of other lymphoid populations in the mice. Despite their correctly restricted expression pattern, presence of the transgenes in the Rag1(-,-) background rescues development of CD4⁺CD8⁺ double positive cells. One interesting feature of the transgenic cells is their constitutive expression of the IL-2 receptor β chain which renders them susceptible to activation by high levels of IL-2. The transgenic cells appear to have a resting phenotype *in vivo*, but are competent to undergo a proliferative response to TCR crosslinking. Attempts to demonstrate reactivity of the transgenic cells to mycobacterial antigens have been unsuccessful.

Introduction

Studies of murine V γ 1 expressing $\gamma\delta$ T cell hybridomas have suggested that this class of cells may respond to mycobacterial antigens (1, 2). In particular, it was reported that these cells recognize mycobacterial Hsp 65, a major antigenic component of PPD (3). One problem with these studies was the high background response of the V γ 1 hybridomas which secrete significant levels of IL-2 in the absence of exogenous antigen. This high background activity complicates the interpretation of antigenic stimulation, raising the possibility that the added antigen is merely non-specifically augmenting an already existing response rather than providing specific antigenic stimulation. The background response of the V γ 1 hybridomas could be an artifact of the fusion process, which results in the $\gamma\delta$ TCR being expressed in the context of a tumor cell line derived from an allogeneic strain.

Previously, a transgenic approach was useful for confirming and extending the analysis of the response of a V γ 4 expressing hybridoma to a TL gene product (4). In this case, primary transgenic cells were shown to respond to the same molecule as the hybridoma from which the γ and δ transgenes were isolated. In addition, the ability to study cells bearing the transgenic receptors *in vivo* provided evidence for positive and negative selection of $\gamma\delta$ T cells (5, 6).

To further study the reactivity of V γ 1 expressing $\gamma\delta$ T cells, we have created mice transgenic for the rearranged γ and δ chain genes from a spontaneously reactive V γ 1, V δ 6 hybridoma, T3.13.1. These mice contain greatly increased numbers of V γ 1 T cells in the thymus and peripheral lymphoid organs, and the vast majority of these cells express the T3.13.1 $\gamma\delta$ TCR clonotype, as indicated by staining with the anti-clonotypic mAb, 1.9. The transgenic $\gamma\delta$ T cells do not appear to be constitutively activated *in vivo*, but despite the absence of background activity, we have been unable to observe a convincing response of these cells to mycobacterial antigens. Several interesting features of the transgenics include the ability of the transgenes to rescue development of CD4⁺CD8⁺ cells in the Rag1(-,-) background, and the constitutive expression of the IL-2 receptor β chain on the transgenic cells.

Materials and Methods

Mice: C57Bl/6 (B6), B6/CBA F1, and B6/DBA2 F1 mice were obtained from Jackson Laboratory (Bar Harbor, ME) or Taconic (Germantown, NY) Rag1 deficient mice have been previously described (7). TCR β deficient mice have been previously described (8).

Antibodies: The mAbs, 145-2C11 and 500 A2 (anti-CD3 ϵ), RM4-5 (anti-CD4), 53-6.7 (anti-CD8), TM- β 1 (anti-CD122), H1.2F3 (anti-CD69) 3C7 (anti-CD25), RA3-6B2 (anti-B220), R6-60.2 (anti-mouse IgM), 53-2.1 (anti-Thy1.2) and GL4 (anti TCR $\gamma\delta$) were purchased from Pharmingen (San Diego, CA) in purified form or as PE, or FITC conjugates. The mAb, 2.11, is described in detail in chapter 3 of this thesis. The mAb, 1.9, is a hamster IgG specific for the T3.13.1 $\gamma\delta$ TCR clonotype. It was purified by protein G affinity chromatography and conjugated to FITC according to standard procedure.

Primers: The primers used for PCR typing of the transgenics were:

V γ 1: 5'-CCGGCAAAAAGCAAAAAGTT-3'

J γ 4(T3.13.1): 5'-CCCATGATGTGCCTGACCAG-3'

V δ 6: 5'-TCAAGTCCATCAGCCTTGTC-3'

J δ 1(T3.13.1): 5'GTCCCGTATCCCTCCGATAG-3'

The primers used for generating PCR fragments for probes were:

V γ 1: 5'-GCTCCTGAGATGGCCCACCTT-3'

J γ 4: 5'-GCAAATATCTTGACCCATGA-3'

V δ 6: 5'-TGTGGCCCAGAAAGTGACCTC-3'

J δ 6: 5'-TTGGTTCCACAGTCACTTGG-3'

Generation of transgene constructs: To isolate genomic DNA fragments containing the rearranged V γ 1 and V δ 6 genes with sufficient flanking sequence to promote expression, a cosmid library was constructed from genomic DNA derived from the T3.13.1 hybridoma. To prepare genomic DNA, cells were resuspended in digestion buffer (100mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K) at 10^8 cells per ml. The samples were incubated overnight at 56° C, followed by extractions with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol. DNA was then ethanol precipitated and resuspended in TE (10 mM tris HCl pH 7.6, 0.1mM EDTA). Special care was taken during extractions and resuspending to avoid shearing the DNA. Purified genomic DNA was then

partially digested with *Sau3A I*, and the partially digested DNA was fractionated over a 1M to 5M gradient of NaCl. Fractions were dialyzed against TE and ethanol precipitated to remove NaCl, and aliquots were run on a 0.3% agarose gel to estimate size. Fractions within the approximate range of 30 - 60 k B were pooled and a portion was ligated into *BamH I* linearized, phosphatased pWE 15 cosmid vector (Stratagene, La Jolla, CA)). The ligation was packaged using the Gigapack gold packaging kit (Stratagene) , and the packaged library was used to infect XL1-Blue MR cells (Stratagene). The library was screened with PCR generated probes spanning the V-J junctions of the *V γ 1* and *V δ 6* genes from the T3.13.1 hybridoma. Positive clones were purified, mapped and used to generate the transgenic constructs. γ 2F was created by ligating a 15 k B *EcoR I-SnaB I* fragment derived from one cosmid with a ~ 30 k B *SnaB I-Not I* fragment from another cosmid and then cloning this ~ 45 k B fragment into pWE15. The δ 10.1 construct was isolated directly from the cosmid library, but it had to be subcloned into a version of pWE15 in which *Pme I* sites had been added to allow for isolation of the entire intact insert for microinjection.

Generation of transgenic mice: Transgene constructs were isolated by agarose gel electrophoresis followed by electroelution. The eluted DNA was extracted twice with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and ethanol preipitated. The DNA was resuspended in Dulbecco's PBS (Gibco/BRL, Gaithersburg, MD) and the two constructs were mixed together with each at a final concentration of 5-10 ng/ μl . This DNA preparation was injected into the pronuclei of fertilized embryos derived from intercrossing B6/CBA F1 mice or the pronuclei of fertilized B6 embryos. Embryos were transferred into pseudopregant B6/DBA2 F1 recipients. Resulting pups were screened by PCR with primers specific for the transgenes. Transgenic founders were bred with B6 mice to establish transgenic lines.

Immunofluorescence staining and flow cytometric analysis: Cells (10^5 - 10^6) were incubated in staining buffer (PBS, 2% FCS, 0.1% NaN $_3$) with the indicated labeled mAbs for 45'-60' at 4 $^\circ$ C. After two washes, viable cells were analyzed using a FACScan flow cytometer. Dead cells were gated out using propidium iodide. For cell sorting, cells were incubated at a concentration of 4×10^7 /ml with the indicated labeled mAb. After two washes, the cells were

resuspended at a concentration of 10^7 /ml and sorted at the MIT Center for Cancer Research cell sorting facility. Dead cells were excluded by propidium iodide staining.

Cell proliferation assays: Cells were cultured under the indicated conditions in 96 well plates. Proliferation assays were done in triplicate. PPD was obtained either from the World Health Organization or purchased from Japan BCG laboratories. M. Bovis Hsp 65 was obtained from the World Health Organization. Monoethyl phosphate and Isopentenyl pyrophosphate were a gift from Craig Morita and the Brenner lab. Recombinant IL-2 was either purchased from Gibco-BRL or obtained as a gift from Ajinomoto. On the indicated days, wells were pulsed for 6 hrs with $1 \mu\text{Ci } ^3\text{H-thymidine}$ per well. Cultures were harvested onto gridded fiberglass filters (Wallac, Turku, Finland) using a Tomtec 96 well plate harvester and filters were counted using a Betaplate liquid scintillation counter (Pharmacia-LKB, Uppsala, Sweden).

Results

Generation of V γ 1, V δ 6 transgenic mice

In order to create transgene constructs encoding a functional V γ 1, V δ 6 $\gamma\delta$ TCR, a cosmid library was generated from genomic DNA isolated from the V γ 1 expressing hybridoma, T3.13.1, and screened with probes specific for the V γ 1 and V δ 6 genes. This hybridoma was chosen primarily because of the availability of an anti-clonotypic mAb, 1-9, specific for the T3.13.1 TCR. The constructs used to create transgenic mice are shown in Fig. 1A. γ 2F was generated by ligating together two fragments derived from independent cosmids containing the T3.13.1 rearranged V γ 1-C γ 4 locus. δ 10.1 is the insert of a single cosmid containing the T3.13.1 rearranged V δ 6-C δ locus.

Initially B6/CBA F2 embryos were coinjected with γ 2F and the ~30kB internal Not1 fragment of δ 10.1. Fig. 1B shows the results of PCR analysis of tail DNA from six founder lines (odd lanes) and littermates (even lanes) using primers specific for the γ and δ transgenes. Among these lines, four contained both the γ and δ transgenes and 2 contained only the γ transgene. The internal Not1 site in δ 10.1 was subsequently shown to lie downstream of the beginning of the V δ 6 region, and therefore, the δ transgene utilized for these injections is not functional. Thus these transgenics derived from the first series of injections can be considered γ only transgenics.

To produce transgenic mice bearing both functional transgenes, B6 embryos were co-injected with γ 2F and the entire ~35 kB δ 10.1 insert. Fig. 1C shows the results of PCR analysis of tail DNA from three founder lines (odd lanes) and littermates (even lanes), using the primers specific for the transgenes. Among these lines, one contained both the γ and δ transgenes and two contained only the δ transgene.

Expression of transgenes

As shown in Fig 2A, the 5-8P mice that are transgenic for the γ 2F transgene and the truncated δ 10.1 transgene contain roughly 10-fold higher numbers of V γ 1 cells in thymus and spleen relative to non-transgenic controls (Fig. 2C). The V γ 1 cells in these mice are all negative for staining

with the anti-clonotype mAb, 1.9 (Fig. 2A). The vast majority of the $\gamma\delta$ cells in these mice express V γ 1 and utilize rearranged endogenous δ chain genes, including the V δ 4 and V δ 6 genes to encode their $\gamma\delta$ TCR (data not shown). Therefore these mice contain an increased and diverse population of V γ 1 cells. The other lines bearing γ 2F alone, or in combination with the truncated δ 10.1 transgene, all displayed increased numbers of V γ 1 cells, similar to 5-8P (data not shown).

The A-2^m mice that are transgenic for the γ 2F transgene and the full length δ 10.1 transgene also contain greatly increased numbers of V γ 1 cells in thymus and spleen relative to non-transgenic control mice (Fig. 2 B and C), and essentially all of these V γ 1 cells express the T3.13.1 TCR as defined by staining with the anti-clonotype mAb, 1.9 (Fig. 2C).

B cell and $\alpha\beta$ T cell populations appear normal in V γ 1 transgenic mice

To determine whether expression of the V γ 1 and V δ 6 transgenes affects the development of other lymphoid populations, thymocytes and splenocytes from 6 wk. old A-2^m mice were stained with several mAbs and analyzed by flow cytometry. As shown in Fig 3A, $\alpha\beta$ thymocyte development appears normal in the A-2^m transgenics as stainings for CD4 versus CD8 are very similar in transgenics and littermates. Similarly, $\alpha\beta$ T cells and B cells in the spleen appear normal in A-2^m transgenic mice as stainings of splenocytes for CD4 versus CD8 and B220 versus IgM are indistinguishable between transgenics and littermates (Fig 3B). Similar results were obtained for the γ only transgenics. (data not shown). The transgenes appear to be expressed specifically in the $\gamma\delta$ lineage, as $\alpha\beta$ cells are negative for transgene expression (data not shown).

The effect of the transgenes on development of endogenous $\gamma\delta$ cells is less well defined. In some of the γ only lines such as 5-8P, $\gamma\delta$ cells are almost exclusively of the V γ 1 class, indicating that the expression of the V γ 1 transgene excludes expression of endogenous $\gamma\delta$ TCRs. However, in the A-2^m line, there are detectable numbers of V γ 1 negative $\gamma\delta$ T cells, suggesting that exclusion of endogenous $\gamma\delta$ TCR expression could be copy number or integration site dependent.

Presence of both $\gamma 2F$ and $\delta 10.1$ transgenes rescues $CD4^+CD8^+$ cells in Rag1 deficient mice

In order to generate mice with a pure population of transgenic T cells, the A-2^m mice were crossed with Rag1 deficient mice to produce A-2^m/Rag1(-,-) mice. The thymi of these mice are noticeably larger than those of non-transgenic Rag1(-,-) mice in which $\alpha\beta$ thymocyte development is blocked at the $CD4^-CD8^-$, double negative stage (7). To determine whether the increased thymus size in the A-2^m/Rag1(-,-) mice reflects development of $CD4^+CD8^+$ cells, thymocytes from A-2^m/Rag1(-,-) mice were stained with several mAbs and analyzed by flow cytometry. Fig. 4 shows an example of one such staining. As expected, a significant population of $CD3^+$ thymocytes, all of which are 1.9⁺, is present in A-2^m/Rag1(-,-) mice (Fig. 4A), while no $CD3^+$ thymocytes are present in non-transgenic Rag(-,-) mice (Fig. 4B). The majority of thymocytes in the A-2^m/Rag1(-,-) mice are $CD4^+CD8^+$, and most if not all of these double positive cells are 1.9⁻ (Fig. 4A). By contrast, no $CD4^+CD8^+$ thymocytes are present in non transgenic Rag1 (-,-) littermates (Fig. 4B). Thus, presence of the $\gamma 2F$ and $\delta 10.1$ transgenes rescues development of $CD4^+CD8^+$ cells in the Rag1(-,-) background.

A-2^m transgenic cells have resting phenotype in vivo

Since many V γ 1 hybridomas, including T3.13.1, appear to be constitutively activated, it was of interest to examine the activation status of A-2^m transgenic cells. Activation of $\alpha\beta$ cells results in expression of surface activation markers, including CD69 and CD25 (9, 10), and in cell proliferation. To examine the activation status of the transgenic cells, 1.9⁺ splenocytes from A-2^m mice were analyzed for surface expression of CD69 and CD25 *ex vivo* and following stimulation *in vitro* with concanavalin A (ConA). As with $\alpha\beta$ T cells, unstimulated A-2^m transgenic cells are negative for surface expression of CD69, and expression is strongly induced following ConA stimulation (Fig. 5A). Similar results were obtained for expression of CD25 (Fig. 5B). To examine the proliferative capacity of A-2^m transgenic cells, Thy 1⁺ cells were sorted from A-2^m/TCR β (-,-) total spleen cells and cultured with 10 U/ml recombinant IL-2, in the absence or presence of $\gamma\delta$ TCR crosslinking. Cells

sorted in this manner are >80% 1.9⁺. As shown in Fig. 5C, transgenic cells do not proliferate in the absence of stimulation, but proliferate strongly in response to $\gamma\delta$ TCR crosslinking. Thus A-2^m transgenic cells appear to be in a resting state *in vivo*, but have the capacity to respond to stimulation through their TCR.

A-2^m transgenic cells express CD122 and proliferate in response to high IL-2 concentration

Although A-2^m cells appear to behave like $\alpha\beta$ cells with respect to expression of the CD69 and CD25 activation markers, they differ from $\alpha\beta$ cells in expression of some other activation markers such as the IL-2 receptor β chain, CD122. As shown in Fig. 6A, most $\alpha\beta$ cells from A-2^m mice are dull or negative for CD122 expression while the majority of 1.9⁺ cells are CD122⁺. Total V γ 1 cells appear to have a bimodal distribution with respect to CD122 expression, as the peak of positive cells has an obvious shoulder representing a population with lower expression (Fig. 6B, top panel). These two populations are also apparent among V γ 1⁺ cells from 5-2^m γ only transgenic mice, in which the V γ 1 chain is uniform and the δ chains are diverse (Fig. 6B, middle panel). In contrast, the homogeneous A-2^m transgenic cells appear to have more uniformly positive expression of CD122 (Fig. 6A and 6B bottom panel).

Some CD8⁺ $\alpha\beta$ T cells and NK cells also express CD122 constitutively (11) and can be activated by exposure to high IL-2 concentration. To determine whether the IL-2 receptor β chain on the A-2^m transgenic cells is functional, purified A-2^m transgenic cells were cultured with increasing concentrations of recombinant IL-2 and assayed for proliferation. As shown in Fig. 7A, A-2^m cells proliferate in response to high IL-2 concentration, with a large increase in proliferative response occurring between 100 and 500 U/ml. This result is consistent with the function of the intermediate affinity IL-2 receptor composed of the β and γ chains. To confirm that the proliferative response to IL-2 is dependent on the IL-2 receptor β chain, A-2^m cells were cultured with high IL-2 concentration in the presence of a series of three-fold dilutions of anti-CD122 mAb, or anti-CD4 mAb as a negative control. As shown in Fig. 7B, the anti-CD122 mAb blocked the proliferative response to IL-2 in a dose-

dependent manner, while the anti-CD4 mAb did not affect the proliferative response. Thus the proliferative response to IL-2 of the A-2^m transgenic cells is dependent on the function of the IL-2 receptor β chain.

A-2^m transgenic cells show modest response to mycobacterial antigens

Previous studies of hybridoma cells have suggested that V γ 1 T cells respond to mycobacterial PPD and specifically to mycobacterial Hsp 65 (1-3). To determine whether A-2^m transgenic cells respond to these antigens, total spleen cells from A-2^m/TCR β (-,-) mice were cultured in the presence of 50 μ g/ml PPD, 25 μ g/ml Hsp 65 or on plastic coated with the anti-V γ 1 mAb, 2-11. As shown in Fig. 8A, A-2^m transgenic cells respond vigorously to stimulation by 2-11, with strong increases in cell size, CD69 expression and CD25 expression. The A-2^m cells exhibit a significant increase in CD69 expression in response to PPD and Hsp 65, however, increases in A-2^m transgenic cell size and CD25 expression in response to PPD and Hsp 65 are modest in comparison to the response to 2-11 stimulation (Fig. 8A). Thus while A-2^m cells may undergo an initial activation in response to these mycobacterial antigens, as evidenced by CD69 induction, the majority of cells do not appear to go on to form blasts. This conclusion is supported by analysis of the proliferative response of A-2^m cells to mycobacterial antigens. As shown in Fig 8B, A-2^m transgenic cells show a modest increase in proliferative response in the presence of PPD or Hsp 65 compared to conditions with no added antigen. However, this response is minor compared to the response of the cells to mAb mediated crosslinking of the $\gamma\delta$ TCR. Thus, it appears that A-2^m transgenic cells do not have a strong antigenic response to PPD or Hsp 65. We have also tested the response of A-2^m cells to the novel antigens for human V γ 2, V δ 2 $\gamma\delta$ cells such as monoethyl phosphate (MEP) (12) and isopentenyl pyrophosphate (IPP) (13). A-2^m cells did not proliferate in response to either MEP or IPP, suggesting that these compounds are not ligands for murine V γ 1, V δ 6 $\gamma\delta$ T cells.

Discussion

We have generated mice transgenic for the rearranged V γ 1 and V δ 6 genes expressed by the T3.13.1 hybridoma. An initial series of injections produced mice that express only the V γ 1 transgene in combination with endogenous rearranged δ chain genes. A second series of injections produced the A-2^m transgenic line that expresses the T3.13.1 clonotype, as evidenced by staining with the anti-clonotypic mAb, 1-9.

The γ only and A-2^m transgenic mice have greatly increased numbers of V γ 1 cells in thymus and spleen, as determined by 2-11 staining. None of the 2-11⁺ cells from the γ only mice are 1-9⁺, while essentially all of the 2-11⁺ cells from the A-2^m mice are 1-9⁺. Development of $\alpha\beta$ cells and B cells appears to be normal in the transgenic mice, as judged by staining analysis for CD4 versus CD8, and B220 versus IgM.

A previous report showed that expression of a transgenic V γ 5,V δ 1 $\gamma\delta$ TCR in the SCID mouse background restored the development of CD4⁺CD8⁺ double positive cells (14). We have found that expression of the A-2^m transgenic $\gamma\delta$ TCR partially restores development of CD4⁺CD8⁺ cells in the Rag1(-,-) background. Unlike the previous report, in which a significant percentage of the double positive cells were $\gamma\delta$ TCR⁺ (14), the vast majority of the double positive cells are $\gamma\delta$ TCR⁻ in our case. We are currently crossing the γ only and δ only transgenics into the Rag1(-,-) background to determine if either gene is sufficient to restore development of CD4⁺CD8⁺ cells, and preliminary results suggest that δ alone is not sufficient. The presence of the rearranged γ and δ transgenes could affect CD4⁺CD8⁺ development either indirectly, or directly through prior expression in the lineage that gives rise to the CD4⁺CD8⁺ cells. Further study of this issue could shed light on the factors involved in development of CD4⁺CD8⁺ cells, and factors involved in lineage determination of $\alpha\beta$ versus $\gamma\delta$ cells.

A large proportion of V γ 1 expressing hybridomas, including T3.13.1, are constitutively activated to secrete IL-2 (2), therefore it was of interest to determine if A-2^m transgenic primary cells are constitutively activated. We found that by several criteria, including CD69 and CD25 expression, and proliferative status, primary A-2^m transgenic cells appear to be in a resting, non-activated state *in vivo*. In addition, A-2^m cells are competent to be

activated by TCR crosslinking as indicated by CD69 and CD25 induction and proliferative response.

We found that transgenic A-2^m cells and a large proportion of total V γ 1 cells constitutively express the IL-2 receptor β chain, and respond to high IL-2 concentration. Recently the β and γ chains of the IL-2 receptor were shown to be components of the IL-15 receptor (15, 16). IL-15 has been shown to bind to NK cells which are positive for IL-2 receptor β chain expression (16). Since transgenic A-2^m cells share expression of IL-2 receptor β with NK cells, and we have found that a significant fraction of A-2^m transgenic cells express NK1.1 (unpublished observations), it was of interest to determine whether the transgenic A-2^m cells respond to IL-15. In preliminary experiments, we were unable to detect a response of A-2^m cells to recombinant human IL-15 either alone or in combination with $\gamma\delta$ TCR crosslinking. The possibility remains that the IL-2 receptor β chain is expressed by the A-2^m transgenic cells as part of a receptor for a novel interleukin other than IL-15. Biochemical analysis of the IL-2 receptor β chain expressed by the A-2^m cells could help to clarify this issue.

We have studied the response of the A-2^m transgenic cells to several mycobacterial antigens. CD69 expression is induced on the transgenic cells by exposure to PPD or Hsp 65, however induction of CD25 expression and proliferative response are small following stimulation with PPD or Hsp 65 compared to stimulation with anti- $\gamma\delta$ TCR mAbs. Thus while the cells display some initial activation in response to these antigens, the response does not proceed to a full blown, proliferative blastic response. The issue of whether the observed responses of the A-2^m transgenic cells to mycobacterial proteins represent specific antigenic responses is open to question. Addition of either PPD or *M. bovis* Hsp 65 to cultured A-2^m transgenic cells results in a major induction of CD69 expression and a lesser, but significant induction of CD25 expression. Furthermore, both PPD and Hsp 65 induce a proliferative response that is roughly three-fold higher than the background. Thus, addition of PPD or Hsp 65 to A-2^m cells induces a number of the hallmark features of T-cell antigenic responses. However, I believe that for a number of reasons, caution is in order in interpreting these data as representing a true antigenic response. First, while the induction of CD69 expression in response to antigen and mAb-mediated crosslinking is comparable, the magnitude of

both the increase in CD25 expression and the proliferative response is much lower following exposure to antigen than mAb-mediated TCR crosslinking. While it is not expected for responses to antigen to be equivalent to the maximal level induced by non-specific TCR crosslinking, the disparity observed here, particularly for increase in cell size and proliferative response is extreme. Second, both PPD and Hsp 65 are potent stimulators of other cells present among the APC's in the cultures. Thus, it is likely that addition of these antigens to cultures induces secretion of a variety of cytokines by the APC's that could indirectly stimulate the A-2^m cells. As described earlier, the A-2^m cells display a significant response to IL-2 in absence of antigen. Finally, these data do not include negative control experiments with a protein antigen that fails to stimulate the transgenic cells. Ideally, assays should be performed with other protein antigens including some that are known to stimulate various cells among the APC population. In the context of a lack of response of the A-2^m cells to various other protein antigens, the results with PPD and Hsp 65 could be interpreted more favorably. In the absence of these experiments, and after working extensively with these cells, it is my bias that the data do not represent true antigenic responses of the A-2^m cells to PPD or Hsp 65. However, the possibility that these results represent antigenic responses remains.

We have tried several different approaches to analyze potential reactivity of the transgenic V γ 1 cells to mycobacterial antigens, including immunization of transgenics with complete Freund's adjuvant, *in vitro* culture of transgenic cells with a variety of mycobacterial preparations and *in vitro* culture of transgenic cells with novel antigens for human $\gamma\delta$ T cells, such as MEP and IPP. We have been unable to observe consistent, convincing responses of the transgenic cells under any of the conditions analyzed, and conclude that these cells do not respond to mycobacterial antigens. Of course this analysis is limited by the fact that we are analyzing a specific population of V γ 1 cells, and by the fact that we do not understand the requirements for antigen presentation of $\gamma\delta$ cells or the nature of the $\gamma\delta$ response to antigenic stimulation. Hopefully these transgenics could contribute to our understanding of these more fundamental issues in $\gamma\delta$ T cell function in the future.

Figure 1. Generation of transgenic mice.

A. Restriction maps of the $\gamma 2F$ and $\delta 10.1$ constructs used to generate transgenic mice. Variable and constant regions are indicated underneath each map. The sizes of the variable and constant regions are approximate. Not all of the restriction sites are necessarily shown. A (Apa 1), F (Fsp 1), K (Kpn 1), N (Not 1), R (EcoR 1), S (SnaB 1).

B. PCR analysis of transgenic lines derived from coinjection of $\gamma 2F$ and the truncated $\delta 10.1$ construct using primers specific for the T3.13.1 rearranged V $\gamma 1$ -J $\gamma 4$ and V $\delta 6$ -J $\delta 1$ genes. Lanes: 1, T3.13.1 genomic DNA; 2, T4.8.1 genomic DNA; 3, 2-1^m tail DNA; 4, l.m. tail DNA; 5, 3-4^P tail DNA; 6, l.m. tail DNA; 7, 5-2^m tail DNA; 8, l.m. tail DNA; 9, 5-5^m tail DNA; 10, l.m. tail DNA; 11, 5-8^P tail DNA; 12, l.m. tail DNA; 13, 7-6^P tail DNA; 14, l.m. tail DNA. T3.13.1 is the hybridoma from which the transgenes were isolated. T4.8.1 is another V $\gamma 1$,V $\delta 6$ hybridoma. Lines 2-1^m, 5-5^m, 5-8^P and 7-6^P are transgenic for $\gamma 2F$ and the truncated $\delta 10.1$ construct. Lines 3-4^P and 5-2^m are transgenic for only the $\gamma 2F$ construct. l.m. (non-transgenic littermate)

C. PCR analysis of transgenic lines derived from coinjection of $\gamma 2F$ and full-length $\delta 10.1$ using primers specific for the transgenes. Lanes: 1, T3.13.1 genomic DNA; 2, T4.8.1 genomic DNA; 3, A-2^m tail DNA; 4, l.m. tail DNA; 5, F-3^P tail DNA; 6, l.m. tail DNA; 7, F-4^P tail DNA; 8, l.m. tail DNA. Line A-2^m is transgenic for both constructs. Lines F-3^P and F-4^P are transgenic for only the δ construct. l.m. (non-transgenic littermate)

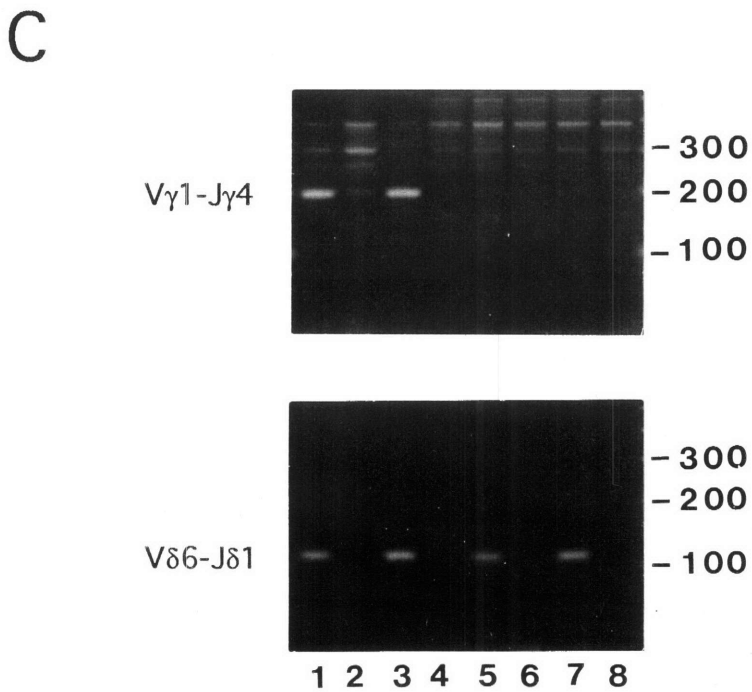
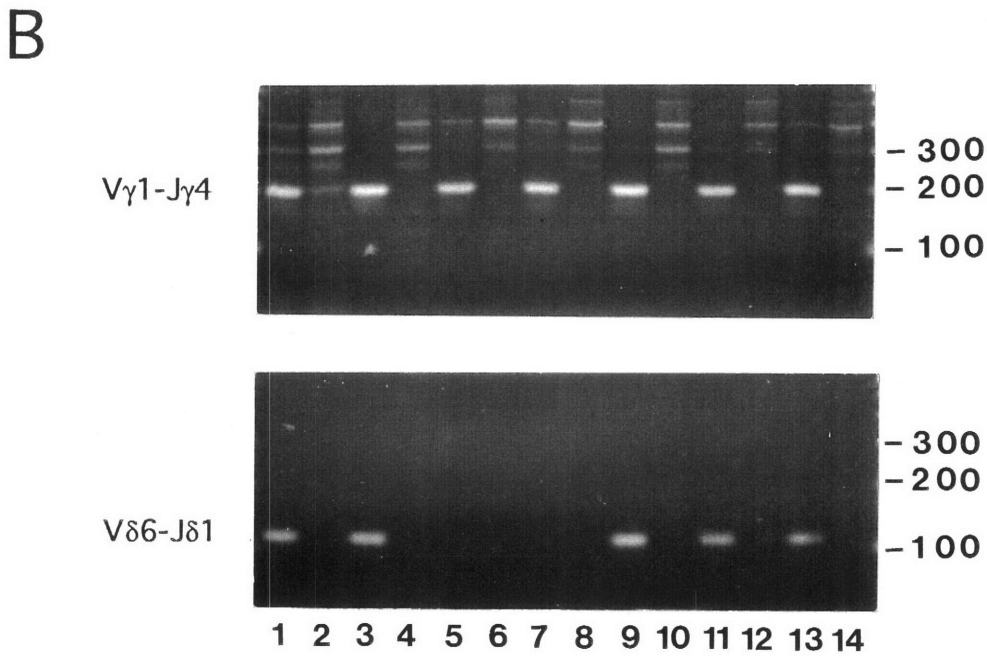
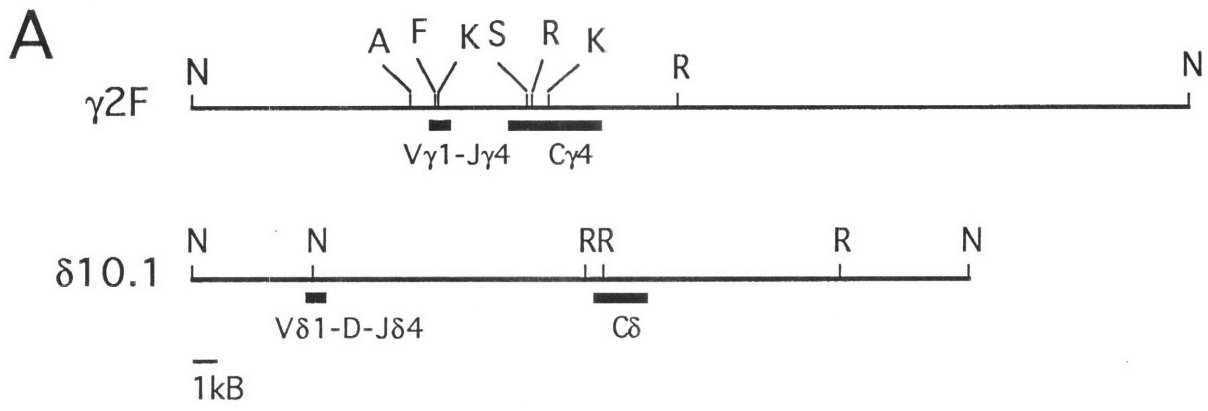


Figure 2. Expression of transgenes.

A. 5-8P thymocytes and splenocytes were labeled with anti-CD3 PE in combination with either 2-11 FITC or 1.9 FITC and analyzed by flow cytometry. 5-8P thymus and spleen each contain an obvious population of 2-11⁺ (V γ 1) cells and no 1.9⁺ (T3.13.1 clonotype) cells.

B. A-2^m thymocytes and splenocytes were labeled with anti-CD3 PE in combination with either 2-11 FITC or 1.9 FITC and analyzed by flow cytometry. A-2^m thymus and spleen each contain an obvious population of 2-11⁺ cells which are also 1.9⁺.

C. Thymocytes and splenocytes from a non-transgenic mouse were labeled with anti-CD3 PE and 2-11 FITC. The percentages of 2-11⁺ cells in both thymus and spleen are considerably lower than those of either transgenic line.

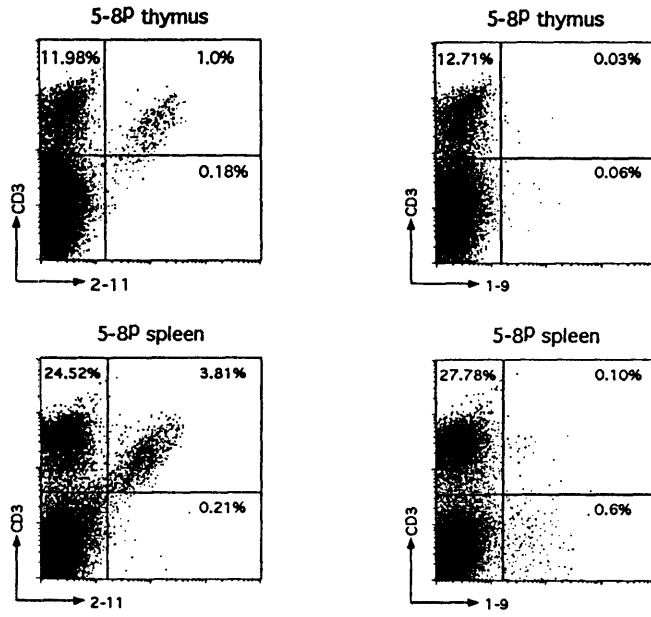
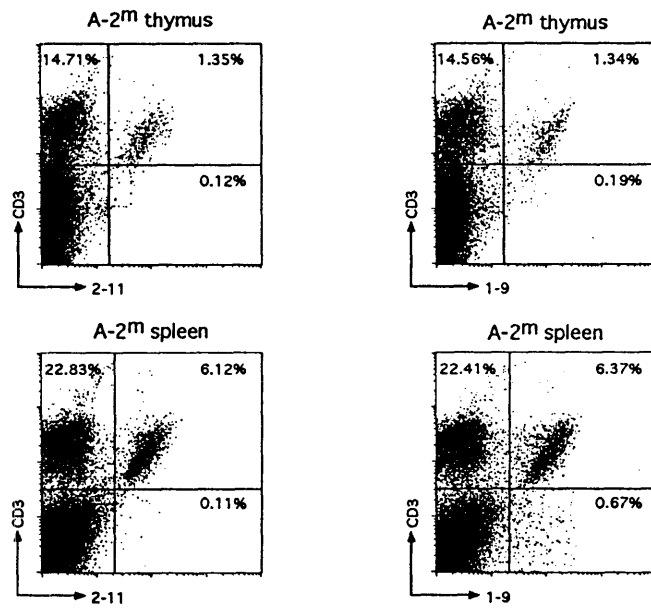
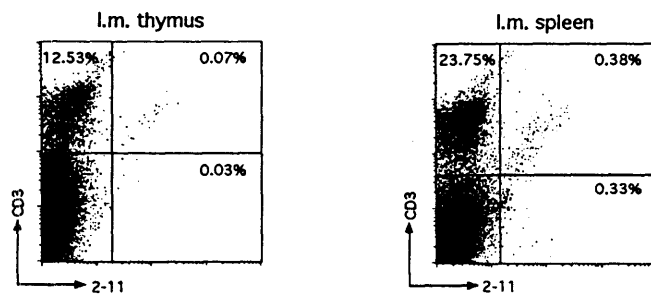
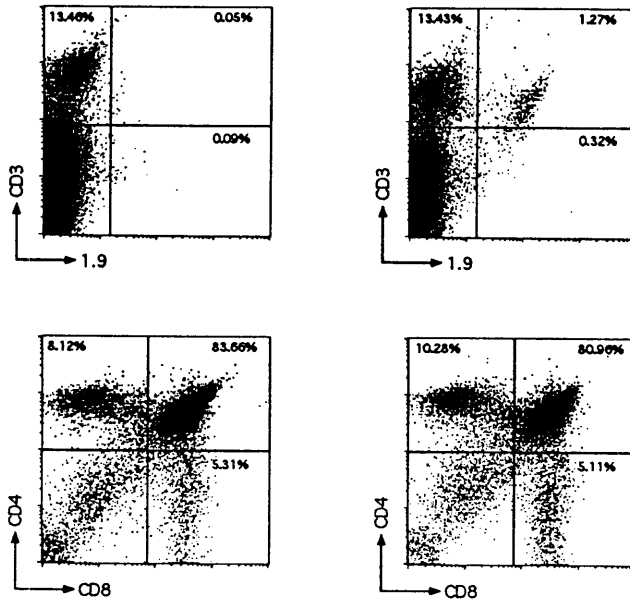
A**B****C**

Figure 3. Normal $\alpha\beta$ T cell and B cell development in A-2^m transgenics.

A. A-2^m and littermate thymocytes were labeled with anti-CD4 PE and anti-CD8 FITC and analyzed by flow cytometry. No difference in any of the four populations defined by expression of CD4 or CD8 was observed between transgenics and littermates.

B. A-2^m and littermate splenocytes were labeled with anti-CD4 PE and anti-CD8 FITC or with anti-B220 PE and anti-IgM FITC and analyzed by flow cytometry. No difference in peripheral $\alpha\beta$ and B cell populations was observed between the transgenics and littermates.

A littermate A-2^m transgenic



B

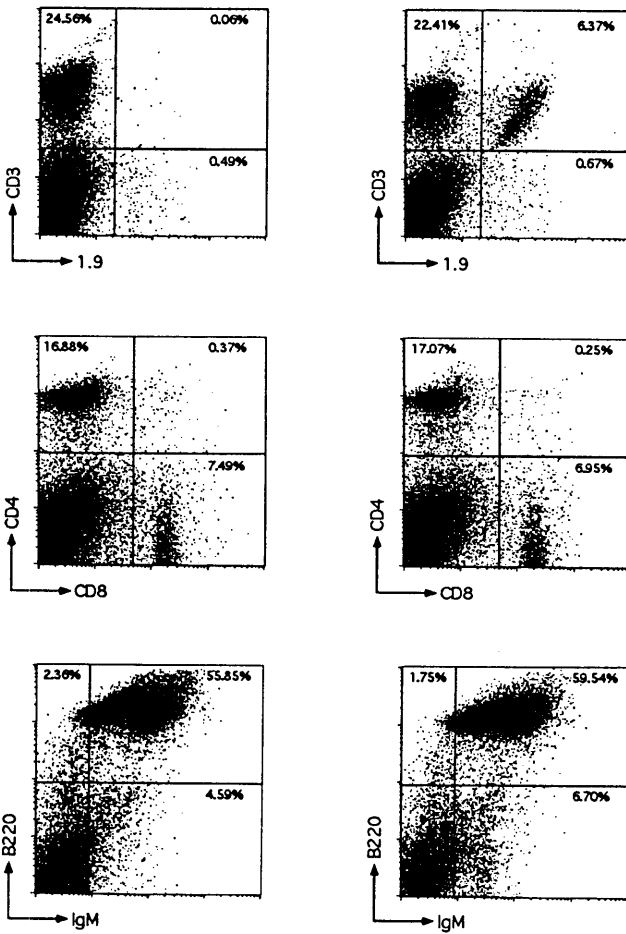
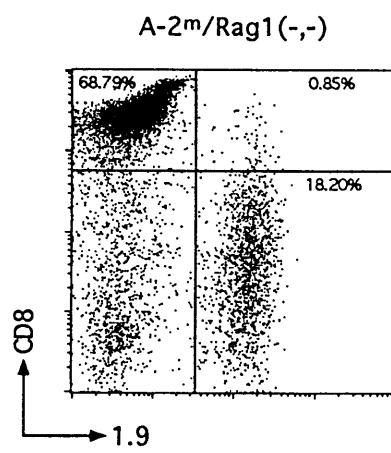
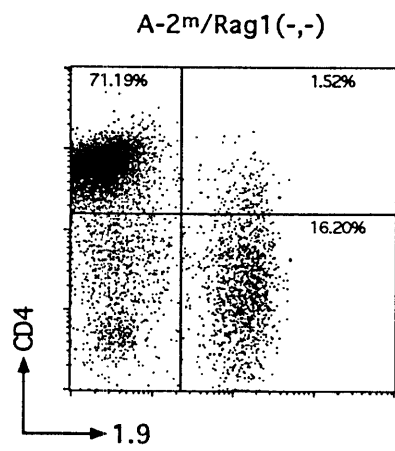
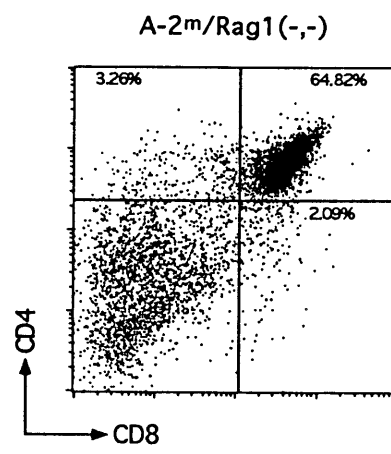
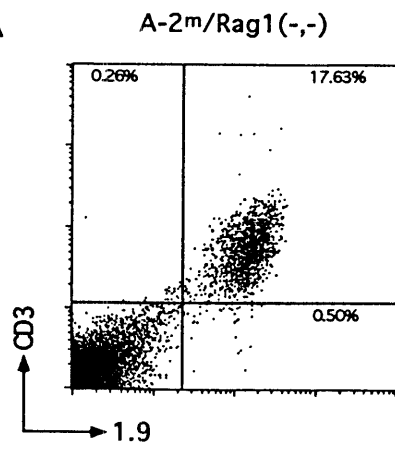


Figure 4. A-2^m transgenes rescue CD4⁺CD8⁺ thymocytes in Rag1 deficient background.

A. Thymocytes from a 4 week old A-2^m/Rag1(-,-) mouse were labeled with anti-CD3 PE and 1.9 FITC (upper left); anti-CD4 PE and anti-CD8 FITC (upper right); anti-CD4 PE and 1.9 FITC lower left and anti-CD8 PE and 1.9 FITC (lower right) and analyzed by flow cytometry. The majority of cells in the A-2^m/Rag1(-,-) thymus are CD4⁺CD8⁺ cells that are negative for $\gamma\delta$ TCR surface expression.

B. Thymocytes from a non-transgenic, Rag1(-,-) littermate were labeled with anti-CD4 PE and anti-CD8 FITC and analyzed by flow cytometry. The non-transgenic Rag1(-,-) thymus contains no CD4⁺CD8⁺ cells. In this experiment the A-2^m/Rag1(-,-) thymus contained 7.1×10^7 total cells and the non-transgenic Rag1(-,-) littermate contained 6.7×10^6 total cells.

A



B

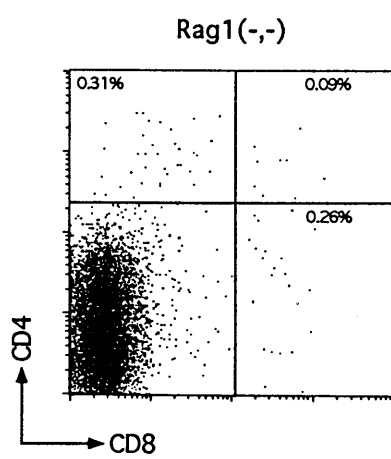
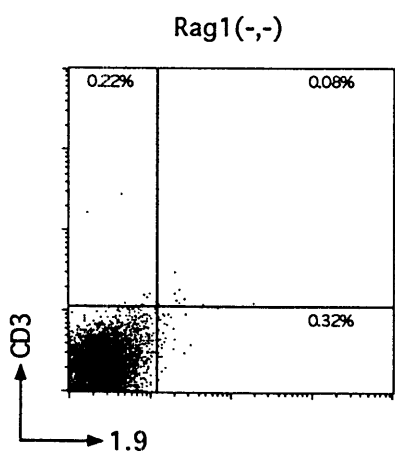


Figure 5. A-2^m transgenic cells have resting phenotype in vivo and are competent for activation following TCR crosslinking.

A-2^m spleen cells were labeled with 1.9 FITC in combination with anti-CD69 PE (A) or anti-CD25 PE (B) either *ex vivo* or following 48 hrs culture at initial density of 10⁶ cells per ml, with 10µg/ml ConA and 10U/ml IL-2, and analyzed by flow cytometry. Shown is histogram analysis for CD69 counts (A) and CD25 counts (B) on 1.9⁺ gated cells. Transgenic cells are initially negative for expression of both activation markers, and expression of both markers is induced following ConA stimulation.

C. Thy1.2⁺ cells were sorted from total A-2^m/TCRβ(-,-) splenocytes. 10,000 cells were cultured per well, in 96 well plates with or without prior coating of the wells with the anti-γδ TCR mAb, GL4. Wells were pulsed for 6 hrs with 1 µCi ³H- thymidine on the indicated days, and cells were harvested and analyzed for incorporation as described in Materials and Methods. The A-2^m cells require TCR stimulation to proliferate indicating that they do not proliferate constitutively. Cell preparations generated by this sorting procedure are >80% 1.9⁺. Error bars represent standard error of the mean.

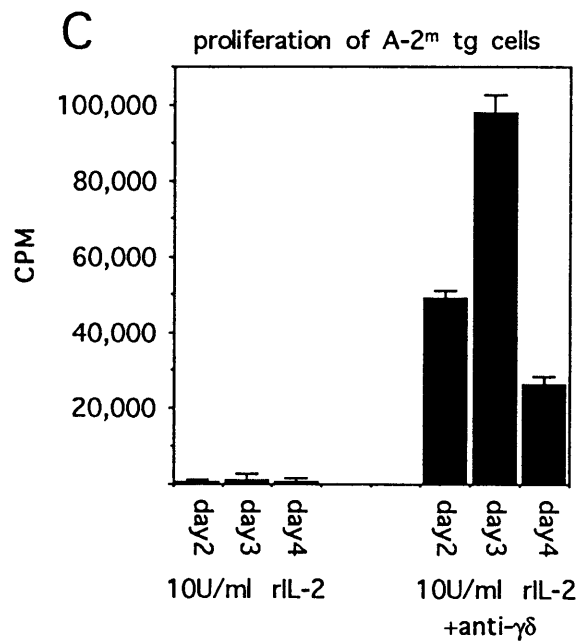
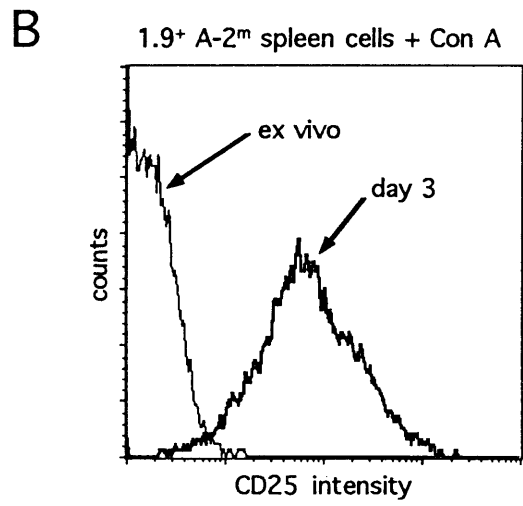
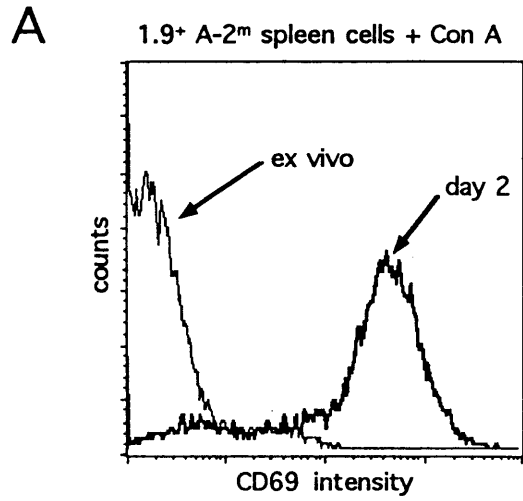
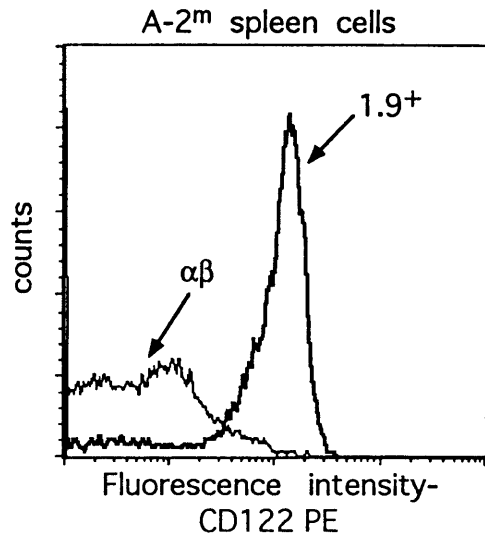


Figure 6. A-2^m transgenic cells and a major proportion of total V γ 1 cells constitutively express the IL-2 receptor β chain.

A. Total spleen cells from an A-2^m transgenic mouse were stained with anti-CD122 PE (IL-2 receptor β) in combination with either anti-TCR β FITC or 1.9 FITC. Histogram analysis of CD122 counts on gated $\alpha\beta^+$ and 1.9⁺ populations is shown. While most $\alpha\beta$ cells are dull or negative for CD122 expression, the vast majority of A-2^m cells express high levels of CD122.

B. Total spleen cells from a TCR $\beta(-,-)$ mouse (upper panel), a 5-2^m/TCR $\beta(-,-)$ mouse (middle panel), and an A-2^m/TCR $\beta(-,-)$ mouse (lower panel) were labeled with CD122 PE in combination with 2-11 FITC and analyzed by flow cytometry. Shown is histogram analysis of CD122 counts on gated 2-11⁺ (V γ 1) cells. Total V γ 1 cells and 5-2^m V γ 1 cells have a bimodal distribution with respect to CD122 expression, while the homogeneous A-2^m transgenic cells have more uniform, positive expression of CD122.

A



B

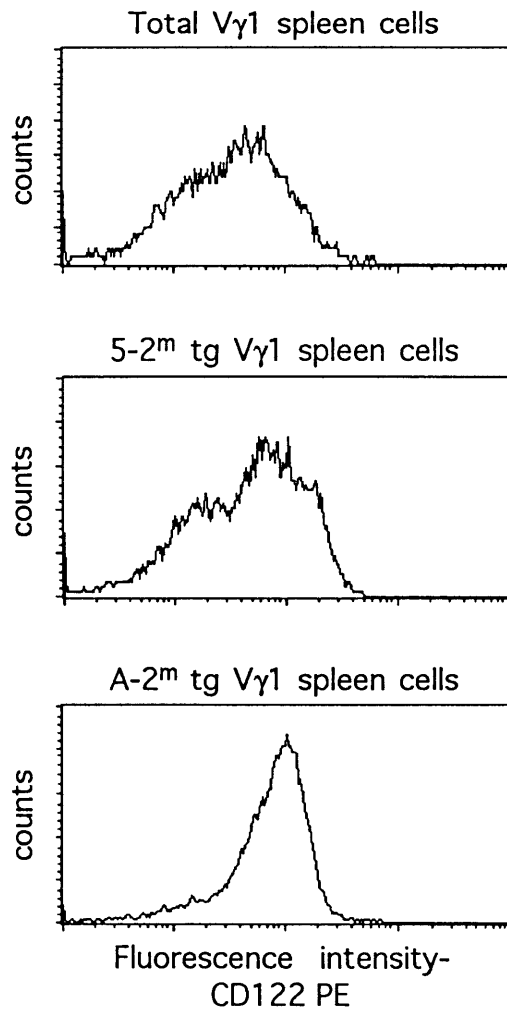


Figure 7. A-2^m transgenic cells proliferate in response to high IL-2 concentration and proliferation is dependent on the IL-2 receptor β chain.

A. Thy1.2⁺ cells were sorted from total A-2^m/TCR β (-,-) splenocytes. 10,000 cells were cultured per well in 96 well plates in the presence of increasing amounts of IL-2. Wells were pulsed at the indicated times for 6 hrs with 1 μ Ci ³H-thymidine, and cells were harvested and analyzed for incorporation as described in Materials and Methods. Significant proliferation was observed in response to 500U/ml IL-2. Error bars indicate standard error of the mean.

B. 10,000 Thy1.2⁺ sorted cells were cultured per well in 96 well plates with 200-500 U/ml IL-2 in the presence of a series of three-fold dilutions of anti-CD122 mAb, or anti-CD4 mAb as a negative control. The highest mAb concentration was 5 μ g/ml. The IL-2 dependent proliferation of the A-2^m transgenic cells was specifically inhibited in a dose-dependent manner by the anti-CD122 mAb. Error bars indicate standard error of the mean.

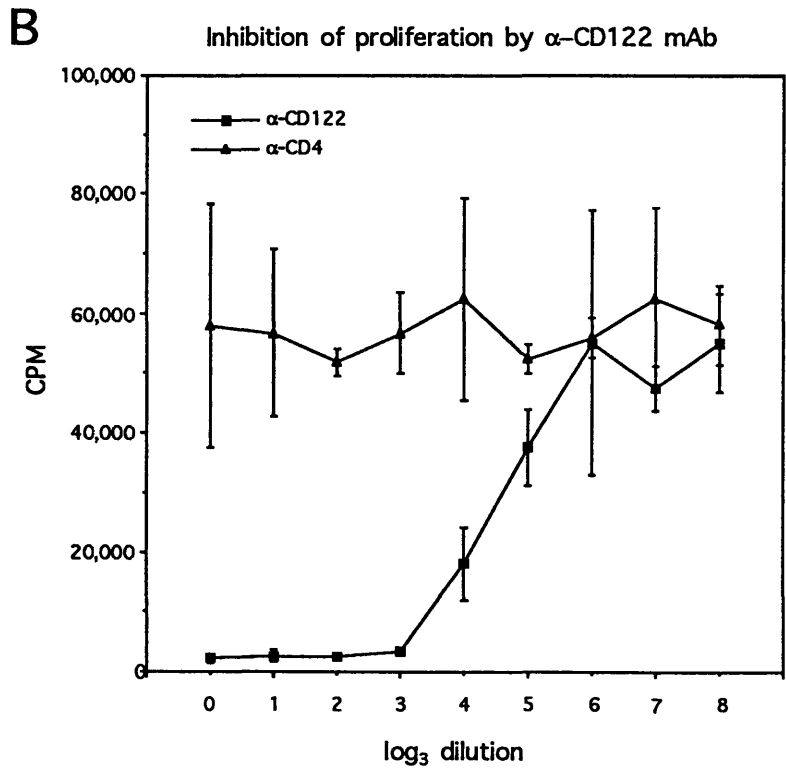
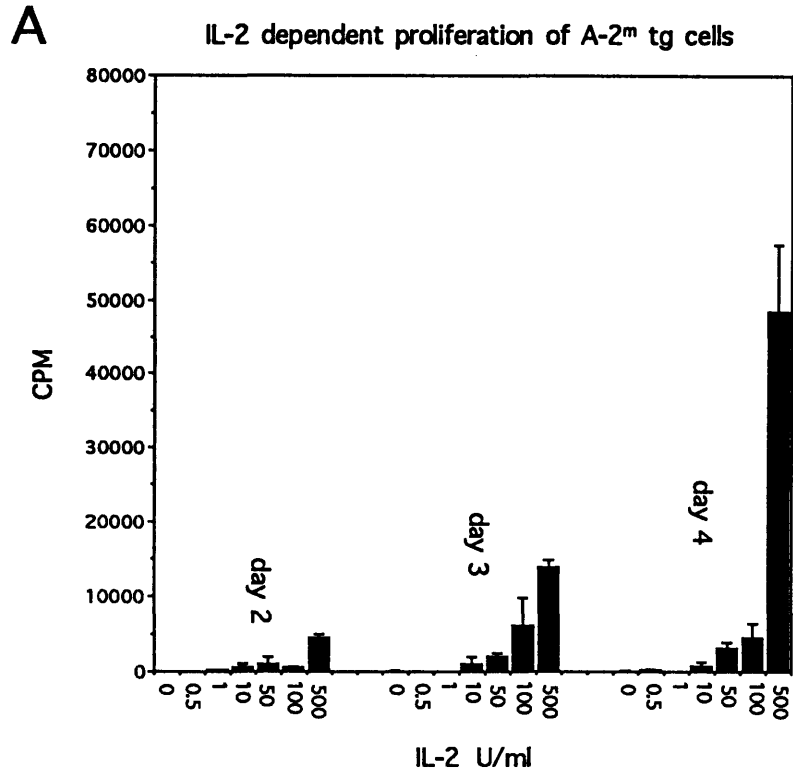


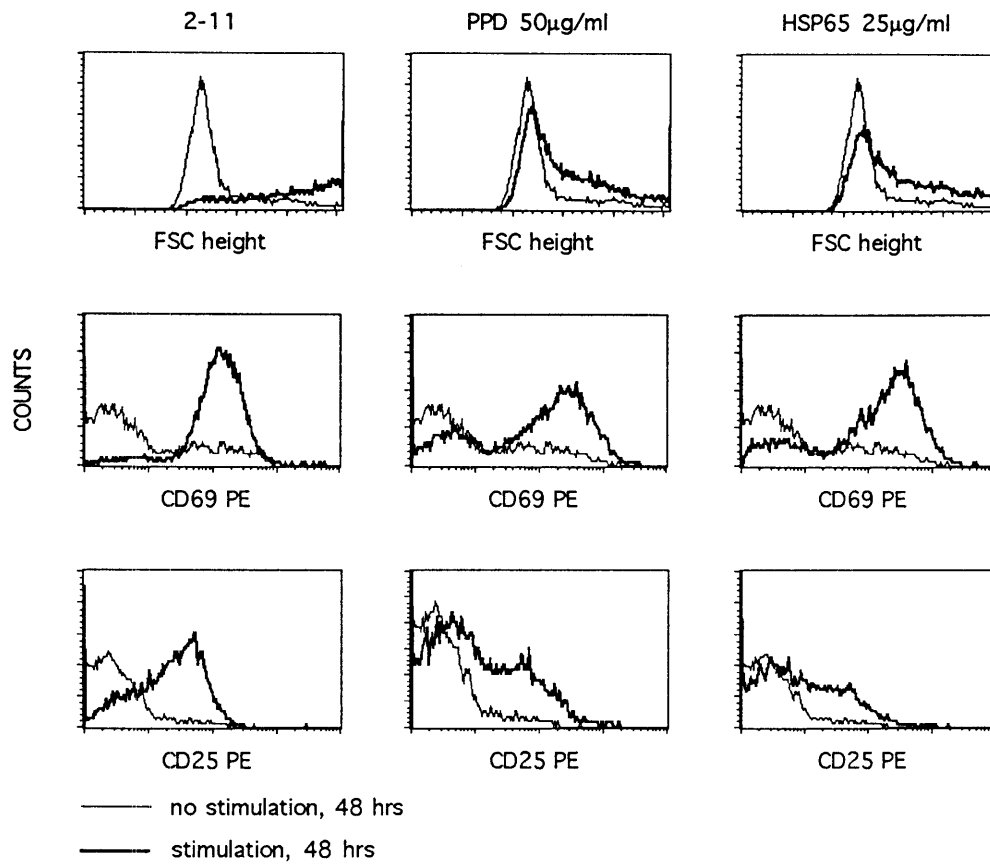
Figure 8. A-2^m cells show only modest response to mycobacterial antigens.

A. Total spleen cells from A-2^m/TCR β (-,-) mice were cultured with 10U/ml IL2 with no stimulation (narrow line, all plots), on plastic coated with 2-11(bold line, left column), with 50 μ g/ml PPD (bold line, middle column), and with 25 μ g/ml (*M. bovis* Hsp 65) (bold line, right column). Cells were analyzed at 48 hrs for size (upper row), CD69 expression (middle row) and CD25 expression (lower row). Cells showed strong increases in size, CD69 expression and CD25 expression in response to stimulation with 2-11. Although expression of CD69 was induced following stimulation with PPD and Hsp 65, increases in cell size and CD25 expression following stimulation with PPD or Hsp 65 were minor compared to those following 2-11 stimulation.

B. Thy1.2⁺ cells were sorted from total A-2^m/TCR β (-,-) splenocytes. 10,000 sorted cells were cultured per well in 96 well plates with 50,000 mitomycin C treated B6 spleen cells (column 1), 50, 000 mitomycin C treated B6 spleen cells and 50 μ g/ml PPD (column 2), 50,000 mitomycin C treated B6 spleen cells and 25 μ g/ml Hsp 65 (column 3), in wells coated with 2-11 (anti-V γ 1) (column 4) and in wells coated with GL4 (anti- $\gamma\delta$ TCR) (column 5). IL-2 was added at 10U/ml to all wells. Although the cells showed a mild proliferative response upon addition of either antigen compared to the no-antigen control. the response was negligible compared to the response to mAb-mediated crosslinking of the TCR. Error bars indicate standard error of the mean.

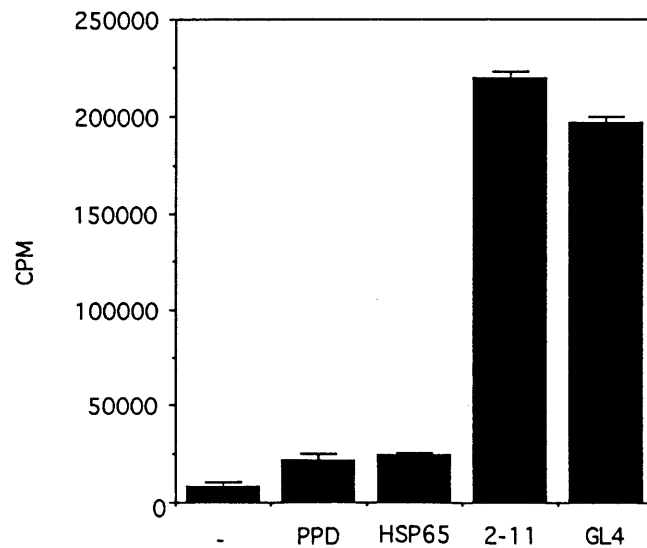
A

Expression of activation markers on A-2^m tg cells in response to mycobacterial antigens



B

Proliferation of A-2^m tg cells in response to mycobacterial antigens



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Conclusions and future directions

Over the past several years it has become evident that mycobacteria contain potent antigens for a major class of human $\gamma\delta$ T cells. This line of investigation has recently culminated with the molecular identification of a novel class of low molecular weight non-peptide ligands for these human $\gamma\delta$ T cells. Although there have been several reports suggesting that murine $\gamma\delta$ T cells respond to mycobacterial antigens such as Hsp 65, this issue has remained cloudy relative to the case of the human $\gamma\delta$ T cell response to mycobacteria.

Initially we attempted to directly address this issue by generating $\gamma\delta$ T cell hybridomas following stimulation of purified $\gamma\delta$ cells with PPD. By this means, we hoped to obtain $\gamma\delta$ cell lines that respond to mycobacterial peptide antigens. This procedure produced a panel of 21 $\gamma\delta$ hybridomas with several interesting features. The majority of these hybridomas were of the V γ 1V δ 6 subset, as all contained a productive V γ 1-J γ 4 rearrangement and most contained a productive V δ 6-J δ 1 rearrangement. Junctional sequences of both the γ and δ chain genes were diverse. In addition, we found that these hybridomas secrete IL-2 constitutively, and this spontaneous IL-2 generation constitutes a significant proportion of the maximal IL-2 production induced by mAb mediated CD3/TCR complex crosslinking. The constitutive activation of the hybridomas rendered study of their antigenic specificity difficult, and only modest increases in IL-2 secretion could be observed in the presence of mycobacterial antigens.

It is noteworthy that the procedure used to generate these hybridomas yielded a group of hybridomas with such uniform expression of the V γ 1V δ 6 TCR. The only two conditions applied in selecting these hybridomas were the stimulation protocol and the requirement that the hybridomas have the capacity to secrete IL-2. Since V γ 4 cells, which are the other major class of $\gamma\delta$ T cells in adult thymus and spleen, have been shown to secrete IL-2, it is likely that this high representation of V γ 1V δ 6 cells among the hybridomas is a consequence of specific activation of these cells prior to the fusion. Further study of conditions required to activate the V γ 1V δ 6 cells in culture could be informative.

The constitutive IL-2 secretion of the V γ 1V δ 6 hybridomas could be blocked by addition of mAbs directed against the CD3/TCR complex, suggesting that this activation depends on an interaction involving the TCR. In order to identify other cell surface molecules involved in this spontaneous activation, including potential coreceptors and cellular ligands for V γ 1V δ 6 T cells, we generated mAbs that block the IL-2 secretion following immunization of hamsters with $\gamma\delta$ hybridoma cells. By this means we generated several useful reagents including a mAb, 2-11, specific for the V γ 1-J γ 4-C γ 4 chain, and several mAbs specific for the murine α v and β 3 integrin chains.

We used the 2-11 mAb to study the development and tissue distribution of V γ 1 expressing $\gamma\delta$ T cells. V γ 1 cells comprise only a minor proportion of $\gamma\delta$ cells in the thymus and peripheral lymphoid organs during fetal and postnatal life, but they form a major population of $\gamma\delta$ cells in the thymus and peripheral lymphoid organs of adult mice. Furthermore, we found that V γ 1 cells constitute a large proportion of the $\gamma\delta$ intestinal intraepithelial lymphocytes (i-IELs) present in all strains of mice tested. This finding was in contrast to the previous notion that the vast majority of $\gamma\delta$ i-IELs are of the V γ 7 subset. The V γ 1 i-IELs were found in athymic (nude) mice and in antigen-free mice, indicating that V γ 1 i-IELs can develop extrathymically, and that their colonization of the intestinal epithelium is independent of the presence of antigens in the gut. The availability of a mAb specific for the V γ 1 chain should prove useful for future study of the function of V γ 1-expressing $\gamma\delta$ T cells, and particularly for determining the contribution of V γ 1 cells to various $\gamma\delta$ responses that have been observed in mice.

Several of the blocking mAbs were initially shown to bind to the α v β 3 vitronectin receptor by immunoprecipitation analysis. Using several approaches, including analysis of expression on platelets, binding competition experiments and immunoprecipitations from lysates derived from cells that express α v in combination with different β chains, we determined that 2 of the mAbs are specific for the α v integrin chain and 2 are specific for the β 3 integrin chain. We used these mAbs to study the expression of α v and β 3 on hematopoietic cells in vivo. Previous studies suggested that α v and β 3 are not expressed on resting thymocytes and splenocytes, and that expression is only detectable following activation or long term in vitro culture. In contrast to

these findings, we observed that αv and $\beta 3$ are expressed on a variety of lymphocyte populations in vivo. Thus, $CD3^+$ thymocytes and peripheral $\alpha\beta$ T cells clearly express both αv and $\beta 3$ chains, while B cells and preB cells clearly express the αv chain and express low but detectable levels of the $\beta 3$ chain. We also observed that $\alpha v\beta 3$ is differentially expressed at discrete stages of thymocyte development. Thus $CD4^-CD8^-$ double negative thymocytes express high levels of $\alpha v\beta 3$ while $CD4^+CD8^+$ thymocytes express much lower levels of $\alpha v\beta 3$ and $CD3^+$ thymocytes and mature T cells again express higher levels of $\alpha v\beta 3$. This modulation of $\alpha v\beta 3$ expression suggests that the $\alpha v\beta 3$ integrin could be involved in thymocyte development. Preliminary experiments in which addition of the αv and $\beta 3$ mAbs to fetal thymic organ culture blocked T cell development at the $CD4^-CD8^-$ double negative stage imply the involvement of $\alpha v\beta 3$ in thymocyte development, however further experiments utilizing the mAbs and various RGD peptides are required to clarify this issue. These mAbs should prove useful for studying the involvement of the αv and $\beta 3$ chains in various cellular processes including adhesion and migration in addition to development.

To further examine the reactivity of $V\gamma 1V\delta 6$ T cells, we generated mice transgenic for the rearranged $V\gamma 1$ and $V\delta 6$ genes from the T3.13.1 $V\gamma 1V\delta 6$ hybridoma. In particular, we wished to address the questions of whether primary $V\gamma 1V\delta 6$ T cells are constitutively activated and whether they respond to PPD. In the process of creating the transgenic mice, we obtained several lines of mice transgenic for the $V\gamma 1$ or $V\delta 6$ genes alone, and one line of mice transgenic for both genes (line A-2^m). The $V\gamma 1$ only transgenics and the A-2^m mice all displayed greatly increased numbers of $V\gamma 1$ cells in the thymus and peripheral lymphoid organs relative to non-transgenic littermates, and the vast majority of the $V\gamma 1$ cells in the A-2^m mice expressed the T3.13.1 TCR clonotype. By several criteria including expression of surface activation markers and proliferative status, the primary $V\gamma 1$ cells do not appear to be constitutively activated. However, they do display the capacity to be activated by TCR crosslinking in the presence of low amounts of exogenous IL-2. Despite the apparent resting state of the transgenic $V\gamma 1$ cells, we were unable to detect a significant response to mycobacterial antigens including H37Ra extract, PPD, Hsp 65, mycobacterial lipids and isopentenyl pyrophosphate. We did detect induction of CD69 expression and minor induction of CD25

expression and cell proliferation in the presence of PPD and Hsp65. While this low level activation may be sufficient to promote successful fusion and generation of V γ 1V δ 6 hybridomas, it does not appear to be a strong response. Of course these experiments were limited by the specific population of transgenic V γ 1 cells that were analyzed, and by the fact that we do not understand the nature of the response of primary murine $\gamma\delta$ cells to antigens. However, based on this work and communication with other investigators in the field, I believe that most murine $\gamma\delta$ cells are not specific for mycobacterial antigens.

In order to generate mice with a pure population of transgenic T cells, the A-2^m transgenes were crossed into the Rag1 deficient background. We observed that the presence of the two transgenes rescued the development of CD4⁺CD8⁺ double positive cells which is disrupted in non-transgenic Rag1 deficient mice. The vast majority of double positive cells were negative for surface expression of the transgenic TCR. There are two possible mechanisms for the restoration of development of double positive cells. Either the rescue is accomplished directly by prior expression of the transgenes in the lineage that gives rise to the double positive cells at a stage when some TCR dependent signalling is required, or the presence of the transgenic V γ 1V δ 6 cells indirectly rescues development of double positive cells by altering the thymic environment. Further experiments could help to clarify this issue. First, it is of interest to determine whether either the γ or δ transgene alone is sufficient to induce development of CD4⁺CD8⁺ cells. To this end we are crossing the γ and δ only transgenics with Rag 1 deficient mice to produce V γ 1/Rag(-,-) and V δ 6/Rag1(-,-) mice. Preliminary results from these crosses suggest that the V δ 6 transgene is not sufficient to rescue development of double positive cells. Second, to investigate whether the transgenic thymocytes indirectly induce development of CD4⁺CD8⁺ cells, it would be informative to produce bone marrow chimeras by injecting bone marrow from Thy1.2⁺, A-2^m/Rag1(-,-) donors into Thy1.1⁺, Rag1(-,-) recipients. Following reconstitution, one could examine whether development of Thy1.1⁺ CD4⁺CD8⁺ cells is induced by the transgenic cells. The presence of Thy1.1⁺ CD4⁺CD8⁺ cells would provide strong evidence for indirect rescue. Direct rescue of CD4⁺CD8⁺ cells by the A-2^m transgenes could have implications for certain models of $\alpha\beta$ versus $\gamma\delta$ lineage determination. Since the transgenes

are not expressed in the CD4⁺CD8⁺ cells, it appears that the lineage determination occurs in the absence of a functional β chain which is in conflict with models in which lineage segregation depends on competition between the $\gamma\delta$ TCR and the $\alpha\beta$ TCR or preTCR.

In analyzing the expression of cell surface activation markers on the A-2^m transgenic cells, we observed that these cells constitutively express the IL-2 receptor β chain. The IL-2 receptor β chain appeared to be functional on these cells as they were shown to proliferate in the presence of high concentrations of IL-2, and this response could be specifically blocked by addition of mAbs against the IL-2 receptor β chain. Recently, it was found that the IL-2 receptor β chain is a component of the IL-15 receptor, therefore, it was of interest to determine whether the A-2^m cells can be activated by IL-15. In preliminary experiments, we were unable to detect activation of the A-2^m transgenic cells by IL-15 in the presence or absence of TCR crosslinking. The possibility remains that the IL-2 receptor β chain is expressed on the $\gamma\delta$ cells as part of a novel receptor for another cytokine. To investigate this possibility, it could be informative to perform immunoprecipitation analysis on lysates of surface labeled A-2^m transgenic cells to determine whether the IL-2 receptor β chain is associated with any novel proteins on these cells.

The A-2^m transgenic cells share expression of the IL-2 receptor β chain with NK cells, and a significant fraction of A-2^m transgenic cells also express NK1.1. It may be interesting to further explore the similarities between this class of $\gamma\delta$ cells and NK cells. A recent report suggested that V γ 1 cells may have anti-tumor activity. In this study, mice transgenic for a V γ 1-J γ 4-C γ 4 cDNA under a heterologous promoter were shown to be resistant to T cell leukaemias. This study was hampered by the facts that the transgene expression was driven by a strong heterologous promoter which could lead to non-physiological distribution and level of transgene expression, and that general effects on T cell development were observed in these mice. Our V γ 1 transgenic mice could be useful for further study of the potential anti-tumor activity of V γ 1 T cells.

Rearrangement and expression of $V_{\gamma}1$, $V_{\gamma}2$ and $V_{\gamma}3$ TCR γ genes in C57BL/6 mice

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Abstract

We have recently described a mAb (2.11) that recognizes the $V_{\gamma}1$ – $J_{\gamma}4$ – $C_{\gamma}4$ chain. With this mAb and an anti- δ mAb we separated $\gamma\delta^{+}$ 2.11⁺ and $\gamma\delta^{+}$ 2.11[−] intraepithelial lymphocytes (i-IEL) by FACS. Transcripts of rearranged TCR $V_{\gamma}1$ and $V_{\gamma}2$ genes in both i-IEL populations were analyzed by PCR followed by sequence analysis of cDNA spanning the junction of the variable (V) and joining (J) genes. Roughly the same number of $V_{\gamma}1$ and $V_{\gamma}2$ transcripts were found in the 2.11⁺ population, while >90% of the transcripts in the 2.11[−] population contained a $V_{\gamma}2$ gene sequence. Furthermore, >80% of the $V_{\gamma}1$ transcripts in the 2.11⁺ population were functional, while only 30–40% of the $V_{\gamma}2$ transcripts in either population contained an in-frame sequence. The observed frequency of in-frame $V_{\gamma}2$ transcripts is what would be expected from cell populations that have not gone through cellular selection mediated by the TCR. Expression of $V_{\gamma}2$ mRNA in TCR $\alpha\beta$ and TCR $\gamma\delta$ lymphocytes was studied by a technique that analyzes populations of transcripts of rearranged genes. In both T cell populations similar levels of $V_{\gamma}2$ transcripts were found and about two out of three transcripts were out-of-frame. During the cloning and sequence analysis, we identified a clone that expresses the $V_{\gamma}3$ segment rearranged to the $J_{\gamma}3$ – $C_{\gamma}3$ region in C57BL/6 mice. Together with the PCR cloning and sequencing of the complete $C_{\gamma}3$ region in C57BL/6 mice, these data demonstrate that the $J_{\gamma}3$ – $C_{\gamma}3$ gene is functional in this strain. Taken together, these studies revealed that: (i) cells expressing the $V_{\gamma}1$ chain are an important subset of the $\gamma\delta$ i-IEL population and that they show extensive junctional diversity; (ii) there is no correlation between expression of in-frame $V_{\gamma}2$ transcripts and expression of $V_{\gamma}2$ chains at the cell surface; and (iii) cells expressing the $V_{\gamma}3$ chain might be a minor subset of the $\gamma\delta$ T cell population in C57BL/6 mice.

Introduction

Among TCR γ genes of mice, there are seven different variable (V) γ genes that can rearrange to four different constant (C) genes, each of which is associated with a junctional (J) element. Four V_{γ} genes, $V_{\gamma}4$, $V_{\gamma}5$, $V_{\gamma}6$ and $V_{\gamma}7$, rearrange mostly to the $J_{\gamma}1$ – $C_{\gamma}1$ gene. The other three genes ($V_{\gamma}1$, $V_{\gamma}2$ and $V_{\gamma}3$) show a very high level of homology and rearrange mostly to the $J_{\gamma}4$ – $C_{\gamma}4$, $J_{\gamma}2$ – $C_{\gamma}2$ and $J_{\gamma}3$ – $C_{\gamma}3$ genes, respectively (reviewed in 1). The region containing the $J_{\gamma}3$ – $C_{\gamma}3$ gene has been deleted in most of the common laboratory strains of mice (2,3), and is believed to be non-functional in BALB/c mice due to a mutation in the splice donor at the end of exon 3, a defective putative polyadenylation signal sequence and a base pair deletion in the $J_{\gamma}3$ region (4,5).

Although a large amount of information is available regard-

ing the onset of appearance during ontogeny, TCR repertoire, tissue distribution and thymus dependence of the $\gamma\delta$ T cell subsets bearing the products of V_{γ} genes that rearrange to the $J_{\gamma}1$ – $C_{\gamma}1$ gene (i.e. $V_{\gamma}4$, $V_{\gamma}5$, $V_{\gamma}6$ and $V_{\gamma}7$ subsets), analogous information regarding other $\gamma\delta$ T cell subsets is limited (reviewed in 6). Sequence analysis of the $V_{\gamma}1$ – $J_{\gamma}4$ junctions of $V_{\gamma}1$ -expressing hybridomas obtained from thymus and spleen and of cDNA obtained from dendritic epidermal cells of nude mice has shown that $V_{\gamma}1$ -bearing cells in these organs are highly diverse (7–9). Recently, we described a mAb (2.11) that recognizes the $V_{\gamma}1$ – $C_{\gamma}4$ protein, and showed that $V_{\gamma}1$ -bearing cells constitute a large fraction of the $\gamma\delta$ T cells not only in the thymus and peripheral lymphoid organs but also in the intestinal epithelium (32). In contrast, previous studies

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have shown a large number of functionally rearranged *V_γ2* genes in intraepithelial lymphocytes (i-IEL) leading to the belief that *V_γ2*-bearing cells constitute a significant population of mouse i-IEL (10,11). However, *V_γ2* gene rearrangement and mRNA have been found in some $\gamma\delta$ T cells that express γ chains other than the *V_γ2*-*C_γ2* protein and even in $\alpha\beta$ T cells. Therefore, the role of the *V₂-J₂-C₂* gene in encoding the TCR for a major $\gamma\delta$ T cell subset has been questioned (1). To clarify this issue and to study the extent of diversity of *V_γ1*⁺ and/or putative *V_γ2*⁺ i-IEL, we have sorted $\gamma\delta$ ⁺ 2.11⁺ and $\gamma\delta$ ⁺ 2.11⁻ i-IEL and analyzed the junctional sequences obtained after PCR amplification of cDNA isolated from both cell populations using primers that recognize both the *V_γ1* and the *V_γ2* genes. Our results show that *V_γ1*-bearing cells constitute a large and diverse population of i-IEL and that *V_γ2*-*J₂-C₂* mRNA is frequently expressed in many T cells without apparent expression of the protein at the cell surface. Furthermore, we show that the *J₃-C₃* gene is functional in C57BL/6 mice and that cells bearing the *V_γ3* chain might represent a minor subset of $\gamma\delta$ lymphocytes in this strain.

Methods

Mice

C57BL/6 (B6) mice were obtained from Iffa-Credo (L'Arbresle, France). Males that were 8–12 weeks old were used.

Cell preparations and cultures

The preparation of i-IEL has been described in detail (12). Purified TCR $\alpha\beta$ or TCR $\gamma\delta$ cells were obtained by stimulation of B6 total or CD4⁺CD8⁻ thymocytes with anti- β mAb (H57; 13) or anti- δ mAb (14) coated to plates and expanded in IL-2 as described (32). Cells activated with H57 were >98% TCR $\alpha\beta$ ⁺ with no detectable TCR $\gamma\delta$ ⁺ cells, and cells activated with 3A10 were >95% TCR $\gamma\delta$ ⁺ with <2% TCR $\alpha\beta$ ⁺ T cells.

Immunofluorescence staining and cell sorting

i-IEL were incubated with FITC-labeled anti-pan $\gamma\delta$ mAb [3A10 (14)] and biotin-labeled anti-*V_γ1*-*C_γ4* mAb [2.11 (32)] at a concentration of 10⁷ cells/ml for 30 min. on ice, washed twice and incubated for 15 min with streptavidin-phycoerythrin (Southern Biotechnology, Birmingham, AL). Cell sorting was carried out in a FACStar Plus (Becton Dickinson, Mountain View, CA).

Nucleic acids

Sorted cells were mixed with 10⁷ P815 mastocytoma cells as a carrier and total RNA was prepared using the guanidinium thiocyanate–CsCl method (15) cDNA was synthesized with 5 μ g of total RNA using OligodT (Pharmacia, Uppsala, Sweden), and the SuperScript reverse transcriptase (Gibco/BRL, Gaithersburg, MD) following the manufacturer's instructions. In some instances, the RNA preparations were treated with DNase prior to cDNA synthesis.

Oligonucleotide primers and PCR conditions

The following oligonucleotide primers were used: *V_γ1* + *V_γ2*: GCTATACATTGGTACCGGCA; *V_γ1* + *V_γ2* labeled: AATCAAC-GACCCTTAGGAGG; *V_γ1*: CCGGCAAAAAGCAAAAAGT;

V_γ2: CGGGCAAAAACAAATCAA; *V_γ3*: TTGAGTATCTAATAT-ATGTCGAG; *J_γ2*: CAGAGGGAATTACTATGA; *J_γ3*: TAAGCT-CATAGTAATTCCTTCT; *J_γ4*: GCAAATATCTTGACCCATGA; pan-*C_γ*: CTTATGGAGATTTGTTTCAGC; and *C_γ3* untranslated region: CAGCTGACTTGCTGTACCAC.

PCR was performed using a GeneAmp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT). Each cycle consisted of incubations at 92°C for 20 s, followed by 55°C for 30 s and 72°C for 30 s. Before the first cycle, a 2 min 94°C denaturation step was included and after the 30th cycle the extension at 72°C was prolonged for 4 min.

Cloning and sequencing

Between 5 and 10 μ l of each PCR reaction was blunted, phosphorylated and cloned into the *NotI* site of the pUC18 vector using the SureClone ligation kit (Pharmacia, Uppsala, Sweden) and following the manufacturer's instructions. Plasmid DNA isolated from ampicillin-resistant white colonies was sequenced by the dideoxy chain-termination method using the sequenase enzyme (US Biochemicals, Cleveland, OH) and the M13 forward and reverse primers. In some instances colonies were screened for the presence of the right insert by PCR.

Population analysis

PCR product (2 μ l) was submitted to a run-off elongation with a fluorescent primer (16) specific for the *V_γ1* and *V_γ2* genes; 2 μ l of the elongation product was mixed with an equal volume of 95% (v/v) formamide/10 mM EDTA and loaded on an 8% denaturing polyacrylamide–urea gel cast on an automated DNA sequencer (Applied Biosystems, Foster City, CA). Size determination of the run-off products was performed with a previously described software (16). This software provides an image of the gel by analyzing each band as a peak, the area of which is proportional to the intensity of fluorescence. The length of the fragments is determined by comparison with a set of size standards run in parallel in each experiment. This set of standards consists of five labeled fragments of known size.

Results

The junctional sequences of *V_γ1*-bearing i-IEL are very diverse

To ensure that the cells recognized by the 2.11 mAb express functional *V_γ1*-*C_γ4* gene products and to analyze the extent of junctional diversity of the *V_γ1*-expressing i-IEL, we cloned and sequenced the PCR products obtained after amplification of cDNA with *V_γ1*/*V_γ2* and pan-*C_γ* primers. The RNA used to prepare the cDNA was isolated from sorted $\gamma\delta$ ⁺ 2.11⁺ i-IEL obtained from B6 mice. The *V_γ1*-*J_γ4* junctional sequences obtained from this population are shown in Fig. 1 and contain several points of interest. First, >80% of the *V_γ1*-*J_γ4* sequences are joined in-frame. Second, the majority of these sequences contain one to 12 non-germline encoded nucleotides and the ends of both the V and the J segments are often shortened, generating considerable diversity in the junctions. This shortening is much more pronounced at the end of the V segment than at the end of the J segment and, as a consequence, P nucleotides are retained much more

	V _γ 1	P	N	P	J _γ 4	Functional
germline V _γ 1	TGT GCA GTC TGG ATA AA	TT				
germline J _γ 4				GA	TCA GGC ACA	
	TGT GCA GTC TGG		C	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		CCGTCCGCC	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG A		A	A	TCA GGC ACA	Yes
	TGT GCA GTC TGG A			GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		G	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		GGTG	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		GT	A	TCA GGC ACA	Yes
	TGT GCA GTC TGG		CGT		TCA GGC ACA	Yes
	TGT GCA GTC TGG ATA AA		ATGGG		CA GGC ACA	Yes
	TGT GCA GTC TGG		CCGA	GA	TCA GGC ACA	Yes
	TGT GCA GTC TGG		TTCCC		C ACA	Yes
	TGT GCA GTC TGG				TCA GGC ACA	Yes
	TGT GCA GTC TGG ATA A		GT		TCA GGC ACA	Yes
	TGT GCA GTC TGG A				CA GGC ACA	Yes
	TGT GCA GTC TG			A	TCA GGC ACA	No
	TGT GCA GTC TGG ATA		GAGGGG	GA	TCA GGC ACA	No
	TGT GCA GTC TGG AT		G		A GGC ACA	No

Fig. 1. V-J junctional sequences of V_γ1 transcripts from B6 i-IEL. Data represent cloned V_γ1-C_γ4 PCR products from sorted γδ⁺ 2.11⁺ i-IEL.

often in the 5' end of the J than in the 3' end of the V. Third, the size of the CDR3 appears quite constant with >80% of the V_γ1⁺ clones having the predicted germline size plus or minus three nucleotides.

Evaluation of the predicted amino acid sequences of the n-frame V_γ1-J_γ4 genes (Fig. 2) indicated that the junctional diversity also applies at the protein level. The increased frequency of a codon for Arg in the CDR3 of the 2.11⁺ population can be explained by the high frequency of clones containing P nucleotides in the 5' end of the J region.

From this experiment we conclude that the TCR_γ repertoire of the V_γ1⁺ i-IEL population is as diverse as that of the V_γ7⁺ i-IEL population (17-19).

Rearrangement and expression of the V_γ2 gene in different γδ i-IEL populations

Analysis with TCR_γ-specific mAb has shown that ~90% of i-IEL express the V_γ1 or the V_γ7 chains (21,32). On the other hand, functionally rearranged V_γ2 gene segments are abundant in i-IEL, suggesting that V_γ2-bearing cells are not a minor i-IEL subpopulation (10,11). This apparent contradiction could be resolved if the 2.11 mAb not only recognizes the V_γ1-C_γ4 protein but also the V_γ2-C_γ2 protein or if many of the functionally rearranged V_γ2 gene segments do not give rise to cell surface expression of the V_γ2-C_γ2 protein. To distinguish between these two possibilities we compared the frequencies of functional and non-functional V_γ1-J_γ4 and V_γ2-J_γ2 rearrangements in sorted 2.11⁺ and 2.11⁻ γδ i-IEL populations. cDNA from the sorted populations was amplified by PCR. The sense primer used in this experiment has a sequence identity to the V_γ1 and V_γ2 genes, while the sequence of the antisense primer is present in all C_γ genes. Thus, this set of primers is likely to amplify the V_γ1-C_γ4 and the V_γ2-C_γ2 cDNAs proportionally to their representation in the total cDNA population.

V _γ 1	P1+N+P2	J _γ 4
AVWI		SGT
AVW	R	SGT
AVW	PSAR	SGT
AVW	K	SGT
AV	R	SGT
AVW	G	SGT
AVW	GG	SGT
AVW	V	SGT
AVWI	KWA	GT
AVW	FR	SGT
AVW	FP	T
AVW		SGT
AVWI	S	SGT
AVW	T	GT
AVW	R	SGT

Fig. 2. Predicted amino acid sequences encoded by the IEL in-frame V_γ1 junctions.

The frequencies of functional and non-functional rearrangements of V_γ1 and V_γ2 genes in the 2.11⁺ and 2.11⁻ δ i-IEL populations are presented in Table 1. Three major points are worth noting. First, most of the functionally rearranged V_γ1 genes are contained in the 2.11⁺ population, showing a correlation between functionally rearranged V_γ1 gene expression and 2.11⁺ phenotype. Second, the frequencies of functional V_γ2 rearrangements in the two cell populations are very similar to each other (33.3% in the 2.11⁺ population and 37.5% in the 2.11⁻ population) and are also very close to the frequency expected by random rearrangement of TCR and/ or Ig genes in the absence of cellular selection (one-third of

Table 1. Expression of functional *V_γ1-J_γ4* and *V_γ2-J_γ2* rearrangements in different i-IEL populations

i-IEL population ^a	Gene rearrangement	No. of clones	No. of functional clones (% of the total)
$\gamma\delta^+$ 2.11 ⁺	<i>V_γ1-J_γ4</i>	17	14 (82.3)
	<i>V_γ2-J_γ2</i>	18	6 (33.3)
$\gamma\delta^+$ 2.11 ⁻	<i>V_γ1-J_γ4</i>	2	1 (50.0)
	<i>V_γ2-J_γ2</i>	24	9 (37.5)

^aSorted $\gamma\delta^+$ 2.11⁺ and $\gamma\delta^+$ 2.11⁻ i-IEL population for B6 mice.

all rearrangements are expected to be in-frame). These data suggest a lack of cellular selection for functional *V_γ2* rearrangements, supporting the notion that cells expressing the *V_γ2* gene product as a part of the TCR on the cell surface are rare, if they exist at all. Third, the fact that we amplified roughly as many *V_γ2* cDNA clones as functionally rearranged *V_γ1* cDNA clones (14 clones were *V_γ1* in-frame and 18 clones were *V_γ2*) in the 2.11⁺ population suggests that most *V_γ1*-bearing cells express mRNA from one rearranged *V_γ2* gene. Assuming that most $\gamma\delta$ T cells do not express two different TCR, these data indicate that there is no correlation between the expression of functionally rearranged *V_γ2-C_γ2* mRNA and expression of the *V_γ2-C_γ2* protein at the cell surface. Thus, the presence of functional *V_γ-C_γ* rearrangements *per se* should not be considered as an indication of the presence of cells expressing this *V_γ-C_γ* protein as part of their TCR.

Population analysis of transcripts from rearranged TCR γ chain genes

A recently developed technique allows population analysis of TCR γ chain rearrangement or mRNA expression without nucleotide sequencing. This technique takes advantage of the fact that different rearrangements between the same gene segments often display length heterogeneity due to the random shortening and/or addition of N nucleotides at the junction. The lengths of productively rearranged genes can differ by multiples of three nucleotides, whereas non-productively rearranged genes will have lengths offset by one or two nucleotides from productively rearranged sequences. PCR amplification, with *V_γ1-J_γ4* or *V_γ2-J_γ2*-specific primers, of DNA or cDNA isolated from a polyclonal T cell population followed by a primer extension reaction with a fluorochrome-labeled nested primer will yield a labeled set of fragments of different lengths. Such fragments, differing in length by as few as a single base pair, can be resolved on denaturing polyacrylamide gels.

A plot of the fluorescence intensity profile versus length for the *V_γ1-J_γ4* and the *V_γ2-J_γ2* amplifications of cDNA isolated from 2.11⁺ i-IEL and for the *V_γ2-J_γ2* amplifications of cDNA isolated from 2.11⁻ i-IEL is shown in Fig. 3. In concordance with the sequence data, most of the detectable fragments obtained after amplification with *V_γ1-J_γ4* primers in the 2.11⁺ population show length intervals of three nucleotides (Fig. 3A), while the patterns observed after amplification of both cell populations with *V_γ2-J_γ2*-specific primers show length

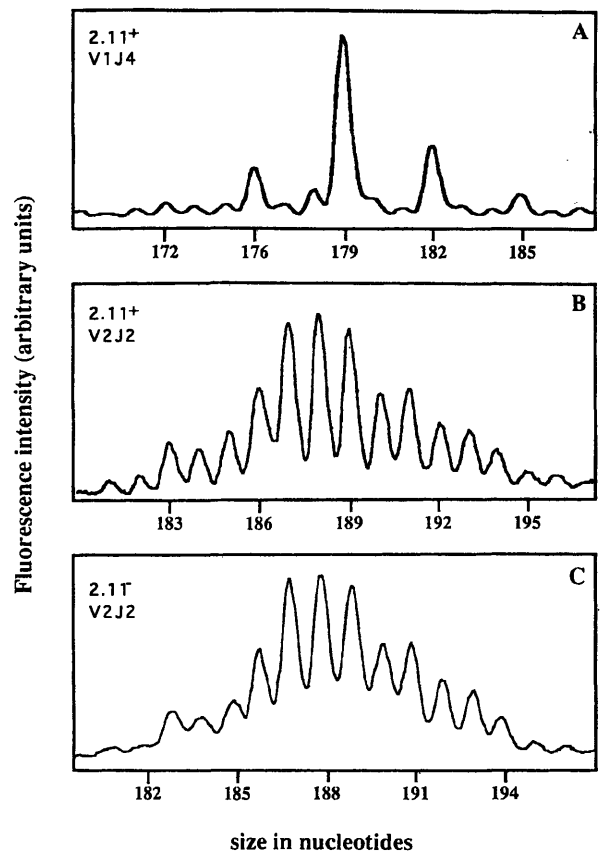


Fig. 3. TCR γ mRNA population analysis from sorted i-IEL populations. Profiles of PCRs performed on cDNA isolated from $\gamma\delta^+$ 2.11⁺ (A and B) or from $\gamma\delta^+$ 2.11⁻ (C) sorted i-IEL populations using *V_γ1-J_γ4*- (A) or *V_γ2-J_γ2*-specific primers (B and C).

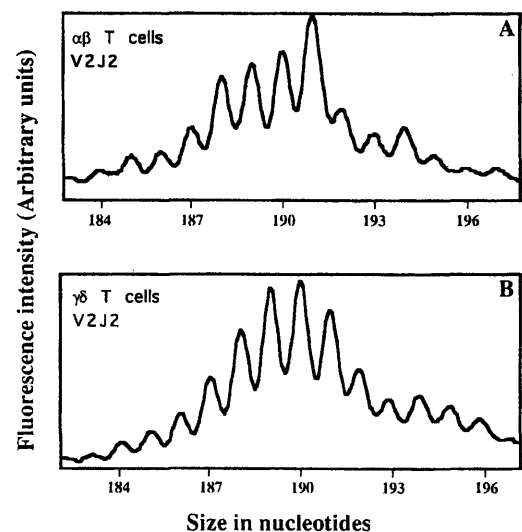


Fig. 4. Analysis of *V_γ2* mRNA expression in different T cell populations. Profiles of PCRs performed on cDNA isolated from TCR $\alpha\beta^+$ (A) and TCR $\gamma\delta^+$ thymocytes. Cells were prepared as described in Methods. The plots represent the length of the amplified fragments versus the fluorescence intensity of each band in arbitrary units.

of the V_γ1-J_γ4 rearrangements were distributed in three major peaks having the predicted germline length plus or minus three nucleotides.

The high level of homology between the V_γ1 and the V_γ2 genes imposed the choice of a reverse primer that would provide specificity for each J-C_γ gene. We decided to use J_γ-specific primers instead of C_γ-specific primers because the C_γ4 sequence from B6 mice is not known. To avoid amplification of rearranged DNA the RNA preparations were treated with DNase before cDNA synthesis. In addition, similar results were obtained when the amplifications were performed with a pan-C_γ primer which does not effectively amplify rearranged genomic DNA (not shown).

Analysis of V_γ2-J_γ2 mRNA was also performed on highly purified αβ⁺ and γδ⁺ thymocytes, and the results are shown in Fig. 4. The pattern observed in both T cell populations was very similar to the one previously found in γδ i-IEL, with length intervals of one nucleotide. These data demonstrate that the unselected rearrangement and expression of the V_γ2-J_γ2 gene is not limited to the γδ i-IEL population, but also takes place in other γδ T cell populations and in αβ T cells.

A functional V_γ3-J_γ3-C_γ3 gene in B6 mice

During the cloning and sequencing of the PCR products presented above, we isolated one clone containing a putative J_γ segment different from all J_γ segments previously described. Comparison of its sequence with published J_γ sequences indicated a very high degree of homology with the J_γ3 segment present in BALB/c mice (5). The J_γ3 segment in BALB/c mice has a single base pair deletion compared with the J_γ2 segment. This base pair deletion causes a frame

shift which, together with the fact that the C_γ3 gene lacks a proper splicing acceptor site, renders the J_γ3-C_γ3 gene non-functional (4,5). The putative J_γ gene found in B6 does not have the single base pair deletion, and with the exception of this difference, is identical in sequence to the BALB/c J_γ2 segment (Fig. 5). Thus, it is very likely that this J_γ segment corresponds to the B6 J_γ3. Analysis of the V region rearranged to the J_γ3 in this clone showed that it is the V_γ3 segment (see Fig. 6). Together with the fact that this clone was obtained from a PCR performed on cDNA, these data suggested that the V_γ3-J_γ3-C_γ3 gene in B6 is functional.

To ascertain that this potential V_γ3-J_γ3-C_γ3 rearrangement was genuine and not a PCR artefact, we decided to further characterize the C region associated with this J segment. We screened B6 hybridomas for the presence of a V_γ3-J_γ2 rearrangement and found one satisfying this criterion. We then prepared cDNA from this hybridoma and amplified it by PCR using primers for the J_γ3 segment and the 3' untranslated region of the BALB/c C_γ3 gene. We then determined the nucleotide sequence of the DNA product obtained. A comparison of the putative B6 V_γ3-J_γ3-C_γ3 with the previously described BALB/c V_γ2-J_γ2-C_γ2 and V_γ3-J_γ3-C_γ3 sequence is shown in Fig. 6. The putative B6 C_γ3 sequence differs from the BALB/c C_γ3 sequence by only 14 of 515 nucleotides (97.3% identity). All but one of the nucleotide differences were located in the first exon. Only five of the 14 nucleotide differences between the putative B6 C_γ3 gene and the BALB/c C_γ3 gene result in amino acid changes and all of these are located in the first exon. Interestingly, four of these five different nucleotides present in the putative B6 C_γ3 sequence are found in the sequence of the BALB/c C_γ2 gene

	V _γ 3	P	N	P	J _γ 3	Functional
germline V _γ 3	TGT GCA GTC TGG ATA AA	TT				
germline J _γ 3				AT	AT AGT TGG GAC TTT	
A) <u>i-IEL</u>						
	TGT GCA GTC TGG ATA		GAGAGGGG	AT	AT AGT TGG GAC TTT	Yes
	TGT GC		GAGGA		AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG			T	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG		GC	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC T		TCAG	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG ATA		CCGGGACTGGA	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA		TGGGG	T	AT AGT TGG GAC TTT	No
	TGT GCA GTC TGG A		A		AT AGT TGG GAC TTT	No
	TGT GCA GTC TGG ATA		CG		GT TGG GAC TTT	No
	TGT GCA GTC TGG ATA AA	TT	GGA	AT	AT AGT TGG GAC TTT	No
	TGT GCA GTC TGG		C	AT	AT AGT TGG GAC TTT	No
B) <u>Thymus</u>						
	TGT GCA GTC TGG			T	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC T		CGG		GT TGG GAC TTT	Yes
	TGT GCA GTC TGG		GG	AT	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG		CAT	T	AT AGT TGG GAC TTT	Yes
	TGT GCA GTC TGG AT		CCCT		T AGT TGG GAC TTT	No
	TGT GCA GTC TGG AT		GTGGGG		AGT TGG GAC TTT	No
	TGT GCA GTC TGG		GGCCTG		T AGT TGG GAC TTT	No

Fig. 7. V-J junctional sequences of V_γ3 transcripts from B6 i-IEL and thymocytes. Data represent cloned V_γ3-C_γ3 PCR products from sorted γδ⁺ i-IEL (A) and *in vitro* activated γδ⁺ thymocytes (B).

In fact, considering only the first exon, the B6 C_γ3 gene is more similar to the BALB/c C_γ2 gene than to the BALB/c C_γ3 gene, and the B6 C_γ3 and BALB/c C_γ2 genes show a higher degree of identity than do the two BALB/c C_γ regions. Thus, the first exon of the B6 C_γ3 gene differs from that of the BALB/c gene by nine nucleotides, resulting in five amino acid substitutions, while the first exons of the BALB/c C_γ2 and C_γ3 genes differ by 13 nucleotides resulting in nine amino acid changes.

The partial sequence of the V_γ3 gene in B6 differs from the V_γ3 gene of BALB/c by two nucleotides, both of which result in amino acid changes, and they are likely to represent an allelic polymorphism. Taken together, these data show that the J_γ3-C_γ3 gene is functional in B6 mice and that the V_γ3 gene rearranges to it.

Expression of the V_γ3-J_γ3-C_γ3 gene in γδ T cells from B6 mice

The fact that the V_γ3-C_γ3 gene is functional in B6 mice raises the issue of whether a subset of γδ T cells expresses the V_γ3-C_γ3 protein as part of their TCR. To analyze this issue we prepared cDNAs from γδ T cells isolated from the thymus and the small intestine of B6 mice, and amplified their junctional sequences with V_γ3 and C_γ-specific primers. The PCR products were then cloned and sequenced, and the V_γ3-J_γ3 junctional sequences are shown in Fig. 7. Six of the 11 clones (54.5%) isolated from the γδ i-IEL and five of the nine clones (55.5%) obtained from the γδ thymocytes contained functional rearrangements. These data are consistent with the possibility that a small fraction of γδ T cells in those organs express the V_γ3 chain.

Discussion

γδ T cell subsets that home to different epithelia are known to exhibit different degrees of diversity in the junctional sequences of their TCR. Previous analysis of V_γ7 and V_δ sequences in γδ i-IEL have shown that these cells exhibit extensive junctional diversity, which is believed to confer on these cells the ability to recognize an array of different antigens (18–20). We found similar junctional diversity in the V_γ1⁺ i-IEL population with no apparent selection for any particular amino acid sequence in the CDR3. The high frequency of a codon for arginine (GGA) in the V_γ1-J_γ4 junctions can be explained by the P nucleotides that are retained in the 5' end of the joined J_γ4 segment and by the preference of the TdT enzyme for purine bases. The very high frequency of a codon for tyrosine that was previously described in V_γ7-J_γ1 junctions (18–20) (and is actually present at the junctions of other V_γ genes with J_γ1) may also be due to P nucleotides: any in-frame sequence containing at least one P nucleotide in the 5' end of the J_γ1 segment will have a tyrosine codon, TAT.

The junctional diversity found in the TCR of γδ i-IEL is in contrast with the limited diversity found in the TCR of αβ i-IEL, in both mice (21) and humans (22–26). It is not clear whether this difference reflects antigenic selection or a difference in the number of precursor T cell clones that give rise to the respective i-IEL subsets. In any case, these data suggest a different function of αβ and γδ i-IEL.

The high expression of transcripts of rearranged V_γ2-C_γ2

genes in γδ T cells known to express other V_γ chains at the cell surface and in αβ T cells poses a question about the cell surface expression of the V_γ2 chain in these T cells. The ratio of functionally versus non-functionally rearranged V_γ2 genes in different γδ and αβ T cell populations shows that there is no selection for cells harboring in-frame rearranged V_γ2 genes. This strongly suggests that the expression of the V_γ2 chain is irrelevant for the fate of these T cells subsets. Although it is clear that a γδ TCR composed of a V_γ2 chain is not expressed at detectable levels on the cell surface of αβ T cells (14) one cannot exclude the possibility that the V_γ2-J_γ2-C_γ2 chain is co-expressed with other V_γ chains on the surface of some γδ T cells. Lack of isotype exclusion of γ chains at the cell surface level has been reported in some γδ T cell lines and hybridomas in which V_γ1-C_γ4 chains are co-expressed with either V_γ2-J_γ2 or V_γ4-J_γ1 chains (27–29). However, in these cases the expression of the V_γ1-J_γ4 chain was much higher than the expression of the other γ chains. Furthermore, double staining analysis with available γ-chain-specific antibodies in normal γδ T cells shows that co-expression of detectable levels of two different γ chains in the same cell occurs rarely, if at all (unpublished results). It appears, therefore, that expression of functionally rearranged V_γ2 transcripts in γδ T cells does not generally correlate with detectable levels of expression of the V_γ2 chain on the cell surface. Nevertheless, in a few instances, γδ T cell lines and clones expressing the V_γ2-J_γ2 chain as part of their TCR have been reported (30,31), suggesting that a relatively small subset of γδ T cells in normal mice expresses the V_γ2 chain. Likewise, according to our data a small fraction of the γδ T cells in B6 mice may express the V_γ3 chain. The fact that the V_γ3-J_γ3-C_γ3 gene has been deleted in most of the laboratory mouse strains and is non-functional in others suggests that there has been a selection against this gene. It is possible that the B6 V_γ3-J_γ3-C_γ3 gene has been subject to a different form of inactivation and that a similar case exists for the V_γ2-J_γ2-C_γ2 gene in general. A common feature of these two genes is the lack of glycosylation sites, that might interfere with the folding, transport or half-life of the molecule in the cytosol.

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**On Somatic Recombination in the Central Nervous
System of Transgenic Mice**

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On Somatic Recombination in the Central Nervous System of Transgenic Mice

Complete immunoglobulin (Ig) and T cell receptor (TCR) genes are generated by developmentally controlled DNA rearrangement referred to as V(D)J recombination or V(D)J joining (1). V(D)J recombination has been thought to be restricted to lymphoid cells and, until recently, there had been no evidence that recombination signal sequence (RSS)-mediated recombination of non-Ig or non-TCR genes occurred during normal vertebrate development. However, it had been speculated that this or similar types of somatic DNA rearrangement might play a role in mammalian cell differentiation (2). Interest in this hypothesis was refueled by the report that RAG-1 transcripts were present, albeit in low numbers, in the central nervous system (3). Furthermore, it was reported that somatic recombination was detectable in the brains (4) of Tg mice that harbored a V(D)J recombination substrate. A second study (5) reported a small amount of V(D)J recombination in the brain, but this was attributed to lymphocyte contamination. We had independently constructed similar Tg mice whose initial analysis (6) generated data consistent with the occurrence of V(D)J recombination in the brain. However, further analysis has led us to a different interpretation of that data.

In order to prepare a Tg V(D)J recombination substrate, we constructed a plasmid pSPH-1 (7). This plasmid contained the transcriptional promoter and enhancer of the mouse phosphoglycerate kinase-1 gene (*pgk-1*) (8), a pair of RSSs derived from the Ig V_{H21C} and J_{H1} gene segments (9), and a reporter gene *lacZ* encoding bacterial β -galactosidase. The reporter gene *lacZ* was placed in an orientation opposite to that of the promoter so that RSS-mediated inversional recombination would activate its expression (Fig. 1). Cells expressing *lacZ* can be detected histochemically after staining with X-gal. Alternatively, inversional recombination can be detected more directly at the molecular level by the polymerase chain reaction (PCR) method (10) with the use of appropriate primers. We used both of these methods.

We generated Tg mice by injecting the pSPH1 insert into C57BL/6J zygotes (11). We analyzed the heterozygous progeny of five Tg lines (1-7, 1-20, 1-21, 1-28, and 1-39) for expression of β -galactosidase activity by histochemistry (12). Liver sections of all Tg lines were negative for β -galactosidase activity. We observed considerable enzyme activity in kidney, spleen, and thymus sections, but also in the non-Tg litter-

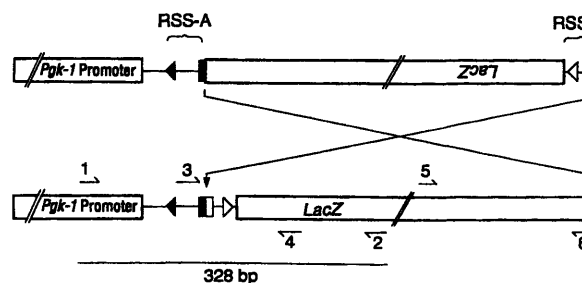
mates (presumably because of endogenous β -galactosidase activity). We observed β -galactosidase activity in the brain of two of the five Tg lines, 1-7 (Fig. 2) and 1-20; we saw no such activity in non-Tg littermates. Regions of the brain in which we saw intense β -galactosidase activity in the Tg line 1-7 (Fig. 2) include the hippocampus (the dentate gyrus and the CA1 and CA3 fields), the cerebral cortex (especially the superficial layers), the superior colliculus (upper layers), nuclei of the dorsal tegmentum, and the cerebellum (especially its molecular and Purkinje cell layers). Sparse β -galactosidase activity also appeared in other sites (Fig. 2A). There was low expression in the striatum and in much of the thalamus. In Tg line 1-20, we observed a roughly similar pattern of X-gal staining, but the staining was weak. In both lines, the X-gal staining was region-specific. Cells with β -galactosidase activity appeared to be neurons in both Tg lines. The staining appeared to be limited to a small (2 to 5 μ m), eccentrically located compartment of the cytoplasm (Fig. 2, C and E). Both we and Matsuoka *et al.* detected X-gal staining in the cerebral cortex and the hippocampus, but the patterns differed considerably in other regions, such as the cerebellum, where Matsuoka *et al.* saw X-gal staining in the granule and Purkinje cell layers and we saw staining primarily in the molecular cell layer.

In order to test whether RSS-mediated inversion had occurred in some tissues of the Tg mice, we analyzed genomic DNA by PCR using primers 1 and 2 (Fig. 1). If the Tg RSSs underwent an inversional V(D)J recombination, a 328-bp DNA fragment containing the joined RSSs would be generated by PCR (Fig. 1). The predicted 328-bp product was observed with the DNA isolated from the thymus of the Tg line 1-7, but not with that from non-Tg

mice or from any of the other four Tg lines (1-20, 1-21, 1-28, and 1-39) (Fig. 3A). We cloned the 328-bp fragment and determined its nucleotide sequence. It contains the precisely head-to-head joined Tg RSSs. We analyzed DNA isolated from additional tissues of each Tg line, but none of the tissues derived from any Tg lines other than line 1-7 produced the 328-bp DNA fragment. In line 1-7, only spleen and thymus produced the 328-bp DNA fragment; liver, cerebral cortex, ovary, muscle, kidney, and lung were negative (Figs. 3, B and C). We also analyzed DNA isolated from cerebral cortex and hippocampus of line 1-7, regions in which β -galactosidase-positive cells were abundant (Fig. 2) and DNA isolated from the striatum, where thymocytes were rare (Fig. 2). We did not detect the 328-bp DNA fragment in these tissues (Figs. 3, B and C).

We estimated the sensitivity of this PCR assay for the detection of V(D)J recombination by analyzing a fixed amount of non-Tg thymus DNA mixed with different amounts of Tg (line 1-7) thymus DNA (Fig. 3C). The 328-bp DNA fragment was detectable in DNA samples in which Tg thymus DNA constituted only 1 part in 1000 of the total DNA. As only a fraction of the thymus cells from the Tg mice would have undergone RSS-mediated recombination, these data indicate that the sensitivity of the PCR method is at least 1 in 10 cells. In some parts of the brain the proportion of β -galactosidase-positive cells among total nucleated cells far exceeded the sensitivity of the PCR method. For instance, we estimated their proportion in the cerebral cortex to be 1 to 10% (13), a figure at least one to two orders of magnitude greater than the PCR detection limit. We therefore conclude that the majority of β -galactosidase-positive cells observed did not result from RSS-mediated inversion. We analyzed the absolute sensitivity of the PCR assay by carrying out a reconstitution experiment (14) in which different amounts of plasmid DNA containing *pgk-1* promoter

Fig. 1. Schematic representations of the Tg V(D)J recombination substrate (top) and the predicted product (bottom) of a V(D)J recombination event. (Top) In the substrate the bacterial β -galactosidase gene, *lacZ*, is oriented inversely to the *pgk-1* promoter. Recombination signal sequences (RSS-A and RSS-B) flank *lacZ* and are comprised of heptamer (rectangle), nonamer (triangle), and spacer elements. (Bottom) V(D)J recombination of the substrate is expected to join the two RSSs precisely and to invert *lacZ*, thereby activating transcription by the *pgk-1* promoter. Oligonucleotide primers used for PCR amplification (small arrows) are numbered. PCR amplification of the predicted product of a V(D)J recombination event with primers 1 and 2 results in the 328-bp product indicated at bottom.



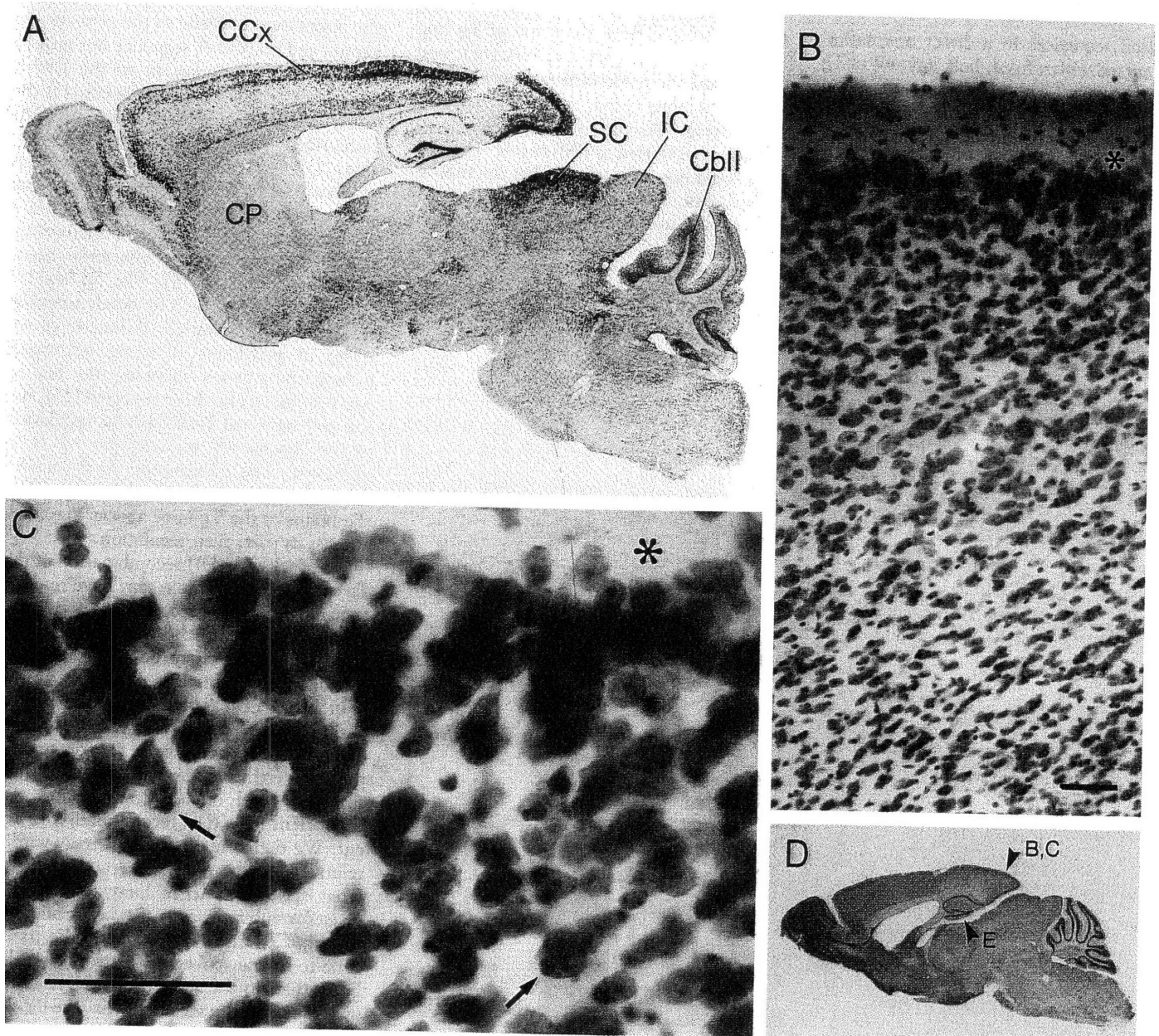
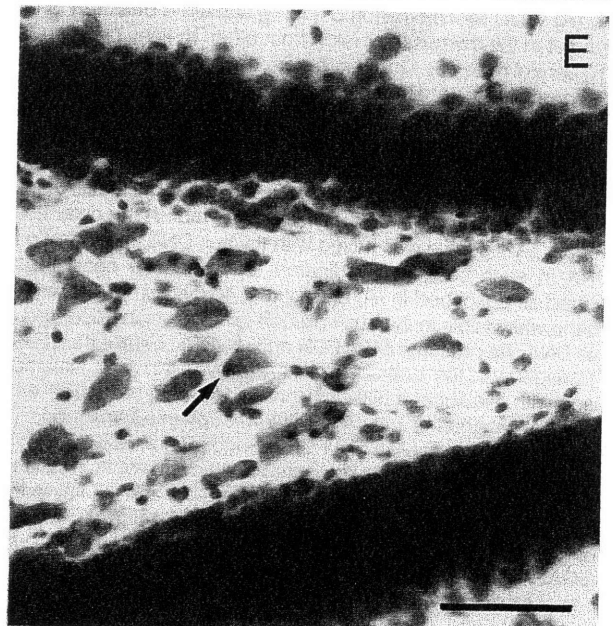


Fig. 2. Patterns of β -galactosidase activity in Tg line 1-7 mouse brain detected with X-gal histochemistry in frozen sections 40 μm thick (12). (A) Photomicrograph of uncounterstained parasagittal section illustrating locations of intense β -galactosidase activity; β -galactosidase-positive cells appear black. There is intense expression in posterior cortex and in ventral frontal cortex, in superior colliculus, and in some ventral forebrain and brainstem nuclei. CCx, cerebral cortex; CP, caudoputamen; SC, superior colliculus; IC, inferior colliculus; Cbll, cerebellum. (B to E) Photomicrographs showing X-gal staining for β -galactosidase activity (blue) in sections counterstained with neutral red (red) to indicate neurons and glia. (B), (C), and (E) show high-magnification views of regions indicated by the same letters in the parasagittal section illustrated in (D). This section was one of those used for cell counting (13). Scale bars in (B), (C), and (E) are 50 μm . (B) Posterior neocortex, with concentration of β -galactosidase-positive cells in superficial layers, fewer β -galactosidase-positive cells in deep layers, and fewest in intermediate layers. (C) Close-up view of superficial cortical layers shown in (B) (asterisks mark corresponding locations). Arrows indicate eccentric position of many of the blue β -galactosidase-positive spots at plasma membranes of neurons. Double staining of β -galactosidase-positive spots with microglial markers was negative. (E) High-magnification view of dentate gyrus of hippocampus. Arrow points to eccentrically located β -galactosidase-positive spot in large neuron.



and *lacZ* sequences in a direct orientation were mixed with a fixed, bulk amount of Tg cerebral cortex DNA. The results suggested that our PCR conditions would detect as few as one to ten recombination events among 10^5 cells. These results confirmed that, if V(D)J recombination occurs in the nonlymphoid tissues that we examined, it is rare.

If not an RSS-mediated inversion, what mechanism allows β -galactosidase to be expressed in the brain? To answer this question, we synthesized cDNA of *lacZ*

mRNA isolated from the brain of Tg line 1-7, cloned the amplified cDNA (15), and determined the nucleotide sequences of the clones. Among nine randomly selected clones (Fig. 4), one (1-2) produced sequences that started at RSS-B (Fig. 1). This cDNA did not indicate whether the reporter *lacZ* gene was rearranged. On the other hand, the sequence of each of the remaining eight cDNA clones began farther upstream, and six of these began within the *pgk-1* promoter. The promoter was used in

the orientation opposite to the conventional one, and no RSS-A sequence was present on these cDNA's adjacent to the RSS-1 sequences, which would be expected if the cDNA had been derived from mRNA transcribed from the inverted *lacZ* gene (Fig. 1). It is likely that these cDNA sequences were derived from mRNAs that were transcribed from the unrearranged *lacZ* gene by using, in the backward orientation the *pgk-1* promoter of the adjacently inserted plasmid copy (Fig. 4). The Tg line 1- contains about ten such tandemly integrated copies of the plasmid. Bidirectional activity of promoters of some eukaryotic housekeeping genes, including the human *pgk-1* promoter, has been reported (16), but the cell type and tissue-specific regulation of the "backward" transcription has been unknown. The patterns of Tg β -galactosidase (*lacZ*) expression that we found in the brains of the Tg mice appear to reflect at least in part, such regulation.

The lack of V(D)J recombination of the Tg substrate in the brain may have resulted from either a lack of recombinase activity or an inaccessibility of the substrate. The latter condition seems to be correlated with the absence of transcripts (17). Our ability to clone the cDNA of *lacZ* mRNA from the brain of the Tg line 1-7 suggested that the Tg substrate is accessible in this organ, but there nevertheless remained the possibility that the level of *lacZ* transcription was insufficient for V(D)J recombination to occur. We therefore used a PCR assay with a relatively low number of reaction cycles (that is, 30) in order to compare the level of *lacZ* transcription in the brain and the spleen of Tg line 1-7. We found the transgene to be transcribed more strongly in the brain than in the spleen (Fig. 3D). These data reinforce the argument that the lack of V(D)J recombination in the brain does not result from the inaccessibility of substrate although we cannot rule out the possibility that the transgene is inaccessible in restricted regions or during developmental stages of the nervous system where or when V(D)J recombinase is available.

Although we focus on a single transgenic line in this study, we believe our conclusions are valid because in this line V(D)J recombination does take place in the lymphoid organs, and therefore the transgene present in this line is fully capable of undergoing V(D)J recombination. We, of course, do not rule out the possibility that a small fraction of the β -galactosidase-positive cells did undergo V(D)J recombination at a frequency that was smaller than the detection limit of the PCR assay. However, this does not change our conclusion that the evidence for somatic V(D)J recombination in the brain, if any indeed occurs, is yet to be obtained.

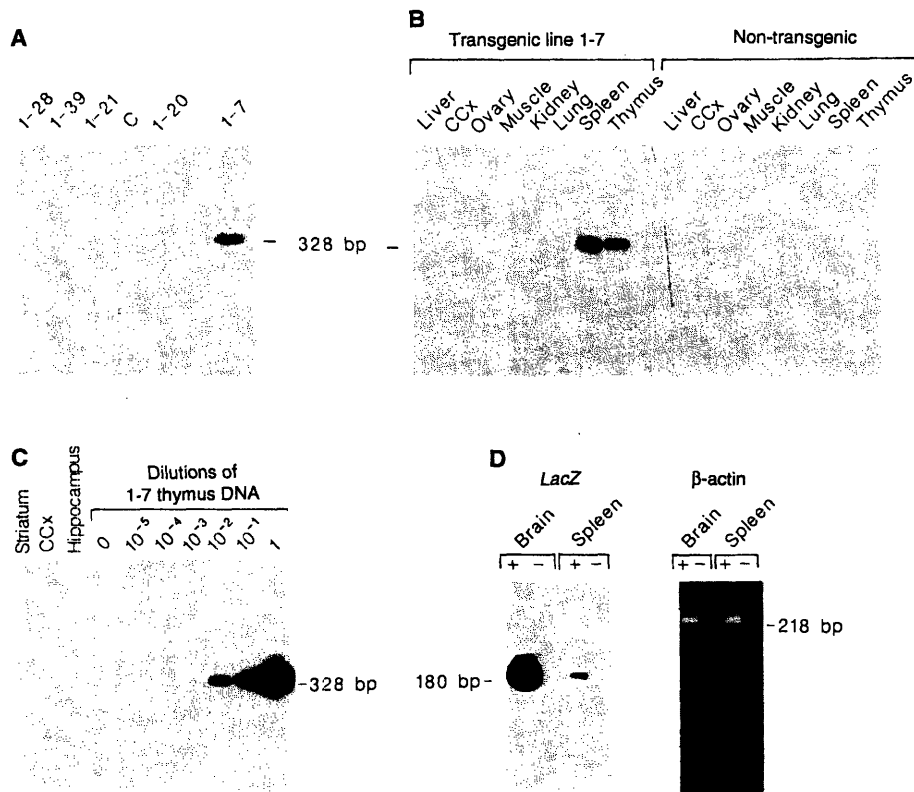
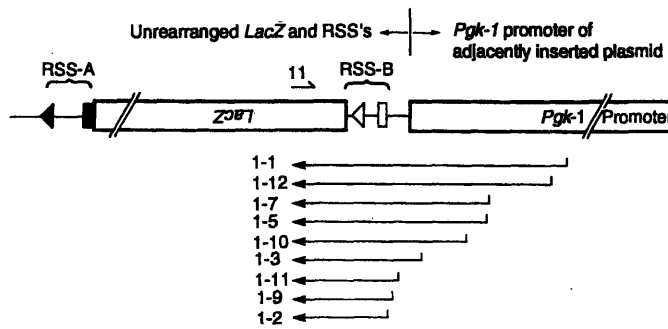


Fig. 3. (A) V(D)J recombination of the Tg substrate detected in the thymuses of mice from Tg line 1-7 but not in the thymuses of mice from other Tg lines (1-28, 1-39, 1-21, 1-20) or in the thymus of non-Tg control mice (C). One microgram of genomic DNA was digested with the restriction enzyme Eco RI, precipitated by ethanol, and then subjected to 30 cycles of PCR (24) with primers 1 and 2 (Fig. 1). Ten percent of the PCR products was then separated on a 2% agarose gel and examined by DNA (Southern) blotting. 32 P-labeled oligonucleotide 29, a 32-bp oligonucleotide that includes RSS-B, was used as a hybridization probe. (B) V(D)J recombination of the Tg substrate, which is limited to the lymphoid organs in mice from Tg line 1-7. Assays performed as in (A). CCx, cerebral cortex. (C) Sensitivity of the PCR assay. To establish the detection limit of this assay, thymus DNA from strain 1-7 Tg mice was mixed with thymus DNA from non-Tg littermates. The proportion of the Tg DNA in each sample is indicated. Each sample consisted of 1 μ g of DNA. Assays were performed as described in (A) except that 40 cycles of PCR were performed instead of 30. A very faint band was visible in the 10^{-5} dilution sample which appeared at very low dilutions of transgene thymus DNA; we presume it is a PCR artifact. CCx, cerebral cortex. (D) Abundant transcription of the *lacZ* transgene in the brain. Poly(A)⁺-positive RNA was isolated (25) from mice that had been perfused with 1% sodium nitrate in phosphate-buffered saline and then with deoxyribonuclease I (26). One microgram of poly(A)⁺ RNA was reverse-transcribed (+), or the reverse transcriptase was omitted (-), to establish that PCR products were derived from RNA and were not contaminating genomic DNA. Samples were subjected to 30 cycles of PCR (24) with primers 5 and 6 (Fig. 1), and then 10% of the PCR products was separated and examined by Southern blotting as in (A). 32 P-labeled oligonucleotide 13 (5'-GTCCAAACTCATCAATGTATCTT-3') was used as a hybridization probe. PCR analysis was also performed with β -actin-specific primers 11 (5'-GGATGCAAGGAGAT TACT-3') and 12 (5'-AAAACGCAGCTCAGTAAACAG-3') under the same conditions in order to establish that equivalent amounts of cDNA were present in the two samples (right). An agarose gel stained with ethidium bromide is illustrated.

Fig. 4. β -galactosidase mRNA detected in the brains of Tg mice from line 1-7 that are not derived from a recombinant transgene. Complementary DNA of mRNA from the brains of line 1-7 Tg mice was amplified by single anchored PCR (16). PCR products were then cloned into the vector Bluescript and sequenced as described.



The *pgk-1* promoter of an adjacent transgene was present at the 5' end of all clones that extended beyond the 5' end of the *lacZ* gene and the adjacent RSS-B. This *pgk-1* promoter, however, is inverted relative to the *lacZ* gene. Individual PCR products are depicted by arrows and are numbered.

This conclusion is at variance with the interpretations drawn by Matsuoka *et al.* (4), who used Tg mice that were constructed independently but with a similar strategy. They also found abundant and region-specific expression of *lacZ* in the brain. However, in contrast to our findings with CR, their PCR assay detected sequences that were apparently produced by inversional recombination of the reporter gene that took place 9 to 138 bp away from the head of the RSSs. Matsuoka *et al.* concluded that "somatic gene rearrangement may be involved in neonatal development" (18). Although it is possible that our conclusion differs from that of Matsuoka *et al.* because of variations in the experimental protocols, including differences in the composition of the Tg plasmids, we believe it is more likely that the different conclusions arise from different interpretations of data.

First, in light of our analysis of β -galactosidase cDNA clones, we concluded that the β -galactosidase expression observed in the brains of our Tg mice is most probably due to backward transcription from the promoter of an adjacent transgene rather than RSS-mediated inversion. We suspect that the same may be true for the Tg mice reported by Matsuoka *et al.* because the arrangement of various sequence motifs in the chicken cytoplasmic β -actin promoter that Matsuoka *et al.* used is similar to that of the chicken skeletal α -actin promoter, a demonstrated bidirectional promoter (19).

Second, we did not detect any evidence of V(D)J recombination with brain DNA using PCR. In contrast, Matsuoka *et al.* cloned PCR products that they interpret as having been derived from "imprecise" (D)J recombination that had occurred at sites 9 to 138 bp away from the heads of the RSSs. There are at least two other interpretations of this finding. Recombination may have occurred *in vitro* during PCR amplification by "PCR mediated recombination" (20). The two parental sequences involved in each recombination event reported by Matsuoka *et al.* carry 10-, 3-, and 2-bp

homologies, respectively, at the recombination sites. Short homology is expected at the site of PCR recombination, whereas it is more an exception than a rule in the noncoding joints of V(D)J recombination. In one published case 12 nucleotides (21), and in another study 7 and 5 nucleotides (22), have been shown to be sufficient for PCR recombination. If PCR recombination accounted for the sequences that Matsuoka *et al.* observed, the apparent tissue specificity of the PCR products (5, figure 2) may have resulted from sample-to-sample variation, which is commonly encountered with PCR artifacts. Another possibility is that the observed joints resulted from rare illegitimate recombination events that took place among the 15 copies of the integrated plasmid that would be unrelated to developmentally meaningful somatic recombination. Transgenes are generally unstable genetic elements, and short stretches of homology of one to five nucleotides at the junction are generally observed in illegitimate recombination (23).

In summary, we suggest that it is premature to conclude whether or not developmentally meaningful somatic recombination occurs in the brain. However, a positive and interesting finding that emerged from our study (and possibly also from that of Matsuoka *et al.*) is that backward transcription in the brain can occur in a highly region- and neuron-specific manner. The physiological role of backward transcription is unknown, but in light of its remarkable tissue or organ specificity, it is possible that backward transcription may participate in the regulation of genes associated with a bidirectional promoter, including genes in the central nervous system.

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7. Plasmid pSPH1 was constructed as follows. Oligonucleotides 29 (5'-TCGAGCACAGTGCTCCAGGGCTAACAAAAACC-3') and 30 (5'-AGCTTGGTTTTTGTTCAGCCCTGGAGCAGCTGTG-3'), which constitute RSS-A, and oligonucleotides 27 (5'-GATCCACAGTGGTAGTACTCCACTGTCTG-GCTGTACAAAAACC-3') and 28 (5'-TCGAGGGTTTTTGTACACCCAGACAGTGGAGTACTACCACAGTG-3'), which constitute RSS-B, were annealed and then ligated to a Bam HI-Hind III fragment of plasmid pCH110 (Pharmacia, Piscataway, NJ) that contains *lacZ*. The ligation product was ligated into the Sal I site of pUC19 to form the plasmid pAA14. A Sma I-Pst I fragment of pAA14 was treated with T4 polymerase and then ligated into Bam HI-Pst I-digested and Klenow enzyme-treated plasmid pKJ (containing the murine *pgk-1* promoter, a gift from M. Rudnicki and M. McBurney) to form the plasmid pK14, in which *lacZ* was inserted in an orientation opposite to the *pgk-1* promoter, and the plasmid p11, in which *lacZ* was inserted in the same orientation as the *pgk-1* promoter. An Eco RI fragment of pK14 that contains the *pgk-1* promoter was subsequently ligated into the Eco RI site of pAA14 to form pSPH1.
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12. C. Walsh and C. L. Cepko, *Science* 241, 1342 (1988). Mice were perfused with 2% paraformaldehyde in PIPES buffer under deep sodium pentobarbital anesthesia (anesthesia procedure according to guidelines of the Massachusetts Institute of Technology Division of Comparative Medicine), and their brains were postfixed for up to 8 hours at 4°C and soaked overnight in phosphate buffer containing 30% sucrose and 2 mM MgCl₂ at 4°C. Parasagittal or transverse sections were cut at 30 or 40 μ m on a sliding microtome and were stained for β -galactosidase with X-gal. Selected sections were counterstained with neutral red.

13. The percentage of β -galactosidase-positive cells in the cerebral cortex of the transgenic line 1-7 mice was estimated as follows. First, to establish the total number of cells per unit volume, a Biocom computer (Les Ulis, France) was used to determine the numbers of neutral red-positive cells visible in cortical samples in parasagittal sections 40 μm thick. Stained cells were counted in rectangles (100 by 150 μm) stacked to cover the full depth of the cortex. Values were calculated as cells per 0.1 mm by 0.15 mm by 0.25 mm = 0.006 cubic millimeters. Counts were made for frontal ($n = 2$), mid-anteroposterior ($n = 2$), and posterior ($n = 1$) cortex in two parasagittal sections. Figure 2D illustrates one such section, in which counts were made of the cortex illustrated in B and C (lettered arrowhead in D). Blue (β -galactosidase-positive) spots (Fig. 2) were counted in the same sections and sample sites. For numbers of neutral red cells, we obtained values of 333,000 to 833,000 cells per cubic millimeter. In order to compare our estimates to figures in the literature, we used the estimate of three neurons to one glial cell for rat (27) and, multiplying by 0.75, obtained an estimate of 250,000 to 625,500 neurons per cubic millimeter. These values are slightly higher but in the same order of magnitude as those in the literature for mouse neocortex (87,000 to 214,000 per cubic millimeter) (28). We estimate that roughly 10% of the cortical cells were β -galactosidase-positive. Some neurons appeared to have more than one X-gal-stained spot associated with them (Fig. 2), and some neutral red-stained cells may have overlapped each other. However, even if we had reduced our estimate by an order of magnitude to take into account these potential sources of error, we still would have obtained a value of 1% of the cortical cells being β -galactosidase-positive.
14. To estimate the absolute sensitivity of the PCR assay used (Fig. 3, A and B), serial dilutions of plasmid p11 (β), which contains directly oriented *pgk-1* and *lacZ* sequences, were added to 1 μg of cerebral DNA, the equivalent of approximately 10^5 genomes. PCR amplification of plasmid p11 with primers 1 and 2 resulted in a 266-bp product that was slightly smaller than the 328-bp product expected after amplification of a V(D)J recombined Tg plasmid. Because of the similarity in size and structure of the 328-bp and 266-bp PCR products, it is likely that the sensitivity of this PCR assay is equivalent for the plasmid p11 and the V(D)J recombined Tg plasmid.
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24. PCR cycle parameters consisted of 60 s at 94°C, 60 s at 50°C, and 90 s at 72°C. Autofluorography was performed on the BiImage Analyzer (Fuji Film). Sequences of oligonucleotide primers used in this assay were as follows: 1, (5'-ATTCTGCACGCTTCAAAAAGCGCA-3'); 2, (5'-CATTGTT-CAGAAGGCATCAGTCG-3'); 3, (5'-ACACCCAGACAGTGAGTACTAC-3'); 4, (5'-AGTGTC-CCAGCTGTTTATCTAC-3'); 5, (5'-TCTCTGTTCTCGCTATT-3'); 6, (5'-AGACATGATAAGATACATTGATG-3').
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29. We thank L. Van Kaer for oligonucleotide synthesis; M. Rudnicki and M. McBurney for plasmid pKJ; G. Holm and D. Major for help with the histology; and H. Hall for the photography. A.M.G. is supported by Javits Award NS25529 and the Human Frontiers Science Program. S.T. is supported by grants from the Howard Hughes Medical Institute and National Institutes of Health. A.A. is an NIH Medical Scientist Training Program predoctoral fellow.

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