Peripheral lymphoid development and function in TCR mutant mice

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Abstract

We describe the development and function of the peripheral lymphoid system of mutant mice rendered deficient in either \(\alpha\beta\) or \(\gamma\delta\) T cells via targeting of TCR genes in embryonic stem cells. In the spleen of \(\alpha\beta\) T cell-deficient mice, \(\gamma\delta\) T cells do not compensate in numbers for the lack of \(\alpha\beta\) T cells, but B cells do. \(\alpha\beta\) T cell-deficient mice are unable to mount an antibody response to ovalbumin and do not reject skin allografts. Natural killer cell function is not impaired in any of the mutant mice. TCR mutant mice will prove useful in dissecting differential functions of \(\alpha\beta\) and \(\gamma\delta\) T cells in vivo.

Introduction

In the vertebrate immune system, recognition of the vast number of foreign antigens is achieved through antigen-specific receptors that are present in a clonally diverse fashion on the surface of lymphocytes, the Ig on B cells and the TCR on T cells. The large diversity of the repertoire of these two types of antigen receptors is generated at the genetic level through DNA rearrangements of various gene segments, in a process known as V(D)J recombination (1,2). The recombination activating gene (RAG)-1 and RAG-2 (3,4) are both necessary in vivo for the activation of V(D)J recombination (5,6).

The TCR was previously thought to be a heterodimer of two clonally diverse polypeptides, the TCR \(\alpha\) and TCR \(\beta\) chains, present on the surface of \(\alpha\beta\) T cells. However, during the search for the genes encoding these TCR subunits, a third rearranging gene called TCR \(\gamma\) was discovered (7). Together with its partner, TCR \(\delta\), a TCR \(\gamma\) chain forms a heterodimeric \(\gamma\delta\) TCR, which is expressed on the surface of \(\gamma\delta\) T cells. In the mouse, \(\gamma\delta\) T cells constitute a minority of the lymphocytes in thymus and receptor lymphoid organs, but they predominate in epithelia (8).

\(\gamma\delta\) T cells remain poorly understood. In an effort to understand the differential roles of \(\alpha\beta\) and \(\gamma\delta\) T cells in vivo, we have generated strains of mutant mice that specifically lack either T cell subset, by targeting TCR genes in embryonic stem cells. Mice with a mutation at the TCR \(\alpha\) or TCR \(\beta\) locus (TCR \(\alpha\) or TCR \(\beta\) mutant mice) are deficient in \(\alpha\beta\) T cells (9,10) and mice with a mutation at the TCR \(\delta\) locus (TCR \(\delta\) mutant mice) are deficient in \(\gamma\delta\) T cells (11). By crossing TCR \(\beta\) mutant mice with TCR \(\delta\) mutant mice, we generated TCR \(\beta\times\delta\) double mutant mice, which are deficient both in \(\alpha\beta\) and \(\gamma\delta\) T cells (10). By mutating RAG-1, we produced mice totally deficient in mature T and B lymphocytes (5).

Analysis of the thymic and peripheral lymphoid system of the
Peripheral lymphoid system of TCR mutant mice

TCR mutant mice indicated that αδ and γδ T cell development occurs in a mutually independent fashion (10,11). We have used the mutant mice to investigate the immune response against infection with the intracellular bacterium Listeria monocytogenes, and found that γδ T cells can confer immunity in the absence of αδ T cells and may have a unique regulatory role in this infectious disease (12). Similarly, we have shown that αδ T cell-deficient mice can be made partially immune to the malaria parasite Plasmodium yoelii by vaccination (13). We have documented the spontaneous development of inflammatory bowel disease in TCR α mutant, TCR β mutant or TCR β × δ double mutant mice; such disease was not detected in RAG-1 mutant mice (14).

In this paper, we describe in detail the development and function of the peripheral immune system of TCR and RAG-1 mutant mice.

Methods

Mice

Details of the generation of TCR α and TCR β mutant mice (9,10), of TCR δ mutant mice (11) and of RAG-1 mutant mice (5) have been reported previously. TCR β × δ double mutant mice were generated by crossing TCR β mutant mice with TCR δ mutant mice (10). Mice were in a mixed (129 × C57BL/6) background unless indicated otherwise. Further information about animal husbandry and health status can be found elsewhere (14).

Flow cytometry

A single cell suspension was prepared and nucleated cells were counted using a hemocytometer. Intestinal intraepithelial lymphocytes (IEL) were isolated as described (15). Between 2 × 10⁶ and 1 × 10⁶ cells were stained in a U-bottom 96-well plate in a total volume of 25 μl consisting of 20 μl staining solution (PBS with 0.1% sodium azide) and 5 μl of an equal mixture of normal rat and hamster serum (Jackson Immunoresearch, West Grove, PA). After 1–2 h, cells were washed two or three times with staining solution, once with propidium iodide-containing PBS and finally resuspended in 100–200 μl of PBS. The staining procedure was carried out at 4°C. Live cells were analyzed using FACScan software on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). The settings for the gates are indicated in each figure legend. Antibodies used were: GL3 for TCR-δ - phycoerythrin (PE), 2C11 for CD3 – FITC, H57-597 for TCR-β – PE, GK1.5 for CD4 – FITC (PharMingen, San Diego, CA). PE-labeled antibody 1120-09 for Ig-δ was purchased from Southern Biotechnologies (Birmingham, AL) and polyconal antiserum M31301 for Ig-μ – FITC was from Caltag (South San Francisco, CA).

Immunizations

One group of mice was immunized i.p. with 100 mg of ovalbumin (OVA, Sigma, St Louis, MO) in complete Freund’s adjuvant and bled on day 15. The titer of OVA-specific IgG1 serum antibodies on day 15 was determined by ELISA. Another group of mice was immunized i.p. with 100 μg trinitrophenyl (TNP) – iodopectosaccharide (LPS, Sigma) at 20 μg TNP/μg LPS (from Escherichia coli 0111:B4), dissolved in normal saline. The titer of TNP-specific IgM antibodies on day 5 was measured by ELISA using TNP – BSA coated plates. Another group of mice was immunized i.p. with 2 × 10⁶ fixed Streptococcus pneumoniae, strain R36A (gift from Dr John Kearney, Birmingham, AL) in normal saline. The titer of phosporycholine (PC)-specific antibodies on day 5 was measured by ELISA using plates coated with PC – BSA (gift from Dr John Kearney).

Skin grafts

Mice were grafted as previously described (16,17). Briefly, 1 cm² pieces of full-thickness trunk skin were applied to the dorsolateral thoracic wall. The dressings were removed 7 days after transplantation, and the grafts were inspected at 24–48 h intervals for signs of inflammation (erythema and edema) and necrosis. The time of rejection was taken as the point when viable epithelium was no longer detectable.

Natural killer (NK) cell assays

The standard NK cell target line YAC-1 is a Molony leukemia virus-induced line of A/Sn background. The RMA and RMA-S cell lines (kindly provided by Dr Klas Kärre, Karolinska Institute, Stockholm, Sweden) were derived from the Rauscher virus induced lymphoma cell line RBL-5 of C57BL/6 background. A detailed characterization of these cell lines has been described before (18). Single cell suspensions of splenocytes depleted from erythocytes by osmotic lysis were used as effector cells in a standard 51Cr-release assay. The IFN inducer tilorone (T8014, Sigma), which augments NK cell activity, was administered per os (0.2 ml of a 10 mg/ml solution per mouse) 24 h before sacrifice and removal of the spleen. Spleens from three to eight mice per group were analyzed separately.

Immunohistological analysis

This was performed as described elsewhere (14).

Results

Peripheral B and T cell development

We determined the numbers of total nucleated cells, of virgin B cells (i.e., cells positive for both surface Ig-μ and Ig-δ), of γδ T cells and of γδ T cells, in the spleen, mesenteric and inguinal lymph nodes of littermates that were offspring of mice either heterozygous or homozygous for both the mutations in TCR β and TCR δ. Such litters contain ‘wild-type’ mice (heterozygous or wild-type for the mutations), TCR δ mutant mice, TCR β mutant mice and TCR β × δ double mutant mice. This analysis allows a more reliable comparison among the three types of mutant mice, as the variations in cell numbers in lymphoid organs are much less among littermates than among mice from different litters. Figure 1 is an example of such flow cytometric analysis. Figure 2 lists numbers for the spleens of 84 mice analyzed at different ages. The total number of splenocytes is slightly reduced in TCR β mutant or TCR β × δ double mutant mice at young age, but becomes similar to the wild-type number at later age. In TCR β mutant mice, the numbers of γδ T cells are increased between 3- and 12-fold compared to wild-type mice, but this increase falls short of compensating for the lack of αδ T cells. This is the case even in αδ T cell-deficient mice that are >1 year old (data not shown). With age, the number of splenic B cells increases, compensating for the absence of αδ T cells. The lymphocytes...
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nodes contain 3- to 10-fold less cells in young TCR δ mutant mice compared to wild-type littersmates, but with age the numbers become similar and more variation is observed (data not shown). This age effect and the variability is probably related to subclinical infections. In mice with inflammatory bowel disease (14), an increase in the cellularity of mesenteric lymph nodes is consistently observed and the numbers in inguinal lymph nodes are elevated in mice with an anorectal prolapse (data not shown). TCR δ mutant mice are indistinguishable from wild-type mice in terms of cell numbers and lymphocyte composition, except, of course, for those of γδ T cells.

Splenics of young mutant mice were also analyzed by immunohistology. Immunohistochemical staining is shown for CD3-ε, to identify T cells (Fig. 3A), and for Ig-μ, to identify B cells (Fig. 3B). The separation of lymphocytes into T cell areas (the paratrabecular lymphoid sheath) and in B cell areas (lymphoid follicles) is still maintained in the TCR mutant mice. The histology of the RAG-1 mutant spleen confirms the total absence of mature T and B cells. Interestingly, we reproducibly observed small numbers of CD3-ε+ cells in spleen and in small and large intestine of TCR δ × δ double mutant mice, but not of RAG-1 mutant mice. The identity of these cells remains unknown.

**CD4+ dull TCR δ+ cells in TCR α mutant mice**

We have previously reported an unusual population of lymphocytes in the peripheral lymphoid organs of TCR α mutant mice (10). These cells are CD4+, stain weakly for CD3-ε and TCR δ, but are negative for TCR α. Analysis of the TCR Vδ repertoire of nine 6d (>6 months) TCR α mutant mice in an inbred 129/SvJ background using antibodies against seven different TCR Vδs showed that it was different from mouse to mouse, without a particular pattern (data not shown), suggesting that this population is oligoclonal. The CD T cell repertoire of aging nude mice has also been shown to be oligoclonal (19), suggesting that peripheral expansion occurred after a rare maturation event.

The expression of the CD4 antigen on these cells raised the issue whether they are dependent on class II MHC for their development. We therefore crossed TCR α mutant mice with...
Peripheral lymphoid system of TCR mutant mice

**SPLEEN**

Fig. 2. Numbers of total nucleated cells, of B cells, of αβ T cells and of γδ T cells in the spleen of wild-type mice, TCR β− mutant mice, TCR β− mutant mice and TCR β × δ double mutant mice. A total of 84 mice, from 13 litters, were analysed. Mice were in a 129/Ola x C57BL/6 mixed background. The age of each litter is rounded off to the nearest week. Each symbol represents the average of the data for one to five litters. If a cell population is absent due to a targeted mutation, no symbol is used. The number of αβ T cells in the 5 week old litter was not determined. Note that the scales are different and that the scale for γδ T cells is logarithmic. Profiles of individual mice from the first litter of 3 weeks are shown in Fig. 1.

In the epithelium of the small intestine of the normal mouse, αβ and γδ T cells are present in comparable numbers (8). Figure 5 shows IEL of wild-type and mutant mice stained for CD3 and TCR β. The minor population of null TCR β− cells described above is also present in the small intestine of the TCR α mutant mouse. αβ T cells are present in an IEL preparation of the TCR δ mutant mouse and γδ T cells can be observed in IEL of the two αδ T cell-deficient mice (revealed as TCR β−, CD3− cells). We determined the cytotoxic activity of αβ and γδ IEL in a redirected lysis assay (15) and found that the remaining T cell subsets had comparable cytotoxic potential (data not shown). The intraepithelial localization of the remaining T cell subsets is confirmed in Fig. 5 by immunoperoxidase staining with antibodies against TCR β or TCR δ. In the TCR α mutant small intestine, again a few weakly TCR β− IEL can be observed. These data demonstrate the mutually independent development of αβ and γδ IEL.

**Humoral and cellular responses**

The humoral immunity of TCR mutant mice was evaluated by immunization with the T-dependent antigen OVA and the T-independent antigen, PC and TNP-LPS. Whereas both TCR α and TCR β mutant mice exhibited a normal antibody response to PC (Fig. 6A and D) and TNP-LPS (Fig. 6B and E), they failed to respond against OVA (Fig. 6C and F). We previously reported a normal anti-OVA response in TCR δ− mutant mice (11). Taken together, these results imply that γδ T cells are neither sufficient nor necessary for help to B cells, at least in response to OVA.

A classical assay for evaluating cellular (as opposed to humoral) immunity is rejection of skin allografts. We grafted trunk skin of C3H/He mice (H-2b) onto TCR mutant or RAG-1 mutant mice, of a mixed 129 x C57BL/6 background (H-2b). The allografts were rejected at 2 weeks in all wild-type and TCR δ− mutant mice, but they remained intact for at least 8 weeks (the latest time point available) in C3H/He mice. RAG-1 mutant mice and TCR β− mutant mice (Table 1). We conclude that γδ T cells are neither sufficient nor necessary for rejection of a skin allograft, at least in the combination of H-2b donor onto H-2b recipient.

**NK cell function**

No significant differences were observed in the number of NK 1.1+ cells in the peripheral blood and spleen of TCR mutant mice compared with wild-type mice (data not shown). The numbers of NK 1.1+ cells in RAG-1 mutant blood or spleen are comparable to those in the other mice, although the relative percentage is increased due to the lack of T and B cells. TCR mutant and RAG-1 mutant mice displayed normal NK activity against YAC-1 and, in addition, retained their ability to discriminate against class I MHC positive RMA (poor killing) and class II MHC deficient RMA-S cells (efficient killing) (Fig. 7).

**Discussion**

**Lymphocyte development**

Our data demonstrate that T cells are not required for a large part of B cell development. Similar observations were recently
**Fig. 3.** Immunperoxicase staining of spleen. WT, wild-type; α, TCR α mutant mouse; β, TCR β mutant mouse; δ, TCR δ mutant mouse; δ × δ, TCR β × δ double mutant mouse; RAG-1, RAG-1 mutant mouse. The mice were between 3 and 5 weeks old. (A) Staining for CD3-ε. The specificity of the staining is demonstrated by the absence of any signal in the RAG-1 mutant spleen. There is no discernible difference in the staining pattern between the wild-type and TCR δ mutant mouse. The scattered, intensely stained cells in the TCR δ mutant sample are γδ T cells, which are present in the per arteriolar sheath (the T cell areas). In the TCR α mutant mouse, intensely stained cells (most likely γδ T cells) can be seen as well as weakly staining cells (most likely CD4+ TCR β+ cells) (objective 20 x). (B) Staining for Ig-μ. B cells are present in lymphoid follicles, which are located around the T cell areas (visible as unstained areas in the center of stained areas). As expected, there is no staining in the RAG-1 mutant spleen (objective 20 x).
made in MHC deficient mice (22). In the spleen of αβ T cell-deficient mice, B cells compensate with age in numbers for αβ T cells. Reduced splenic B cell numbers were reported in mice with antibody-mediated depletion of αβ T cells (23), but this is likely to be a side-effect of the antibody treatment. It is interesting to note that in TCRα mutant, TCRβ mutant or TCRα × β double mutant mice, immunohistology revealed the presence of IgA producing B cells (data not shown), indicating that T cells are not absolutely required to direct Ig class switching in B cells. In the absence of αβ T cells, B cells are still able to mount an antibody response against T-independent antigens, but not against the T-dependent antigen OVA.

This report shows clearly that peripheral development of γδ T cells does not require the presence of αβ T cells and vice versa. Previous studies was suggested in work on αβ T cell depleted mice (23,24), and in another strain of TCRα mutant mice (25). The independent development of T cell subsets is demonstrated most easily by flow cytometric analysis of IELs, where both T cell subsets are equally visible in numbers in the wild-type mouse. We have previously demonstrated the mutual independence during thymic development (10,11). Observations have been made on a possible auxiliary role of γδ T cells for thymic or peripheral αβ T cell development, for instance on the basis of their earlier appearance during thymic ontogeny (26,27) or of dramatic disturbances of αβ T cells in TCRγ transgenic mice (28). The normal development of αβ T cells in TCRβ mutant mice shows that such interactions do not take place. At least, they are not essential.

γδ T cells do not 'take over' in αβ T cell-deficient mice, although the numbers are ~10-fold increased. This expansion is insufficient to replace the αβ T cells in numbers. In the spleen of αβ T cell-deficient mice, γδ T cells can be found predominantly in the T cell areas (the periarteriolar lymphoid sheath) (Fig. 3A). In the small intestine of αβ T cell-deficient mice, γδ T cells still occupy the intraepithelial compartment (Fig. 3B). We conclude that γδ T cells have their own 'niche' and that the homeostatic mechanisms that count and regulate T cell numbers (29) discriminate between αβ and γδ T cells. The age-related increase in the number of γδ T cells in the αβ T cell-deficient mice could be due to stimulation by subclinical infections or inflammatory bowel disease (14). It will be interesting to determine if this increase also occurs in germ-free mice.

Lymphocyte function

This study confirms the central role of αβ T cells in the immune system. In αβ T cell-deficient mice humoral and cellular immune
Fig. 5. IEL (Top) Flow cytometric analysis of IEL purified of the small intestine of WT, wild-type mice (3.8 × 10^6 cells); α, TCR α mutant mouse (2.0 × 10^6 cells); β, TCR β mutant mouse (2.2 × 10^6 cells); δ, TCR δ mutant mouse (4.2 × 10^6 cells). Staining with CD3ε-FITC and TCR β-PE. Mice are between 8 and 9 weeks old. A small population of weakly staining cells on the diagonal is seen in the TCR α mutant sample. The cells in quadrant 4 are presumably γδ T cells. The gates are narrow and include only the lymphocytes. (Middle) Immunoperoxidase staining for TCR β and (bottom) for TCR δ of small intestine of the same four types of mice as the flow cytometric profiles. Samples are from different mice than in the top part. Mice are between 3 and 5 weeks old. Some of the stained cells are located inside the epithelium, adjacent to the basal side of the epithelial cells and just above the lamina propria (objective 40×).
responses are deficient, as exemplified by the deficient antibody response to OVA and the acceptance of skin allografts. The mice suffer from inflammatory bowel disease and often from pneumonia with the opportunistic pathogen *Pneumocystis carinii* as well (14). γδ T cells appear to be unable to substitute for αβ T cells to sustain the general health, even in the sheltered environment of a specific pathogen-free facility where bedding, food and drinking water are autoclaved. However, a reason for this failure could be that the numbers of γδ T cells in the peripheral lymphoid organs of αβ T cell-deficient mice are below a critical threshold for many immune responses. For proper comparison, one would need to study mice with 10-fold less αβ T cells: these mice could be immunocompromised as well. Our studies of infection with the intracellular bacterium *L. monocytogenes* (12) have indicated, however, that even these relatively small numbers of γδ T cells are sufficient for bacterial clearance in a primary infection and

Fig. 6. Antibody responses to immunizations. (Top) TCR α mutant mice (129/Sv x C57BL/6) and control littermates. (Bottom) TCR β mutant mice (129/Ola x BALB/c) and control littermates. Antigens are: PC (A and D), TNP-LPS (B and E) and OVA (C and F). Mice were immunized; p.p. serum was isolated on the day indicated, and the antibody titer was determined using ELISA. Stippled lines, mutant mice; solid lines, heterozygous or wild-type littermates.
Table 1. Rejection of skin allografts

<table>
<thead>
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<th>Strain</th>
<th>No. of mice that rejected</th>
<th>Survival time</th>
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<tr>
<td>C3H/He</td>
<td>0/4</td>
<td>&gt;8 weeks</td>
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<tr>
<td>wild-type</td>
<td>4/4</td>
<td>10 days</td>
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<td>TCR δ</td>
<td>7/7</td>
<td>11-12 days</td>
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<td>TCR β</td>
<td>0/7</td>
<td>&gt;8 weeks</td>
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<tr>
<td>RAG-1</td>
<td>0/5</td>
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C3H/He (H-2b) trunk skin was grafted onto mice of a mixed (129 x C57BL/6) (H-2b) background. All strains of mice were grafted in parallel. The difference in day of rejection between wild-type and TCR δ mutant mice is not significant.

Fig. 7. Cytotoxic activity of natural killer cells from TCR and RAG-1 mutant mice. NK cell cytotoxicity of splenocytes from wild-type (WT), TCR α mutant (α), TCR β mutant (β), TCR δ mutant (δ), TCR β x δ double mutant (β x δ) and RAG-1 mutant (RAG-1) mice was tested against 51Cr-labeled YAC-1 (▲), RMA (▲) and RMA-S (▲) target cells. Effector to target ratios are indicated on the x-axis and percent specific lysis is indicated on the y-axis. Mice were bled 4 and 8 weeks old. The average cell yield of erythrocyte-depleted and washed cells was 105 cells per cell. The results are presented as the means ± SD of triplicate determinations. The NK activity in the RAG-1 mutant samples was similar to that in the other mice.

References


