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## Crucial role of the pre-T-cell receptor $\alpha$ gene in development of $\alpha\beta$ but not $\gamma\delta$ T cells

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### ► To cite this version:

Hans Jörg Fehling, Anna Krotkova, Claude Saint-Ruf, Harald von Boehmer. Crucial role of the pre-T-cell receptor  $\alpha$  gene in development of  $\alpha\beta$  but not  $\gamma\delta$  T cells. *Nature*, 1995, 375 (6534), pp.795-798. 10.1038/375795a0 . inserm-02179382

**HAL Id: inserm-02179382**

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Submitted on 10 Jul 2019

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ment resumed. In this sense, it is important to note that after the zeugopodal defect, limb development does proceed in the double-mutant mouse, albeit with some dysmorphology. But clearly the autopod is less affected than the zeugopod. The autopodal phenotypes distal to the major zeugopodal defects are likely to be the result of a coupling between proximodistal and anteroposterior patterning<sup>24–28</sup>. This coupling may be genetic, with group 11 genes directly influencing the timing of activation of group 12 and group 13 genes. Alternatively, morphological coupling may account for these defects. The elimination of both *hoxa-11* and *hoxd-11* gene products may reduce the overall mesenchymal cell population available to form the zeugopod and posterior carpals. A deficiency of cells may also alter the timing ('heterochrony') of formation of the rest of the limb. The second crisis would therefore be revealed at the end of autopod formation, when cartilaginous elements are competing to recruit the few remaining cells. This heterochrony would explain the delay in digit condensation seen during embryogenesis of the double mutant and account for the more severe phalangeal defects seen in digits II and V, which are the last elements formed in the last digits specified.

It is interesting that in the double mutants the forelimbs are more affected than the hindlimbs. Also, whereas (aa; Dd) and (Aa; dd) mice show an intermediate forelimb phenotype, the presence of a third mutant allele does not alter the hindlimb phenotype beyond what is seen in (aa; DD) or (AA; dd) mice. These differences may be a consequence of a third paralogue, *hoxc-11*, which is expressed more strongly in the hindlimbs<sup>29</sup> and could provide a compensatory function. This hypothesis would predict that in (*hoxa-11*, *hoxc-11*, *hoxd-11*) triple mutants, the tibia and fibula would be completely eliminated and show quantitative dependence on the dosage of mutant alleles. Also, the last vestige of the forelimb radius and ulna observed in the double mutant would be predicted to be absent.

Our model is supported by the *hoxd-13* mutant mouse in which the mutant limb phenotype is restricted to the autopod<sup>13</sup>, and predicts that in a (*hoxa-13*, *hoxd-13*) double mutant the forepaw should be absent. *hoxd-12* has no paralogue in the *Hox A* family and may therefore act on its own (or in conjunction with *hoxc-12*) to influence formation of the carpals and tarsals. Alternatively, *hoxd-12* may recruit its closest *Hox A* paralogue (*hoxa-11* or *hoxa-13*) to help specify this region. Single-mutant mice for *hoxa-10*, *hoxc-10* and *hoxd-10* should show subtle defects in the humerus and femur, but double and triple mutants should affect strongly the entire stylopod and initiate a cascade of less severe phenotypes downstream through the zeugopod and autopod. Consistent with the model, individual *hoxd-10* and *hoxc-10* mutant homozygotes show mild malformations of the humerus and femur (E. M. Carpenter, J. M. Goddard and M.R.C., unpublished results; S. L. Hostikka and M.R.C., unpublished results). From this model, the appendicular ground state would be achieved in an animal lacking all of the *Hox* 10–13 cognate genes. This should be a limbless mouse. □

Received 10 April; accepted 18 May 1995.

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ACKNOWLEDGEMENTS. A.P.D. was supported by an NSF predoctoral fellowship and an NIH genetics training grant. We thank K. Saalfeld, T. Smith and C. Lewis for technical support, and L. Oswald for help with the preparation of the manuscript.

## Crucial role of the pre-T-cell receptor $\alpha$ gene in development of $\alpha\beta$ but not $\gamma\delta$ T cells

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In T-cell precursors, the T-cell-receptor  $\beta$  chain is expressed before the T-cell-receptor  $\alpha$  chain<sup>1,2</sup> and is sufficient to advance T-cell development in the absence of T-cell receptor  $\alpha$  chains<sup>3–7</sup>. In immature T cells, the T-cell-receptor  $\beta$  protein can form disulphide-linked heterodimers with the pre-T-cell-receptor  $\alpha$  chain<sup>8,9</sup> and associate with signal-transducing CD3 molecules<sup>5</sup>. The recently cloned pre-T-cell-receptor  $\alpha$  gene encodes a transmembrane protein that is expressed in immature but not mature T cells<sup>9,10</sup>. Here we show that  $\alpha\beta$ , but not  $\gamma\delta$ , cell development is severely hampered in pre-T-cell-receptor  $\alpha$ -gene-deficient mice, which establishes a crucial role for the pre-T-cell receptor in early thymocyte development.

Intrathymic T-cell development proceeds from CD4<sup>-</sup>8<sup>-</sup> precursors through CD4<sup>+</sup>8<sup>+</sup> intermediates into CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>-</sup>8<sup>+</sup> mature thymocytes<sup>3,11</sup>. In rearrangement-deficient mice, thymocyte development is arrested at the CD4<sup>-</sup>8<sup>low</sup>25<sup>+</sup> stage<sup>6,7,12</sup>. Productive T-cell-receptor (TCR)- $\beta$  transgenes can partly relieve the developmental block, allowing the accumulation of immature CD4<sup>+</sup>8<sup>+</sup> thymocytes but not mature CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> T cells<sup>3–7</sup> that require positive selection by TCR $\alpha\beta$  (ref. 13). In T-cell precursors the TCR- $\beta$  chain forms disulphide-linked heterodimers with the pre-TCR $\alpha$  (pT $\alpha$ ) chain, and can associate with signal-transducing CD3 molecules<sup>5,8,9</sup>.

Here we report on the role of the TCR $\beta$ -pT $\alpha$  heterodimer in development, based on experiments with pT $\alpha$ -deficient mice. These animals were generated by gene targeting in embryonic stem (ES) cells using a deletion-type targeting vector. On homologous recombination, this construct eliminated exons 3 and 4 of the pT $\alpha$  gene encoding the connecting peptide, which contains the cysteine required for heterodimer formation, the transmembrane region, the cytoplasmic tail and most of the 3' untranslated region (Fig. 1). Homologous recombination in ES cells and the absence of the deleted gene segment in pT $\alpha$ <sup>-/-</sup> mice was verified by Southern blotting with appropriate probes (Fig. 1, and results not shown). Offspring from intercrosses of pT $\alpha$ <sup>+/-</sup>



TABLE 1 Absolute number of thymocytes with different phenotypes

Phenotypes	$pT\alpha^+$ ( $\times 10^{-6}$ )	$pT\alpha^{-/-}$ ( $\times 10^{-6}$ )
$CD4^-8^-3^{low}25^+$	25.6	54.0
	22.0	40.4
$CD4^-8^-\delta^+$	12.4	55.1
	14.6	32.0
$CD4^+8^+\delta^+$	10.0	6.5
	14.5	11.0
$CD4^+8^+$	34.54	1.19
	34.62	1.13
$CD4^+8^+TCR\beta^{int.}$	14.50	0.24
	13.75	0.23
$CD4^+8^-TCR\beta^{high}$	3.51	0.13
	3.05	0.13
$CD4^-8^+TCR\beta^{high}$	0.81	0.09
	1.39	0.13

Numbers were obtained from two different mice of each genotype from a 5-day-old litter.

mice were killed, thymus and bone marrow removed, and single cell suspensions prepared and analysed by cytofluorometry. There were no significant differences between numbers of marrow cells from age-matched mice and thymocytes from  $pT\alpha^{+/+}$  and  $pT\alpha^{-/-}$  age-matched mice, whereas the number of thymocytes in  $pT\alpha$ -deficient animals was reduced to less than 10%.

Figure 2 shows subsets from thymus of  $pT\alpha^+$  and  $pT\alpha^{-/-}$  mice only, because there was no difference in subsets of bone marrow and because lymphoid organs from  $pT\alpha^{+/+}$  and  $pT\alpha^{-/-}$  mice did not differ. Both  $pT\alpha^+$  and  $pT\alpha^{-/-}$  mice contain  $CD4^-8^-$ ,  $CD4^+8^+$  and single-positive  $CD4^+8^-$  and  $CD4^-8^+$  thymocytes, but cells with CD4 and CD8 co-receptors are proportionally under-represented in  $pT\alpha^{-/-}$  mice whereas the proportion of  $CD4^-8^-25^+$  cells is drastically increased (Fig. 2). Both types of mice contain CD4 and CD8 co-receptor expressing cells with low, intermediate and high levels of TCR- $\beta$  chain on

TABLE 2 Subsets among  $CD4^-8^-3^{low}$  thymocytes

Phenotypes	$pT\alpha^+$ (%)	$pT\alpha^{-/-}$ (%)
$CD44^+25^-$	12.1	11.0
$CD44^+25^+$	2.20	2.30
$CD44^-25^+$	40.7	86.6
$CD44^-25^-$	45.0	0.10

Percentages were calculated from the same litter as described in Fig. 2 and Table 1.

the cell surface. The fraction of  $CD4^-8^-$  thymocytes with TCR $\delta$  chains on the cell surface was more prominent in  $pT\alpha^{-/-}$  mice, and both TCR $\beta$  and TCR $\delta$  chains were stoichiometrically associated with CD3 molecules. The data also show that the TCR $\beta$  chains were associated with TCR $\alpha$  chains, as revealed by double-staining with TCR $\beta$  and TCR Va2, 3.2, 8 and 11 antibodies (Fig. 2, lower right). From triple stainings with various antibodies listed in Fig. 2 and thymocyte numbers, we calculated the absolute number of cells belonging to various thymocyte subsets as shown in Table 1; the data show that the pre-TCR is not required to generate normal numbers of  $CD4^-8^-3^{low}25^+$  precursors of  $\alpha\beta$  T cells. In fact, their number is increased in  $pT\alpha^{-/-}$  mice, probably because of the developmental arrest in these mice (see below). There is likewise no decrease in the number of  $CD4^-8^-$  or  $CD4$  and  $CD8$  co-receptor expressing  $\gamma\delta$  T cells (Table 1). Table 2 shows that among  $CD4^-8^-3^{low}$  cells,  $pT\alpha^{-/-}$  mice have normal or increased numbers of  $CD44^+25^-$ ,  $CD44^+25^+$  and  $CD44^-25^+$  cells, but no  $CD44^-25^-$  cells, which represent 45% in  $pT\alpha^+$  mice. As most thymocyte expansion occurs in this subset<sup>11</sup>, it is not surprising that the absolute number of  $CD4^+8^+$  cells as well as mature TCR  $\alpha\beta^{high}$   $CD4^+8^-$  and  $CD4^-8^+$  cells is much lower in  $pT\alpha^{-/-}$  than in  $pT\alpha^+$  mice. Also, the proportion of TCR- $\alpha\beta$ -positive cells among  $CD4^+8^+$  cells is lower in  $pT\alpha^{-/-}$  than in  $pT\alpha^+$  mice. Lymph-node cells from 28-day-old  $pT\alpha^{-/-}$  mice contain mature single positive  $CD4$  and  $CD8$  cells representing about 5% of numbers found in  $pT\alpha^+$  littermates (results not shown).

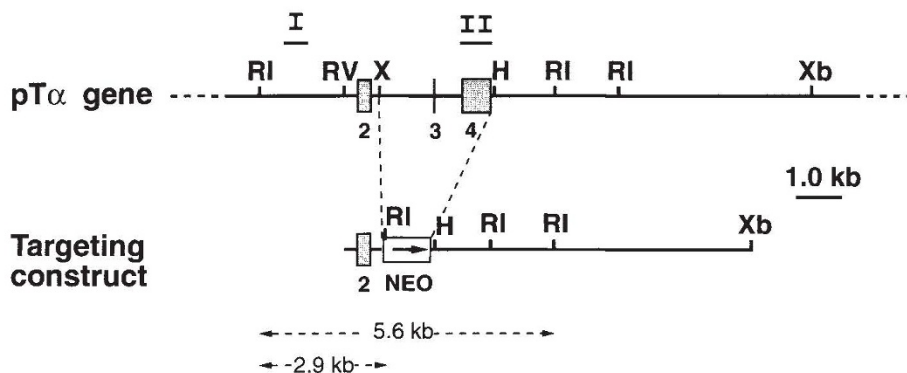


FIG. 1 Disruption of the  $pT\alpha$  gene by homologous recombination: partial organization of the  $pT\alpha$  locus<sup>22</sup> and structure of the targeting vector. The  $pT\alpha$  gene was cloned from a 129/Ola-derived genomic library (kind gift of A. Berns, Amsterdam). The targeting vector was constructed by replacing approximately 2.6 kilobases (kb) of the  $pT\alpha$  sequence between the unique  $XhoI$  site in intron 2 and a  $BglIII$  site ~60 base pairs (bp) upstream of the AATAAATAA polyadenylation site with a 1.2-kb  $XhoI$ - $BamHI$  fragment of pMC1neopA (Stratagene) carrying the neomycin resistance gene (*neo*). The isogenic targeting construct was electroporated into E14.1 embryonic stem cells as described<sup>18</sup>. ES colonies surviving G418 selection were analysed by the polymerase chain reaction (PCR) in pools of 12 using primers specific for the *neo* cassette within the *tk* promoter (ATTGCCAATGACAAGACGCTGC) and for the  $pT\alpha$  gene just upstream of the  $EcoRV$  site (GTTGGATGTTATTGGTTACTCTCTGA), respectively. Colonies within positive pools were re-screened individually by PCR and eventually by Southern analysis using

*EcoRI*-digested DNA and probe I (a 470-bp PCR fragment specific for  $pT\alpha$  sequences outside the targeting construct, ~1.3 kb upstream of exon 2 (primers: TAGGTTTGAACCTCAGAT; TGATTTCTCTGTAGC)). Out of ~1,600 colonies screened, 3 had undergone homologous recombination, and one of these clones (pT355) gave rise to chimaeric mice. Chimaeric males were backcrossed with (C57BL/6  $\times$  DBA/2) $F_1$  females and heterozygous offspring carrying a mutant  $pT\alpha$  allele were intercrossed to obtain mice deficient in  $pT\alpha$ . The absence of the deleted  $pT\alpha$  sequences in homozygous knockout mice was confirmed by Southern blotting of *EcoRI*-digested genomic tail DNA and hybridization with a 310-bp *Apal/BspEI* complementary DNA fragment spanning the region of exon 4 that encodes the transmembrane portion, the cytoplasmic tail and 120 nucleotides of 3' untranslated sequence. Abbreviations for restriction sites: RI, *EcoRI*; RV, *EcoRV*; X, *XhoI*; H, *HindIII*; Xb, *XbaI*.



The above data indicate that  $pT\alpha$  has no role in the development of most  $\gamma\delta$  T cells for one of three reasons: (1) because it is not expressed in the  $\gamma\delta$  lineage; (2) it cannot pair with the  $\gamma$  chain; or (3) because there is no need for a putative TCR $\gamma$ - $pT\alpha$  heterodimer in  $\gamma\delta$  T-cell development. Although this issue requires further investigation, we have been unable to detect RNA for  $pT\alpha$  in thymocytes of TCR $\delta$  surface-positive thymocytes<sup>10</sup>. The increase in the number of  $\gamma\delta$  cells could depend on the availability of space, and/or the possibility that ongoing  $\gamma\delta$  rearrangement is not terminated by the pre-TCR. The fact that a few TCR $\alpha\beta$ -positive CD4/8-co-receptor-expressing thymocytes can be generated in the absence of the pre-TCR is consistent with an earlier finding of TCR $\alpha$  rearrange-

ments in TCR $\beta^{-/-}$  mice deficient in pre-T-cell-receptors<sup>6</sup>. The authors argued that CD4<sup>+</sup>8<sup>+</sup> cells in TCR $\beta^{-/-}$  mice (which are often claimed erroneously not to contain CD4<sup>+</sup>8<sup>+</sup> cells) could be of the  $\delta$  lineage because they were absent in TCR $\beta^{-/-}$  × TCR $\delta^{-/-}$  mice. We find that in  $pT\alpha^{-/-}$  mice CD4<sup>+</sup>8<sup>+</sup>3<sup>low</sup>25<sup>+</sup> precursors of  $\alpha\beta$  T cells can differentiate, albeit inefficiently, into CD4<sup>+</sup>8<sup>+</sup> cells with TCR $\alpha\beta$  on the surface. We therefore propose that the pre-TCR is sufficient and necessary for the generation of CD4<sup>+</sup>8<sup>+</sup> precursors when no other TCR-expressing cells are present, and that in the presence of TCR-positive cells, the pre-TCR is required for the transition of CD4<sup>+</sup>8<sup>+</sup>25<sup>+</sup>  $\alpha\beta$  T-cell precursors, through rapidly dividing CD4<sup>+</sup>8<sup>+</sup>25<sup>-</sup> cells into TCR $\alpha\beta$ -expressing CD4<sup>+</sup>8<sup>+</sup> thymocytes.

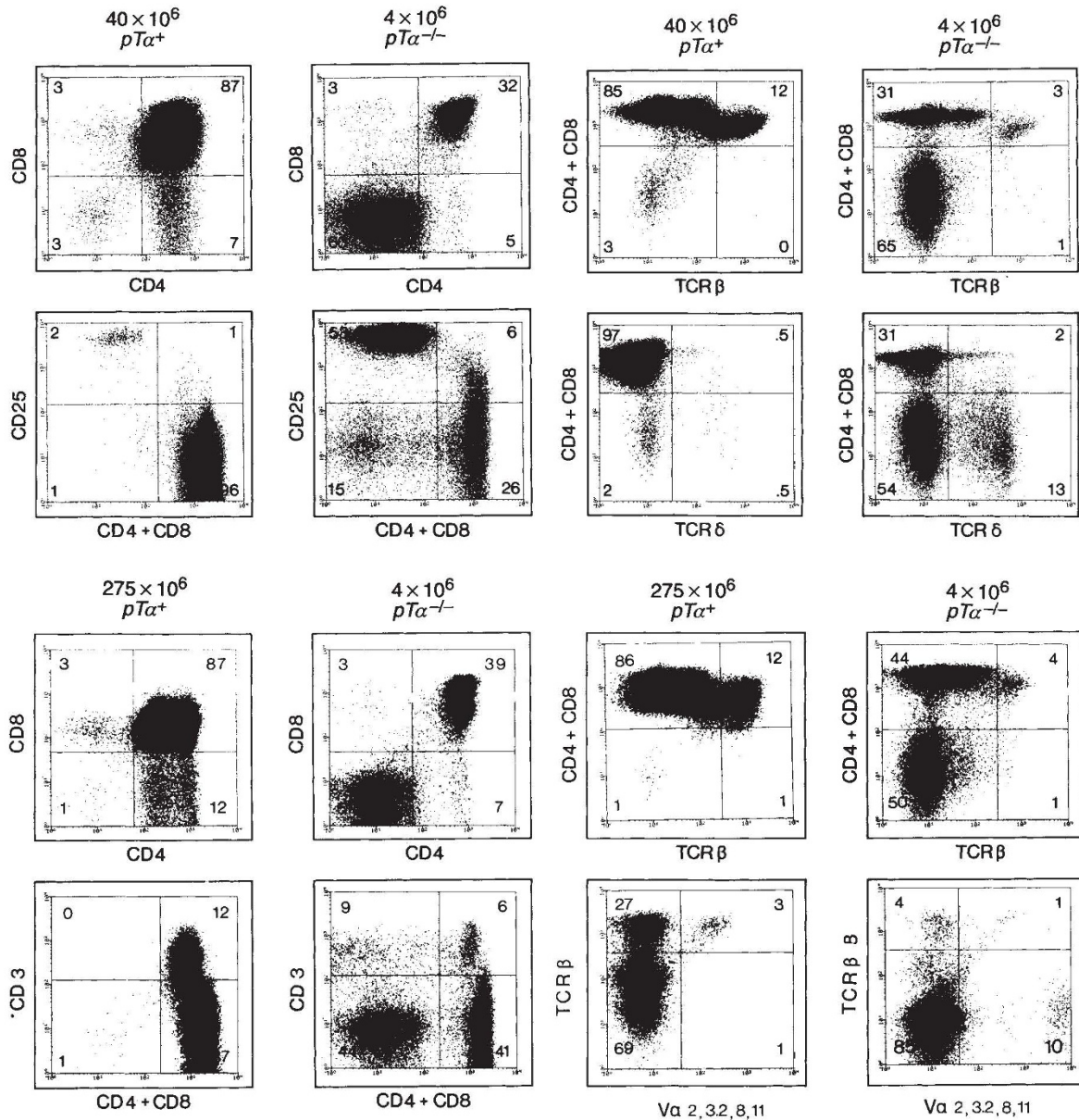


FIG. 2 Thymocyte subsets from  $pT\alpha^+$  and  $pT\alpha^{-/-}$  mice. Staining of thymocytes from 5-day-old (top two rows) and 28-day-old (bottom two rows) litters with CD4, CD8, TCR $\beta$ , TCR $\delta$  and TCR-V $\alpha$  antibodies. All stainings were three-colour stainings with the following antibody-conjugates: anti-CD3-biotin and anti-CD3-FITC conjugates (500 A2 (ref. 19)), anti-TCR $\beta$ -biotin and anti-TCR $\beta$ -FITC (H57 (ref. 20)), anti-CD4-phycoerythrin (H129.19 Gibco), anti-CD8-biotin and anti-CD8-phycoerythrin (53-6.7 Pharmingen, San Diego), Anti-CD25-biotin (Pharmingen, San Diego), anti-CD44-phycoerythrin (ATCC collection KM81), anti-HSA-biotin (M1169 (ref. 21)), and anti-TCR $\delta$ -FITC (G3, Pharmingen, San

Diego). A cocktail of TCR-V $\alpha$  antibodies, namely anti-V $\alpha$ 2 (B20.1), anti-V $\alpha$ 3.2 (RR3.16), anti-V $\alpha$ 8 (KT50) and anti-V $\alpha$ 11 (RR8.1) was obtained from D. Mathis, Strasbourg. The biotin conjugates were revealed by streptavidin-Tricolor, (Caltag, San Francisco). For each of the three colour stainings  $\sim 4 \times 10^5$  cells were incubated with various reagents. In each case,  $10^5$  events were acquired for fluorescence-activated cell sorting by FACScan. The bright staining in the thymus (lower right) is due to thymic B cells that stain brightly with sheep anti-mouse Ig-FITC reagent, which was used to reveal the V $\alpha$  antibodies.



This view is consistent with data showing that TCR-positive thymocytes can induce the development of CD4<sup>+</sup>8<sup>+</sup> cells when injected into rearrangement-deficient mice<sup>14,15</sup> and explains the absence of CD4<sup>+</sup>8<sup>+</sup> thymocytes in rearrangement-deficient<sup>6,7</sup>, TCR-negative<sup>6</sup> as well as CD3-negative mice (B. Malissen, personal communication). Differentiation through the CD4<sup>-</sup>8<sup>-</sup>25<sup>-</sup> subset requires cell-autonomous signals delivered by the pre-TCR, and for that reason in normal mice almost all CD4<sup>+</sup>8<sup>+</sup> T cells contain productive TCR- $\beta$  genes<sup>16</sup>, whereas in the absence of the pre-TCR, only ~2% of TCR $\alpha\beta$ -positive CD4<sup>+</sup>8<sup>+</sup> precursors are generated by some aberrant differentiation. Nevertheless, positive selection will operate on these cells and generate mature  $\alpha\beta$  T cells whose number is regulated by homeostasis independent of the pre-TCR<sup>17</sup>. □

Received 12 May; accepted 8 June 1995.

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ACKNOWLEDGEMENTS. We thank U. Müller for injection and implantation of blastocysts; C. Laplace and K. Hafen for technical assistance and C. Geiger for typing; D. Mathis and C. Benoist (Strasbourg, France) for the gift of TCR-V $\alpha$  antibodies, and K. Karjalainen (Basel, Switzerland) for critical reading of the manuscript. The Basel Institute for Immunology is supported by F. Hoffmann-La Roche Ltd, Basel. The work was also supported by INSERM through the Unité INSERM 373. H.V.B. is supported by the Human Frontier Science Program Organization.

## The small heat-shock protein $\alpha$ B-crystallin as candidate autoantigen in multiple sclerosis

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**THE identification of key antigens in human autoimmune diseases is a crucial step towards the development of specific intervention. The autoantigen(s) relevant to multiple sclerosis (MS) probably reside in myelin of the central nervous system, the target of the disease<sup>1</sup>. Here we examine proliferative responses of human peripheral blood T cells to the complete collection of myelin proteins fractionated by reversed-phase high-performance liquid chromatography. Myelin isolated from MS-affected brain contained a single protein fraction to which T cells from MS patients and from healthy controls showed dominant responses. This highly immunogenic protein was identified as  $\alpha$ B-crystallin, a small heat-shock protein. Immunohistochemical examination of MS lesions revealed the presence of oligodendrocytes and astrocytes with raised  $\alpha$ B-crystallin expression, which were not found in unaffected myelin. Our findings indicate that  $\alpha$ B-crystallin serves as immunodominant myelin antigen to human T cells when expressed at the elevated levels found in active MS lesions.**

To examine responses of human T cells to the complete collection of myelin proteins from the central nervous system (CNS), short-term cultures were raised *in vitro* by priming peripheral blood mononuclear cells (PBMC) from 24 HLA-typed donors with total myelin protein. Proteins used for this purpose were

prepared from either healthy control myelin or from myelin purified from MS-affected white matter. Five PBMC donors were clinically defined MS patients and the other nineteen were healthy control subjects. After one cycle of restimulation with total myelin protein, T cells were collected and tested for proliferative responses to each of the forty reversed-phase high-performance liquid chromatography (RP-HPLC) fractions prepared from the same protein preparation as that used for priming. Figure 1a shows the RP-HPLC profile of fractionated total myelin protein from MS-affected brain, which is virtually identical to that from control material. Data from the proliferation assays were essentially the same in all cases, irrespective of HLA-type or clinical status of the donor. A representative set of data from five donors is shown in Fig. 1b.

The data in Fig. 1 illustrate two points. First, bulk T-cell responses after priming with all CNS myelin proteins were always directed primarily at the various minor myelin proteins contained in HPLC fractions 7–20. In contrast, responses to the major components proteolipid protein or myelin basic protein were barely significant. These findings extend our previous observations that the relative immunogenicity of individual CNS myelin proteins to human T cells bears no apparent relationship to their relative abundance in myelin<sup>2</sup>. Second, the data in Fig. 1 reveal a distinct difference between responses to proteins derived from either healthy myelin or MS-affected tissue. In the latter case, a consistently predominant response was detected against the contents of RP-HPLC fraction 8. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that this fraction contained only one detectable protein, with an apparent relative molecular mass ( $M_r$ ) of about 23K (Fig. 2). The same protein was detected as the single component in the corresponding fraction derived from the control preparation but in smaller amounts.

To identify the 23K protein in fraction 8, the protein was purified from MS-affected white matter by RP-HPLC. Direct amino-acid sequencing of the purified preparation was unproductive, indicative of an N-terminal modification. Thus, the 23K protein was trypsinized and a selection of the resulting fragments was purified by RP-HPLC and sequenced. As a result, four internal sequences were identified that were identical to sequences of human  $\alpha$ B-crystallin. The amino-acid composition of the purified 23K protein was consistent with the full sequence of human  $\alpha$ B-crystallin. In line with this the behaviour of purified human 23K protein and bovine  $\alpha$ B-crystallin was identical in RP-HPLC and in SDS-PAGE. Furthermore, polyclonal rabbit antibodies