

neurons<sup>10,12</sup>. Gating may be site-specific, occurring only where the cutaneous receptive field of the recorded cell is not engaged during tactile discrimination behaviour<sup>13,14</sup>. These data<sup>10,14</sup> suggest suppression of sensory transmission from cutaneous receptive fields where behaviourally significant sensory stimuli are not anticipated and preservation of transmission in fields where such stimuli are expected. This pattern is similar to the blood flow decreases we observe only in somatosensory fields representing unattended skin sites. Our PET data show extensive activity suppression that was especially prominent in cortex ipsilateral to the attended skin location. Furthermore, the sites with the greatest decreases in flow suggest sensory suppression may particularly involve SI regions for structures with the highest inner-

vation densities (mouth, hand) that are the main somatosensory discriminative organs.

Gating information from unattended receptive fields could limit transmission of sensory input to higher cortical levels. Modulation in the form of suppressing background activity from spatial locations that are not attended to may thus facilitate processing of signals expected to carry greater behavioural significance<sup>1</sup>. Nevertheless, gating may have an associated cost of diminished ability to detect weak stimuli if unexpected stimuli occur in an unattended receptive field<sup>15-18</sup>. Similarly, it has been demonstrated that poorer performance in detecting subtle somatosensory stimulus changes occurs when subjects are cued to focus attention upon a finger other than the one where a target appeared<sup>19</sup>. □

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## The site and stage of anti-DNA B-cell deletion

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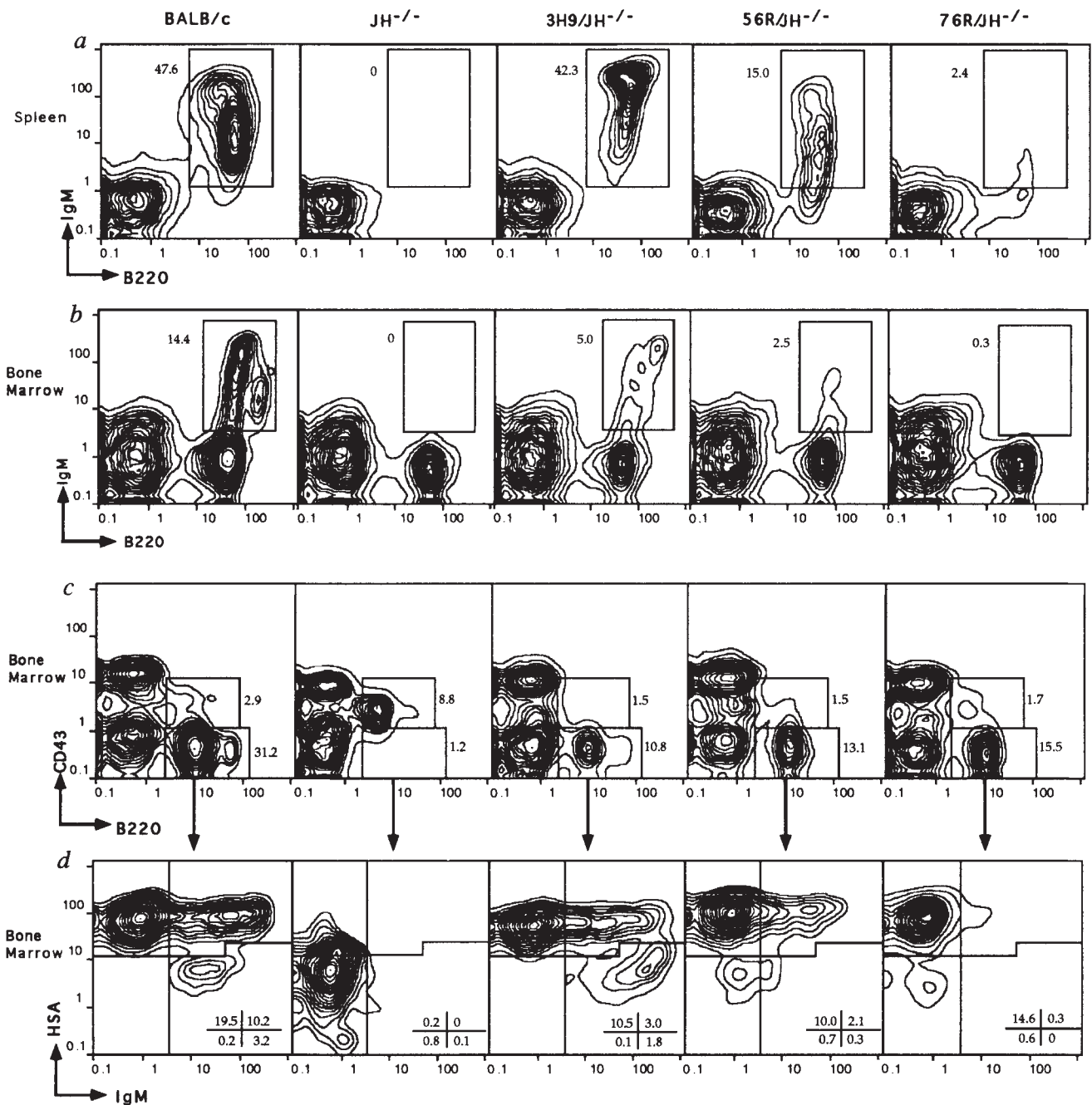
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ANTIBODIES to DNA and nucleoproteins are found in sera of individuals with systemic autoimmune disease. In the population (and in the autoimmune mouse strain MRL/lpr) there is a great variety of such antinuclear antibodies, but individuals with systemic lupus erythematosus or single MRL mice express a subset only of the antinuclear specificities found in the population. These observations have been interpreted to mean that these antibodies arise by immunization<sup>1</sup>. The oligoclonal nature of the autoantibody response and the evidence of selection acting on somatically mutated autoantibodies favour this interpretation<sup>2,3</sup>. Specific activation of autoantibodies in disease implies either that autoantibodies are regulated in non-diseased individuals or that autoantigen availability is variable. The former has been demonstrated in anti-DNA transgenic mice. In normal mice, transgene-encoded antibodies against double-stranded (ds) DNA are not expressed in serum or on B cells<sup>4-6</sup>. Here we describe modified anti-dsDNA transgenic mice which allow us to study the site and developmental stage at which such B-cell regulation occurs. This model shows that in normal mice B cells expressing anti-DNA specificity are deleted in the bone marrow at a pre-B to immature B transitional stage.

Our model relies on a transgene coding for the heavy chain (H chain) of an anti-DNA antibody (3H9) from an autoimmune MRL/lpr mouse<sup>3</sup>. The 3H9 heavy-chain variable region (VH) has the characteristic of anti-DNAs seen in autoimmune disease: somatic mutations that introduced an arginine residue in a complementarity determining region (CDR), generating affinity for dsDNA<sup>7</sup>. The 3H9 VH has a dominant role in determining DNA activity. It is used repeatedly in spontaneous anti-DNA antibodies from autoimmune mice and can be combined with different light chains (L chains) to yield anti-DNA antibodies<sup>8</sup>. Thus the 3H9 H chain transgenic mouse as such is informative as we are

FIG. 1 Flow cytometry analysis. a, Spleen cells from BALB/c, JH<sup>-/-</sup> and the three tg<sup>+</sup>/JH<sup>-/-</sup> mice were stained with anti-B220, anti-IgM and anti-Ly-1<sup>6</sup>. The window contains B220<sup>+</sup>IgM<sup>+</sup> B cells and the number of these cells is shown as the percentage of total spleen lymphocytes. While the JH<sup>-/-</sup> mouse does not have B cells in the spleen, the 3H9/JH<sup>-/-</sup> mouse has a nearly normal frequency of splenic B cells. The frequency of B cells in 56R/JH<sup>-/-</sup> mouse is reduced to about one third of the control and most of the B cells are of low surface IgM density. The 76R/JH<sup>-/-</sup> has only a few B220<sup>+</sup>, IgM<sup>low</sup> B cells. While the frequencies of the B cells in JH<sup>-/-</sup> and tg<sup>+</sup>/JH<sup>-/-</sup> mice range from none to nearly normal, the T-cell frequencies in these mice (as determined by Ly-1<sup>+</sup>) are equivalent to or higher than the BALB/c control (not shown). b, Bone marrow cells were analysed for expression of B220 and IgM. The number in the window represents the percentage of B220<sup>+</sup>IgM<sup>+</sup> cells in the bone marrow lymphocyte gating. As expected, the JH<sup>-/-</sup> mouse has no IgM<sup>+</sup> cells, the 56R/JH<sup>-/-</sup> has greatly reduced frequency and the 76R/JH<sup>-/-</sup> has few, if any, IgM<sup>+</sup> cells in the bone marrow, indicating deletion in this organ. c, Bone marrow cells were stained with anti-CD43 (S7) and anti-B220 (a different staining reagent from that in b). Frequencies of B220<sup>+</sup>CD43<sup>+</sup> and B220<sup>+</sup>CD43<sup>-</sup> cells are shown by windows as the percentage of the bone marrow cells in the lymphocyte gating. The JH<sup>-/-</sup> mouse has all the B cells arrested in the pre-B (B220<sup>+</sup>CD43<sup>+</sup>) stage whereas all three tg<sup>+</sup>/JH<sup>-/-</sup> mice have significant numbers (from 1/3 to 1/2 of the control) of more mature CD43<sup>-</sup> B cells. d, Cells from the B220<sup>+</sup>CD43<sup>-</sup> window of c were analysed for expression of IgM and HSA. The upper left quadrant represents pre-B, the upper right quadrant depicts immature B and the lower right quadrant represents mature B cells. The cells in the lower left quadrant of the JH<sup>-/-</sup> mouse are probably the earliest B cells (pre-pro-B)<sup>13</sup> which



are CD43<sup>low</sup> and overgated in the CD43<sup>-</sup> window whereas the cells in lower left quadrant of the 56R/JH<sup>-/-</sup> and 76R/JH<sup>-/-</sup> mice may represent more mature B cells which were derived from the IgM<sup>+</sup>HSA<sup>high</sup> immature B cells and had their surface immunoglobulin (slg) downregulated. Numbers indicate the cells in each quadrant as a percentage of total bone marrow lymphocytes.

**METHODS.** Transgenic mice expressing 3H9, 56R or 76R H chain transgenes were crossed to JH<sup>-/-</sup> mice<sup>12</sup> until tg<sup>+</sup>/JH<sup>-/-</sup> mice were derived. H chain transgenes were identified as described previously<sup>4</sup>. The JH<sup>-/-</sup> genotype was typed by polymerase chain reaction (PCR) using the JH-specific primers: 5'-GGACCAGGGGCTCAGGTCAGG-3' and 5'-GAGGAGACGGTGACCGTGGTCCCTGC-3'. Amplification of a 400 bp fragment indicates a JH<sup>+/+</sup> or a JH<sup>+/-</sup> genotype whereas the absence of any amplified band identifies the JH<sup>-/-</sup> mice. Spleen cells were stained with fluorescein-conjugated anti-IgM (Fisher Biotech), Cy5-conjugated B220 (6B2), and biotin-conjugated Ly-1 (PharMingen) simultaneously. Bone marrow cells were stained with fluorescein-conjugated anti-CD43 (S7), phycoerythrin-conjugated anti-IgM, allophycocyanin or Cy5-conjugated B220 and biotin-anti-HSA. The flow cytometric analyses were performed on a dual-laser FACStar Plus. Dead cells were excluded by propidium iodide staining whenever possible. The contour plots are

taken from a representative experiment of several. The number of animals examined and the frequency of individual cell populations (expressed as the mean and s.d.) are as follows: for spleen B cells (B220<sup>+</sup>IgM<sup>+</sup>), eight BALB/c, 46.8±3.9; eleven JH<sup>-/-</sup>, 0; nine 3H9/JH<sup>-/-</sup>, 41.3±7.8; seven 56R/JH<sup>-/-</sup>, 12.0±2.8; ten 76R/JH<sup>-/-</sup>, 3.4±2.6. For bone marrow slg<sup>+</sup> B cells (B220<sup>+</sup>IgM<sup>+</sup>), eight BALB/c, 19.5±4.3; ten JH<sup>-/-</sup>, 0; nine 3H9/JH<sup>-/-</sup>, 8.1±2.3; seven 56R/JH<sup>-/-</sup>, 3.0±1.2; ten 76R/JH<sup>-/-</sup>, 2.0±1.0. For bone marrow pro-B cells (B220<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>), six BALB/c, 8.2±3.9; eight JH<sup>-/-</sup>, 15.8±8.0; five 3H9/JH<sup>-/-</sup>, 5.4±3.2; three 56R/JH<sup>-/-</sup>, 6.7±4.5; six 76R/JH<sup>-/-</sup>, 6.5±4.1. For bone marrow pre-B cells (B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>), six BALB/c, 25.6±5.4; eight JH<sup>-/-</sup>, 0.4±0.4; five 3H9/JH<sup>-/-</sup>, 12.2±4.7; three 56R/JH<sup>-/-</sup>, 21.0±8.0; six 76R/JH<sup>-/-</sup>, 18.9±7.4. For bone marrow immature B cells (B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>+</sup>HSA<sup>high</sup>), six BALB/c, 12.6±3.8; eight JH<sup>-/-</sup>, 0; five 3H9/JH<sup>-/-</sup>, 3.4±1.1; three 56R/JH<sup>-/-</sup>, 2.5±1.5; six 76R/JH<sup>-/-</sup>, 0.9±0.7. For bone marrow mature B cells (B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>+</sup>HSA<sup>low</sup>), six BALB/c, 6.2±3.5; eight JH<sup>-/-</sup>, 0; five 3H9/JH<sup>-/-</sup>, 2.2±0.7; three 56R/JH<sup>-/-</sup>, 0.7±0.3; six 76R/JH<sup>-/-</sup>, 0. The age of the mice ranged from 2.5 to 5 months. Three different founders of 76R/JH<sup>-/-</sup> mice and two different founders of 56R/JH<sup>-/-</sup> mice were included in this study.

guaranteed that at least some endogenous L chains when paired with this H chain will produce anti-DNA B cells. But surveys of hybridomas derived from splenic B cells of 3H9 H chain transgenics showed no examples with dsDNA activity<sup>4</sup>. We interpreted this to mean that dsDNA-specific B cells are deleted in normal animals.

One might expect that deletion would lead to a depleted B-cell population. However, the 3H9 H chain transgenics have nearly normal numbers of B cells in spleen and bone marrow. This is because the effect of deletion on B cell number is obscured by editing, a process that replaces V genes coding for autoreactive receptors with V genes coding for functional, non-autoreactive antibodies<sup>5,9,10</sup>. V genes can be edited in several ways, one of which is by secondary rearrangement. Here, an L chain that contributes to an autoreactivity such as DNA binding is replaced by an L chain that does not sustain DNA binding<sup>10</sup>. To interfere with this editing process, another anti-DNA transgenic (56R) was generated in which the affinity for dsDNA of the 3H9 H chain was increased by the addition of an arginine to CDR2 (position 56). This modification reduces the number of L chains capable of blocking dsDNA binding<sup>11</sup>. The 56R B cells, however, used a different editing mechanism, by which the transgene is deleted. This allows the expression of an innocuous endogenous H chain<sup>6</sup>.

Editing reconstitutes the B-cell population of these transgenics and thereby presents an obstacle to pinpointing the site and stage of negative selection. Consequently we further modified our model to preclude editing. First, we added an additional arginine to the 56R VH at position 76. This '76R' antibody has even higher affinity for DNA<sup>11</sup> and, according to modelling studies, should yield anti-dsDNA regardless of the L chain with which it is paired. Second, editing by way of endogenous H chain expression has been subverted by crossing the 76R transgene into mice with an inactivated H chain locus ( $J_H^{-/-}$ )<sup>12</sup>. If H chain transgenes can rescue B cells in the  $J_H^{-/-}$  mice, then we should have an opportunity to study the extent and site of deletion of anti-dsDNA B cells.

76R was first compared with 56R and 3H9 H chain transgenics on a normal BALB/c background (Table 1). A progressive decline of peripheral B cell number was observed in the three transgenics: the 3H9 retained more than 75% of the normal B cell frequency, the 56R had about 40% and the 76R had the fewest B cells. In addition, the transgene has been deleted in all the B-cell hybridomas from 76R mice (none of which binds dsDNA, data not shown). This supports the idea that few, if

any, L chains can abrogate the anti-DNA phenotype conferred by the 76R H chain. The major route by which these B cells escape tolerance in the 76R transgenic is deletion of the transgenic H chain and expression of endogenous H chains, as was observed previously for the 56R transgene.

When the transgene-deletion pathway of editing was blocked by crossing the H chain transgenes onto mice whose  $J_H$  loci had been deleted, B-cell depletion was profound. Flow cytometric analysis of the peripheral B- and T-cell compartments was carried out on 3H9/ $J_H^{-/-}$ , 56R/ $J_H^{-/-}$  and 76R/ $J_H^{-/-}$  mice (Fig. 1a). Whereas  $J_H^{-/-}$  mice have normal numbers of T cells but no B cells ( $B220^+IgM^+$ ) (Fig. 1 and ref. 12), the 3H9/ $J_H^{-/-}$  mice had nearly normal numbers of B cells in the spleen, demonstrating that this transgenic H chain can reconstitute the B cell compartment of  $J_H^{-/-}$  mice. On the other hand, the 56R/ $J_H^{-/-}$  mice had greatly reduced B cell numbers and the 76R/ $J_H^{-/-}$  mice had almost no B cells. This is due to a deletion of anti-DNA B cells, not an incomplete rescue of B-cell development by these transgenes for the following reasons: (1) 56R and 76R mice on a non- $J_H^{-/-}$  background (Table 1) have B cells (of course, none of the hybridomas recovered from these mice had dsDNA activity, indicating a selective deletion); (2) as discussed below, rescue of B cells at stages before surface immunoglobulin expression is as efficient in 56R and 76R as in 3H9.

Given the extent of deletion of B cells in 76R/ $J_H^{-/-}$  mice, we can now determine where these B cells are eliminated. Bone marrow cells from individual  $tg^+/J_H^{-/-}$  mice were analysed for surface phenotypes B220, IgM and  $\kappa$ . Decreasing levels of  $B220^+IgM^+$  B cells were observed in the three  $tg^+/J_H^{-/-}$  mouse lines with 76R/ $J_H^{-/-}$  mice having virtually no  $IgM^+$  (or  $\kappa^+$ ) B cells in the bone marrow (Fig. 1b). These results show that anti-DNA B cells are deleted in the bone marrow in non-autoimmune animals and imply that the autoantigens (DNA or DNA-protein complexes) are present in the bone marrow and have access to developing B cells.

The fact that few  $IgM^+$  B cells are detected in the 76R/ $J_H^{-/-}$  bone marrow (Fig. 1b) suggests a deletion in the immature B cells. To address this issue further, bone marrow B cells are divided into two populations based on the expression of the CD43 surface marker as recognized by the monoclonal antibody, S7<sup>13</sup>. The  $B220^+CD43^+$  population comprises very early B cells (pro-B) whereas the  $B220^+CD43^-$  population includes more mature B cells<sup>13</sup>. Within the latter population, three subsets can be distinguished on the basis of the expression of sIgM and heat-stable antigen (HSA)<sup>13,14</sup>:  $IgM^-HSA^{hi}$  (pre-B),  $IgM^+HSA^{hi}$  (immature B) and  $IgM^+HSA^{low}$  (mature B). Bone marrow cells of  $J_H^{-/-}$ ,  $tg^+/J_H^{-/-}$  and  $J_H^{+/+}$  control mice were stained with antibodies against B220, CD43, HSA and IgM. The  $J_H^{-/-}$  mice show a developmental block at  $B220^+CD43^+$  pro-B stage (Fig. 1c), as reported earlier<sup>12,15</sup>. However, all three  $tg^+/J_H^{-/-}$  mice have large numbers of  $B220^+CD43^-$  B cells (30–50% of the control), indicating that the introduction of a rearranged immunoglobulin H chain transgene promotes B-cell development from the  $CD43^+$  to the  $CD43^-$  stage. By further subdividing the  $B220^+CD43^-$  population, major differences among the three transgenic lines can be seen (Fig. 1d): the 3H9 mice showed reduced but significant levels of the three subsets of B cells (pre-B, immature-B and mature-B); 56R mice had greatly reduced numbers of immature B and mature B cells; and the 76R mice had few, if any, immature and mature B cells. These results indicate that anti-DNA B cell deletion occurs at the pre-B to B transitional stage or just after expression of the surface immunoglobulin.

A small but detectable population of B cells with very low surface Ig density is present in the periphery of 56R/ $J_H^{-/-}$  and even 76R/ $J_H^{-/-}$  mice (Fig. 1a). An analogous population was also observed in the bone marrow (Fig. 1d). We believe that these cells are derived from  $HSA^{hi}IgM^+$  immature B cells through the downregulation of surface immunoglobulin and are analogous to anergic B cells<sup>16</sup>. The L chains used by these cells

TABLE 1 Peripheral B-cell frequencies and hybridoma analysis of transgenic mice on a normal background

Mice	B-cell frequency		Hybridomas			
	B220 <sup>+</sup> , IgM <sup>+</sup> splenocytes	Total hybrids analysed	tgH <sup>+</sup>	tgH <sup>-</sup>	ssDNA	dsDNA
76R	11.5 ± 3.0	125	0	125	NA	NA
56R	18.3 ± 2.4	36	17	19	16	1*
3H9	34.3 ± 2.5	85	85	0	44	0
BALB/c	45.7 ± 3.8	ND	ND	ND	NA	NA

3H9 and 56R transgenic mice were described previously<sup>4,6</sup>. 76R transgenic mice analysed in this table include two founders, each of which have two to four copies of the transgene. Mice were 2–4 months old and all were at 4–8 backcrosses of the transgene onto BALB/c background. Spleen cells were stained with anti-B220, anti-IgM and anti-Ly-1. The percentage of B cells was defined as  $B220^+IgM^+$  cells. Data from 3–6 independent experiments are presented as the mean ± s.d. B-cell frequency decreased progressively from 3H9 to 56R to 76R. All the mice had normal numbers of T (Ly-1<sup>+</sup>) cells (data not shown). Hybridomas were derived using LPS-stimulated spleen cells of the three H chain transgenics<sup>5</sup>. Only the immunoglobulin-secreting hybrids were analysed here. The presence (IgH<sup>+</sup>) and absence (IgH<sup>-</sup>) of the H chain transgenes in the genome of the hybrids were identified by Southern analysis and PCR assays<sup>6</sup>. Antibodies produced by the hybridomas were assayed for their binding to ssDNA and dsDNA by a solution phase ELISA<sup>8</sup>. Only hybrids which retained the transgene DNA were included in the binding analysis. NA, not applicable; ND, not done.

\* This hybrid also expresses an endogenous H chain.

may form antibodies with relatively low affinity for DNA or the H chain transgenes in these cells may be mutated in such a way as to preclude autoreactivity.

Transgenic mice bearing immunoglobulin genes coding for antibodies directed to facultative self antigens have demonstrated two ways of regulating B cells: anergy and deletion. In the case of a soluble self antigen, hen egg lysozyme (HEL), anti-HEL B cells are inactivated<sup>16</sup>; for an antigen expressed on the cell surface, H-2<sup>k</sup>, anti-H-2<sup>k</sup> B cells are deleted<sup>17</sup>. These different ways of manifesting tolerance are not simply due to the different intrinsic affinity for self antigen as conversion of soluble HEL to a membrane-associated form leads to deletion of anti-HEL B cells<sup>18</sup> and the anti-H-2<sup>k</sup> B cells are deleted, even by products of H-2 alleles for which the anti-H-2<sup>k</sup> antibody has low affinity<sup>19</sup>. Instead, the potential of membrane-associated self antigen to crosslink self-reactive receptors may be the feature that leads to deletion. In these models, while the B cells specific for membrane-bound self-antigens are absent in peripheral lymphoid organs, large numbers of immature B cells (B220<sup>low</sup>IgM<sup>+</sup>) with reduced surface immunoglobulin density have been observed in the bone marrow<sup>9,20</sup>. This contrasts with our finding that few, if any, IgM<sup>+</sup> B cells are detectable in the bone marrow of 76R/JH<sup>-/-</sup> mice (Fig. 1b, d). B cells specific for the constitutive self antigen, dsDNA, may be deleted on encountering the antigen. Alternatively, surface IgM of immature B cells may be greatly modulated or masked before deletion as a result of crosslinking by the self antigen. In either case, the manner by which the antigen is presented to lymphocytes may account for the difference. Nucleoprotein complexes are included in blebs present on the surface of apoptotic (dying) cells<sup>21</sup> and organs such as the bone marrow and thymus are sites of cell death. Lymphocytes in such an environment may repeatedly encounter highly concentrated DNA-containing antigens. In particular, anti-DNA B cells undergoing cell death will be fratricidal, resulting in efficient clonal abortion.

Regulation of anti-DNAs at early stages of B-cell development may be vital. Although affinity for dsDNA can be determined by somatic mutation<sup>3</sup>, anti-DNAs must also arise as part of the germline repertoire. Indeed, we would estimate that the germline frequency of anti-DNA B cells is very high. Several V<sub>H</sub> and V<sub>L</sub> genes are found recurrently among anti-DNAs suggesting that some germline-encoded antibodies have a propensity for binding DNA<sup>22,23</sup>. Certain ways of rearranging or translating D<sub>H</sub> genes are common among anti-DNAs<sup>25,24</sup>. A theme relating anti-DNAs is enrichment in amino-acid residues such as arginine and asparagine that favour electrostatic interactions with DNA. If a limited number of such interactions is sufficient to create DNA specificity, then both the germline and somatic repertoires will include many anti-DNAs. The bone marrow may represent a critical filter for preventing these autoantibodies from entering the body. □

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## Differential production of interferon- $\gamma$ and interleukin-4 in response to Th1- and Th2-stimulating pathogens by $\gamma\delta$ T cells *in vivo*

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EXPOSURE to various pathogens can stimulate at least two patterns of cytokine production by CD4<sup>+</sup> T cells<sup>1–4</sup>. Responses that result in secretion of interferon- $\gamma$  (IFN- $\gamma$ ), lymphotxin and interleukin-2 (IL-2) are classified as T-helper-1 (Th1)<sup>5,6</sup>; CD4<sup>+</sup> T-cell production of IL-4, IL-5, IL-9, IL-10 and IL-13 is called a T-helper-2 response (Th2)<sup>5,6</sup>. Differentiation of CD4<sup>+</sup> T cells into either Th1 or Th2 cells is influenced by the cytokine milieu in which the initial antigen priming occurs<sup>7–9</sup>. Here we use flow cytometry to identify the presence of intracellular cytokines (cytoflow) and analyse T-cell production of IFN- $\gamma$  and IL-4 from mice infected with *Listeria monocytogenes* or *Nippostrongylus brasiliensis*. We show that T cells bearing  $\gamma\delta$  receptors discriminate early in infection between these two pathogens by producing cytokines associated with the appropriate T-helper response. Our results demonstrate that  $\gamma\delta$  T cells are involved in establishing primary immune responses.

The conditions leading to a Th1 or Th2 response are interesting because under some circumstances the successful elimination of infectious agents depends on expansion of the appropriate CD4<sup>+</sup> T-cell subset<sup>10–14</sup>. In general, Th1 cells are responsible for generating strong cellular immunity against intracellular pathogens; Th2 cells promote the development of humoral responses that direct effector functions using specific antibodies against extracellular pathogens.

Because several lines of evidence suggesting a 'first line of defence'<sup>15,16</sup> and a protective role<sup>17–19</sup> for  $\gamma\delta$  T cells, we tested whether these cells could differentially produce cytokines known to induce and effect Th1 and Th2 responses. We developed a flow cytometric technique for identifying cytokine proteins intracellularly in order to measure more accurately the *in vivo* situation. C3H/HeJ mice were injected intraperitoneally (i.p.) with either *Listeria monocytogenes*, an intracellular bacterium that invades the spleen and liver and promotes development of Th1 cells<sup>12</sup>, or the extracellular parasite, *Nippostrongylus brasiliensis*, a potent inducer of Th2 cells<sup>20</sup> which migrates through the vasculature to the lungs and the intestinal lumen<sup>21</sup>. These pathogens are normally eliminated at around day 10 after primary exposure<sup>16,22</sup>.