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Cross-linking of T-cell receptors on double-positive thymocytes induces a cytokine-mediated stromal activation process linked to cell death

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To investigate molecular events associated with the intrathymic process of negative selection, we established an *in vivo* system using an anti-CD3ε monoclonal antibody to induce synchronous apoptosis in the thymus of AND T-cell receptor (TCR) transgenic RAG-2$^{-/-}$ mice in a non-selecting haplotype. This model eliminates endogenous negative selection as well as gene activation in the mature thymocyte compartment, offering an ideal source of tester (anti-CD3ε-treated) and driver (untreated) thymus RNA for representational difference analysis (RDA). Fourteen mRNA sequences that are up-regulated in the thymuses of such mice 2–6 h after anti-CD3ε treatment were identified. Surprisingly, the majority of these transcripts were derived from stromal cells rather than the TCR-cross-linked CD4$^+$CD8$^+$TCR^{low} thymocytes including the macrophage products IL-1, the chemokine Mig and the transcription factor LRG-21. IFN-γ secretion from the CD4$^+$CD8$^+$TCR^{low} thymocytes regulates macrophage Mig production. Three other cytokines (IL-4, GM-CSF and TNF-α), known to activate a variety of stromal cells, are also induced in the same thymocyte population undergoing apoptosis. Expression of a TNF-α-inducible gene, B94, in stromal cells after TCR ligation further supports the notion of cross-talk between thymocytes and stroma. Thus, TCR-triggered immature thymocytes elaborate cytokines which may regulate the delivery of further signals from stromal cells required for apoptosis.

Keywords: apoptosis/cytokine/stroma/TCR/thymus

Introduction

T lineage cells bearing autoreactive T-cell receptors (TCRs) are deleted in the thymus, a process termed negative selection (Kappler *et al.*, 1988; MacDonald *et al.*, 1988). Negative selection is known to occur by apoptosis, a morphologically stereotyped form of programmed cell death (Surh and Sprent, 1994). Although models of thymocyte apoptosis studied thus far have suggested that this form of programmed cell death can be blocked by inhibitors of new gene expression, knowledge of the identity of the up-regulated genes responsible for cell death remains incomplete (Cohen and Duke, 1984; D'Adamio *et al.*, 1992).

Progress in analysis of apoptosis signalling pathways in the thymus has been hampered by the lack of a simple model system for negative selection. TCR transgenic mice reduce the complexity of thymocyte TCR specificities but their analysis is complicated by the presence of mature CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ thymocytes which undergo activation following stimuli which induce apoptosis among the less mature CD4$^+$CD8$^+$ thymocytes. When subtractive hybridization procedures are applied to cDNA derived from thymocytes triggered through the TCR, transcripts from activation genes are enriched simultaneously with those from genes associated with the putative apoptosis pathway. A further and equally important liability of utilizing TCR transgenic mice in a positively selecting haplotype is that ongoing negative selection due to endogenous peptides in the thymuses used for preparation of driver RNA may be of sufficient magnitude to subtract putative negative selection transcripts from the target RNA population.

In order to avoid the complicating elements of mature thymocyte activation and ongoing negative selection, as well as to avoid the possibility that apoptosis *in vitro* does not resemble bona fide negative selection in its molecular details, we have chosen to study an *in vivo* model of negative selection in RAG-2$^{-/-}$ I-E^k-restricted AND TCR transgenic mice in a non-selecting haplotype. Treatment of these animals with intraperitoneal (i.p.) anti-CD3ε induces synchronous and profound apoptosis of thymocytes which are arrested at a susceptible DP stage. These mice have drastically reduced numbers of mature cells in either the thymus or the periphery, capable of responding with simple activation and proliferation. To study anti-CD3ε-induced thymocyte apoptosis, we have utilized the technique of representational difference analysis (RDA), a PCR-based subtractive hybridization protocol (Hubank and Schatz, 1993; Lisitsyn *et al.*, 1993). To our surprise, the majority of the genes identified appear to be derived from thymic stromal cells rather than the targeted thymocyte population. We present evidence that prior to undergoing apoptosis, these immature thymocytes secrete cytokines which activate the surrounding thymic stroma. Aside from providing novel molecular insights into the phenomenon of thymocyte–stroma cross-talk, the data suggest that the thymic stroma may be involved in the negative selection process.

Results

ANDtg RAG-2$^{-/-}$ H-2^d thymocyte development is blocked at the CD4$^+$ CD8$^+$ TCR^{low} stage

An I-E^k restricted pigeon cytochrome *c*-specific T-cell receptor transgenic strain of mice was crossed with RAG-2

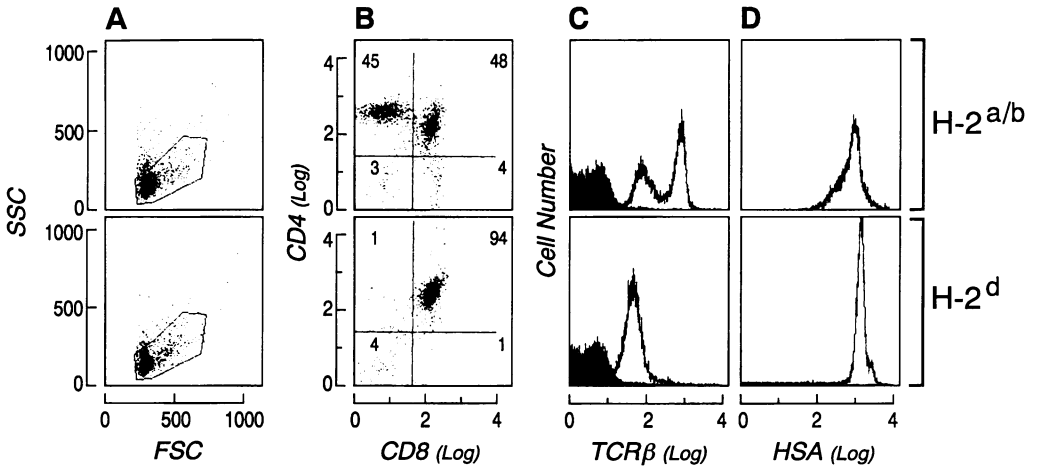

Fig. 1. Analysis of ANDtg RAG-2$^{-/-}$ T-cell development in a positively-selecting (H-2$^{a/b}$) or non-selecting (H-2^{d}) haplotype. Flow cytometric analysis of 20 000 thymocytes per histogram. (**A**) The forward and side scatter gate of the thymocytes analyzed is shown. (**B**) Two-color analysis utilizing FITC-labelled anti-CD4 and PE-conjugated anti-CD8. (**C** and **D**) Single-color analysis of FITC-labelled anti-TCRβ and FITC-labelled anti-HSA expression on gated thymocytes. An isotype-matched control antibody (shaded) is shown with the TCRβ histograms. The thymocytes were from 3-month-old mice.

mutant mice to generate mice in which endogenous TCR rearrangement was blocked (ANDtg RAG-2$^{-/-}$ mice) (Kaye *et al.*, 1989; Shinkai *et al.*, 1992). Hence, all TCR-expressing thymocytes were of a single specificity employing Vα11.1-Jα84 and Vβ3-Jβ1.2. In the H-2$^{a/b}$ haplotype, the AND TCR undergoes strong positive selection by the I-A^{b} and I-E^{k} molecules such that >40% of thymocytes are mature CD4^{+}CD8^{-} cells (CD4 SP) (Figure 1B, top). Although not shown, intravenous (i.v.) injection of cytochrome *c* induced depletion of DP thymocytes but activation of the CD4 SP population, with marked up-regulation of CD69 on these mature thymocytes. In contrast, when we crossed the ANDtg RAG-2$^{-/-}$ transgenic mice into a non-selecting H-2^{d} background (Kaye *et al.*, 1992), thymic development was blocked. From 90 to 95% of the thymocytes were DP and the remainder were DN (Figure 1B, bottom); ≤1% SP thymocytes were detected. Consistent with their developmental stage, the DP thymocytes expressed a low level of TCR (Figure 1C, bottom) and a high level of heat-stable antigen (HSA) (Figure 1D, bottom). Note that in the H-2$^{a/b}$ haplotype, both TCR^{low} and TCR^{high} thymocytes are detected and the expression of HSA is more heterogeneous (Figure 1C and D, top).

Anti-CD3ε-induced thymocyte apoptosis

In the non-selecting haplotype, it should be possible to induce synchronous cell death in the absence of a background of spontaneously occurring negative selection. Given that cytochrome *c* neither activates nor induces apoptosis in the H-2^{d} haplotype, we induced thymocyte apoptosis by cross-linking thymocyte TCRs with the anti-CD3ε-specific mAb 2C11 given i.p. (Smith *et al.*, 1989). At 20 h after administration, FACS analysis documented profound depletion of the DP population with residual DN thymocytes (Figure 2A). An 80–95% depletion in the number of cells that could be harvested from such thymuses as a single cell suspension was observed (data not shown).

Immunohistologic analysis confirmed 'cortical lysis' (Figure 3). Thymuses from the untreated animals consisted almost entirely of cortical CD4^{+}CD8^{+} thymocytes within a network of cortical epithelium defined with the mAb

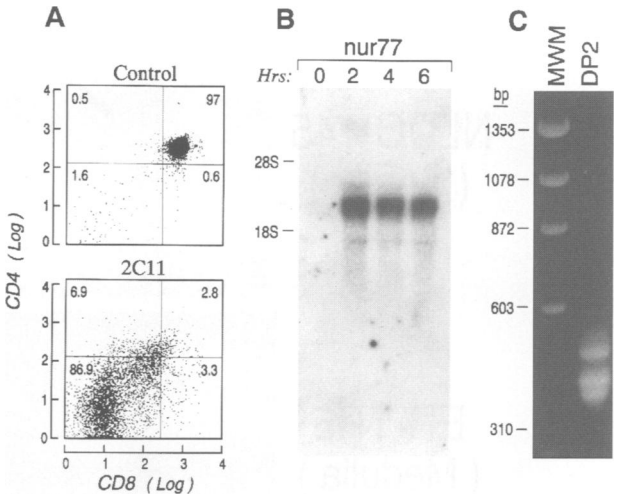

Fig. 2. Anti-CD3ε treatment of ANDtg RAG-2$^{-/-}$ H-2^{d} mice induces depletion of DP TCR^{low} thymocytes. (**A**) CD4 and CD8 expression on gated thymocytes (as per Figure 1) from a control animal and 20 h following i.p. injection of 200 μg of 2C11 mAb. Total thymocyte cell number was reduced 95% in the treated animal. (**B**) Expression of the orphan steroid receptor nur77 by Northern analysis in thymic RNA from an uninjected mouse and 2, 4 or 6 h after 2C11 administration. 10 μg of total RNA was loaded in each lane. Equal loading and transfer of RNA was confirmed by hybridization with GAPDH (data not shown). (**C**) Agarose gel electrophoresis of the second 'difference product' (DP2) derived from representational difference analysis of thymic cDNA from 2C11-injected and uninjected ANDtg RAG-2$^{-/-}$ H-2^{d} mice.

NLDC-145 (Kraal *et al.*, 1986). A small medulla was readily defined by ER-TR5, a mAb reactive with medullary epithelium (van Vliet *et al.*, 1984). At 20 h after injection of 2C11, treated animals showed a dramatic depletion of cortical thymocytes with loss of cell-associated CD4 and CD8. The loss of thymocytes led to collapse of the cortical epithelium, which resulted in increased staining with NLDC-145. TUNEL assays confirmed that the cell depletion was due to apoptosis.

These data suggest that the ANDtg RAG-2$^{-/-}$ H-2^{d} animals represent a unique tool with which to examine

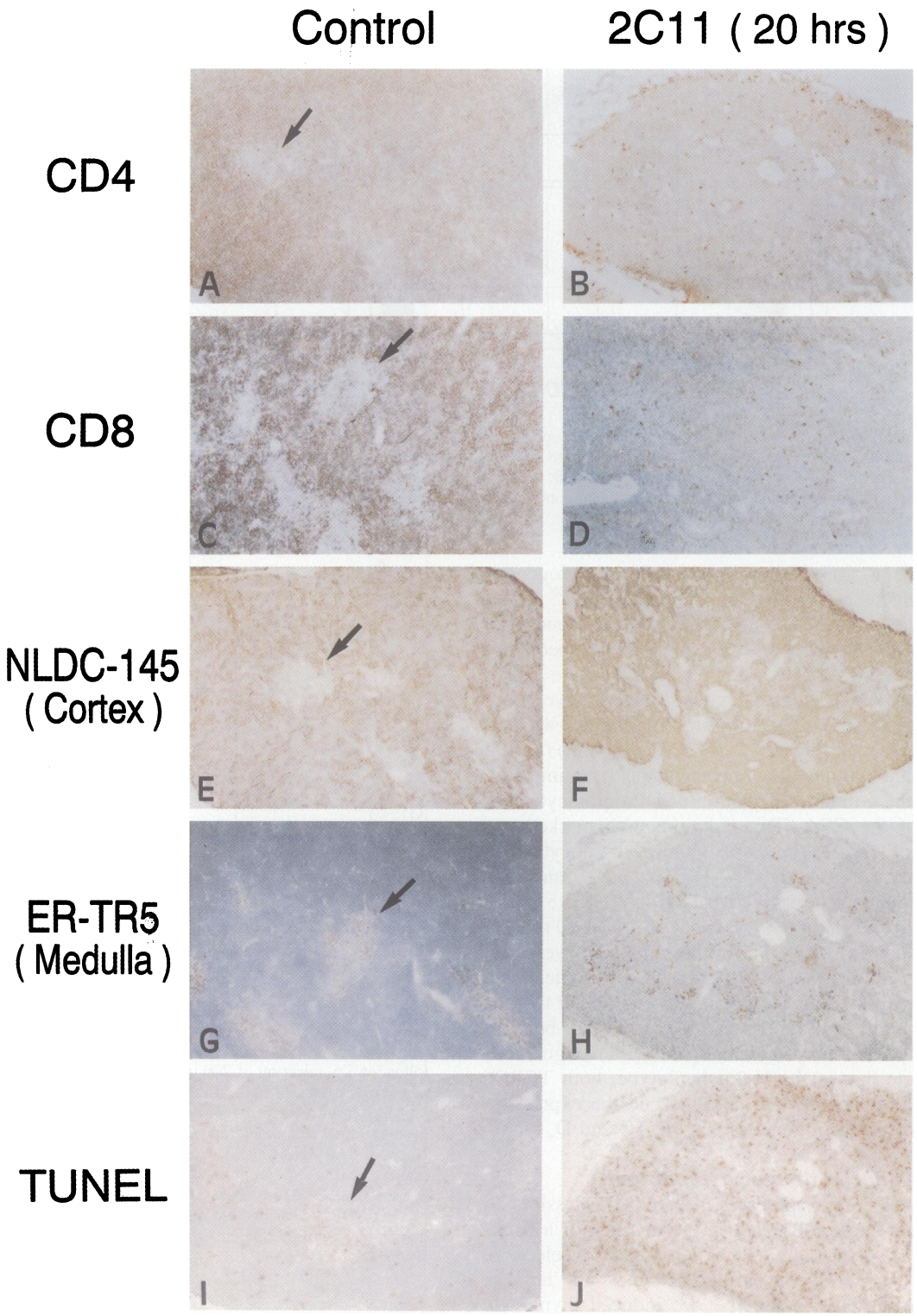

Fig. 3. Histologic analysis of ANDtg RAG-2$^{-/-}$ H-2^{d} thymuses prior to and 20 h following 2C11 treatment confirms 'cortical lysis'. Panels **A**, **C**, **E**, **G** and **I** represent analyses of control ANDtg RAG-2$^{-/-}$ H-2^{d} animals while panels **B**, **D**, **F**, **H** and **J** represent analyses of 2C11 anti-CD3ε mAb-injected ANDtg RAG-2$^{-/-}$ H-2^{d} animals with the specified reagents. Immunohistologic studies were performed as described in Materials and methods. The arrows indicate the position of representative medullary regions. A 10× objective was used in all panels.

the molecular basis of TCR-triggered apoptosis in the thymus. Consequently, we harvested total thymic RNA from these mice 0, 2, 4 and 6 h after injection with 200 μg of 2C11. Previous experiments suggested that DNA fragmentation was first visible by 4 h and became prominent by 6 h after injection (data not shown). Northern blot analysis of RNA from harvested thymuses using the orphan steroid receptor nur77 as a probe confirmed that the TCR of the thymocytes had been cross-linked *in vivo* (Liu *et al.*, 1994) (Figure 2B).

cDNA transcripts unique to the 2C11-treated thymuses were then identified by the recently described technique of

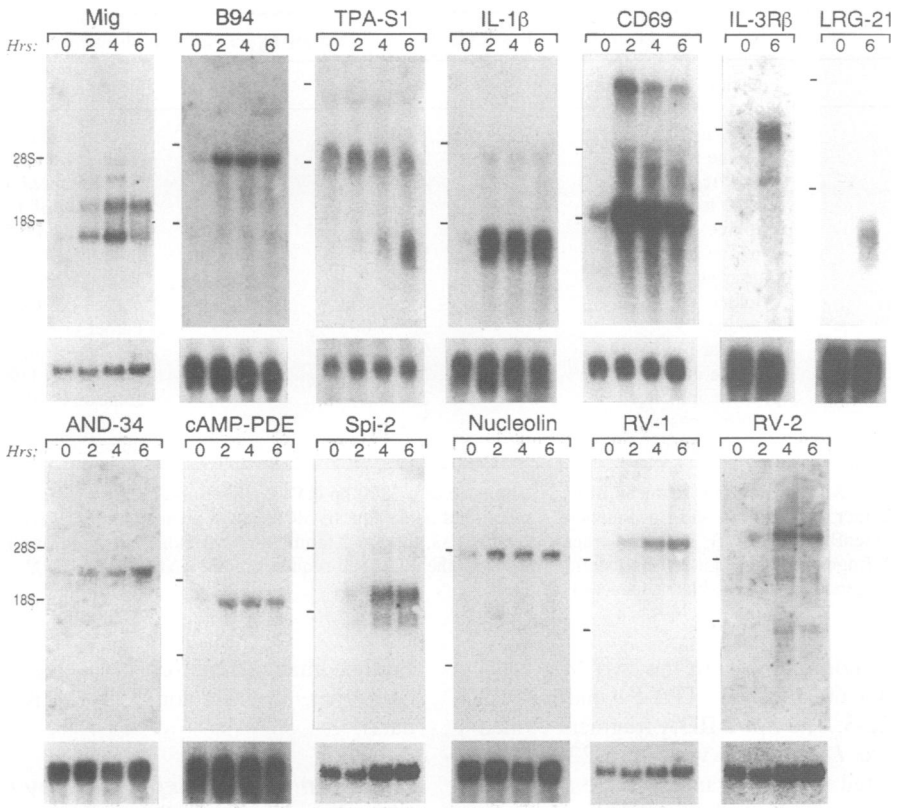

Fig. 4. Northern analysis of ANDtg RAG-2$^{-/-}$ H-2^{d} thymic RNA demonstrates 2C11-induced up-regulation of mRNAs hybridizing to cloned RDA fragments. RNA was harvested from thymuses prior to or 2, 4 or 6 h following 2C11 administration. 5 μg of polyadenylated RNA was utilized for LRG-21 and IL-3Rβ; all other Northerns used 10 μg of total RNA. Equal loading and transfer is confirmed by hybridization with GAPDH (seen in bottom panels below each Northern). Exposure time ranged from 12 h to 4 days.

representational difference analysis (Hubank and Schatz, 1993). cDNA 'representations' were initially produced from 5 μg of polyadenylated thymic RNA from either untreated mice or mice injected 2, 4 or 6 h earlier with anti CD3ε (1:1:1 mix). Two cycles of subtraction were carried out at tester:driver ratios of 1:100 and 1:800. Following these two cycles of subtraction, the second PCR-amplified 'DP2' product showed several clear bands superimposed on a background 'smear' (Figure 2C). Two attempts at producing DP3 with a 1:400 000 subtraction yielded no PCR product. The DP2 product was then gel purified, subcloned into a plasmid vector and transformed into bacteria. Insert containing plasmid DNAs were chosen at random and subjected to Northern analysis. Because of the known high levels of expression of one previously characterized differentially expressed transcript, nur77, we prescreened the initial 30 clones with this cDNA; one hybridizing clone was identified and was not further characterized.

Analysis of RDA-derived cDNA clones

The subtracted library was moderately complex; random sequencing of 41 clones yielded 33 different sequences. Among the 25 RDA clones subsequently examined by Northern blot analysis, 14 were differentially expressed (Figure 4). Insert size varied from 300 to 1200 bp (the smaller PCR products were not included during gel purification). Most of the clones were derived from transcripts expressed at intermediate levels (detectable with 10 μg total RNA); only two clones required poly(A) Northerns for identification.

The majority of the differentially expressed RDA fragments were derived from previously characterized transcripts (Table I; see references therein). Four fragments were derived from cytokines or cytokine receptors (two fragments from the CXC chemokine Mig and one fragment each from IL-1β and the IL-3 receptor β chain). Two were related to protease inhibitors (TPA-S1 and Spi-2). Two were homologous to retroviruses and may be derived from endogenous transcription of genomic defective retroviral elements (RV-1 and RV-2). The remaining transcripts include a monocyte leucine-zipper-containing transcription factor (LRG-21), a signal transduction regulatory enzyme [cAMP phosphodiesterase (PDE)], a cell surface C-type lectin activation molecule (CD69), a nucleolar protein (nucleolin) and a TNF-α-inducible cytoplasmic protein of unknown function (B94). One transcript was homologous only to an 'expressed sequence tag' (EST) (AND-34).

Among the RDA fragments derived from known genes, the size of the hybridizing mRNAs determined by Northern analysis was consistent with those reported in the literature. The kinetics of transcript induction varied among the cloned fragments, as would be expected given that the starting material consisted of a mix of cDNAs derived from mice injected with 2C11, 2, 4 or 6 h previously. The sequences of the RDA fragments corresponded in most cases to known *Dpn*II restriction enzyme sites in the published gene sequences (Table I). The majority were located within the 3′ portion of the transcript. The clones

Table I. Thymic cDNA fragments cloned from 2C11-treated ANDtg RAG-2$^{-/-}$ H-2^{d} mice by representational difference analysis

Gene	Function	Site	Reference
AND 34	unknown	1901–2239	A.Lerner *et al.* (unpublished)
B94	cytokine response	2685–3039	Wolf *et al.* (1994)
cAMP PDE4	cAMP regulation	436–829	Swinnen *et al.* (1989)
CD69	C-type lectin	185–803	Ziegler *et al.* (1993)
IL-1β	cytokine	33–684	Gray *et al.* (1986)
IL-3Rβ	cytokine receptor	NA	Itoh *et al.* (1990)
LRG-21	transcription factor	1305–1719	Drysdale *et al.* (1996)
Mig 1	cytokine	745–1071	Farber (1990)
Mig 2	cytokine	367–745	Farber (1990)
Nucleolin	nucleolar protein	9855–10738	Bourbon *et al.* (1988)
Spi-2	protease inhibitor	NA	Inglis *et al.* (1991)
16C8/TPA-S1	protease inhibitor	219–547	Johnson *et al.* (1987)
RV-1	retrotransposon	NA	Hodgson *et al.* (1990)
RV-2	retrotransposon	NA	Stoye and Coffin (1987)

NA, not applicable. The AND-34 *Dpn*II site is deduced from the sequence of a 3270 bp cDNA clone subsequently isolated using the RDA cDNA fragment as a probe (A.Lerner, L.K.Clayton and E.L.Reinherz, unpublished data). The IL-3R β-like sequence is most likely from the 3′ UTR of β_c, which is not available in GenBank (see text). The RDA fragment of EB22-3, the Spi-2 family member cloned here, did not have *Dpn*II sites (see text). The nucleolin RDA fragment included a 331 bp deletion relative to the published sequence. RV-1 and RV-2 are highly homologous but not identical to published endogenous retrovirus-like sequences (see text).

which showed 100% homology were the two Mig fragments, B94, IL-1β, CD69, LRG-21, TPA-S1 and a Spi-2 family member, EB22-3. The Spi-2 RDA fragment cloned did not correspond to *Dpn*II sites within EB22-3. This clone may have resulted from aberrant cleavage at a non-standard restriction site. The nucleolin RDA fragment showed 100% homology but included a 331 bp deletion in the 3′ UTR with GT/AT boundaries consistent with an alternate splicing event.

One RDA fragment showed 93% identity to rat cAMP phosphodiesterase 4, a PDE isoform widely expressed in that animal (Swinnen *et al.*, 1989). The fragment was derived from the central coding region of the transcript, a region which is known to be highly homologous to the other three known rat cAMP PDE isoforms. There is no comparable sequence information in mice for cAMP PDE isoforms to allow definitive identification of this fragment. Two of the RDA fragments were homologous to retroviral sequences. RV-1 (B6) showed 80% overall homology to the retrotransposon BVL-1 (Hodgson *et al.*, 1990). The homology occurred in a region related to *gag* genes. The homology was 99% in the first 350 bp, then declined to 60% over the last 170 bp. RV-2 (E2) is more than 95% homologous to the LTR of a modified polytropic murine retrovirus, pMX33 (Stoye and Coffin, 1987).

One RDA fragment showed 70% homology to the 3′ UTR of the beta chain of the murine IL-3 receptor (β_{IL-3} or AIC2A). While the mouse has two highly homologous beta chains which form two functional IL-3 receptors, the distal 3′ UTR of the second beta chain (β_c or AIC2B) which also serves as the β chain of the receptors for GM-CSF and IL-5, is not available in GenBank. Our cloned fragment most likely is derived from this transcript. Blast searches of GenBank yielded only homology to EST sequences for clone AND-34. An 'organ Northern' demonstrated that expression of this transcript is widespread in tissues including brain, kidney, spleen and lymph node (data not shown). Sequence analysis of subsequently isolated cDNA clones demonstrated a 2.5 kb open reading frame encoding an SH2 domain in its amino-terminal region but without further homology to known sequences. Further characterization of this transcript is in progress (A.Lerner, L.K.Clayton and E.L.Reinherz, unpublished data).

Induction of gene expression by peptide ligands in class I restricted TCR tg mice

Antibodies directed against cell surface proteins can induce Fc-receptor-mediated activation of macrophages (Ravetch and Kinet, 1991). In order to determine whether the observed up-regulation of the transcripts identified by the RDA cDNA fragments resulted from Fc-receptor cross-linking by 2C11 mAb, we examined thymocyte activation mediated by specific peptide antigen. Given that cytochrome *c* cannot activate the AND TCR in the H-2^{d} haplotype and since interpretation of antigen activation in the ANDtg H-2$^{a/b}$ mice is complicated by the large number of SP thymocytes (≥40%, Figure 1B, top), we used mice transgenic for the H-2^{b}-restricted VSV-specific N15 TCR in a RAG-2$^{-/-}$ H-2^{b} background (Chang *et al.*, 1994; Y.Ghendler, R.E.Hussey, T.Witte, E.Mizoguchi, L.K.Clayton, A.K.Bhan, S.Koyasu, H.-C.Chang and E.L.Reinherz, in preparation). The TCR of these mice recognizes a nuclear protein octapeptide derived from the vesicular stomatitis virus (VSV-8) in the context of H-2^{b}, exclusively (Shibata *et al.*, 1992). The thymus of the H-2^{b} N15 RAG-2$^{-/-}$ mice comprised 70–90% DP and 5–15% CD8 SP (Y.Ghendler, R.E.Hussey, T.Witte, E.Mizoguchi, L.K.Clayton, A.K.Bhan, S.Koyasu, H.-C.Chang and E.L.Reinherz, in preparation). When Mig and B94 were used as probes, their respective transcripts showed up-regulation within the thymus 3 h following i.v. injection of the VSV-8 peptide, but not a control peptide (Figure 5). Thus, the observed up-regulation of these transcripts reflects signal transduction via the T-cell receptor, rather than an Fc-receptor-mediated effect in macrophages.

Gene inductions predominate in the thymic stromal compartment

Given that the initial tester cDNA was derived from total thymic RNA, the observed transcripts might derive from thymocytes, stroma or both populations. In order to define

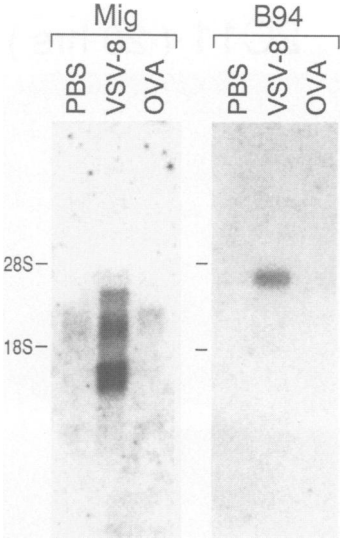

Fig. 5. Thymic mRNAs identified by RDA are up-regulated following i.v. injection of specific VSV-8 peptide antigen in N15 TCRtg H-2^b RAG-$2^{-/-}$ mice. RNA was harvested from thymuses 3 h after i.v. injection of 250 µl of PBS, 24 µg of the cognate VSV-8 peptide or 24 µg of a control ovalbumin peptide in PBS. 10 µg of total RNA was utilized for Northern analysis.

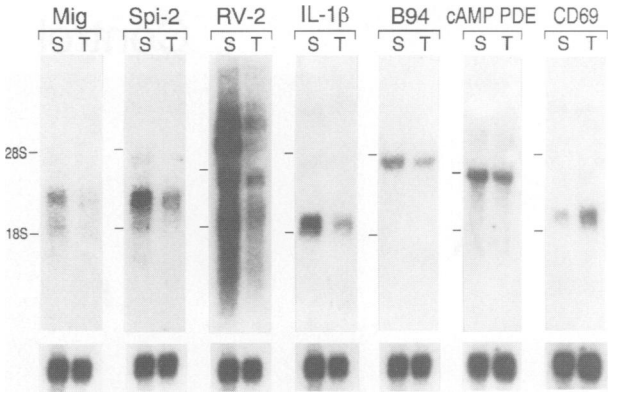

Fig. 6. Determination of the stromal versus thymocyte origin of seven 2C11-induced thymic mRNAs identified by RDA. At 6 h after i.p. injection with 2C11, thymuses of ANDtg RAG-$2^{-/-}$ H-2^d mice were harvested and ground between two glass slides to produce a single cell suspension that was >95% Thy-1^+ (data not shown). These cells were used for thymocyte RNA preparation (T). The remaining tissue, which is predominantly stromal but may contain residual lymphoid cells, was used for 'stromal' RNA preparation (S). 5 µg of total RNA was loaded in each lane and the Northern blots probed with the indicated cloned RDA fragments. Confirmation of equal loading and transfer was obtained by hybridization with GAPDH (seen in panels below each Northern).

the cell compartment from which the transcripts originated, we performed Northern analysis using RNA isolated 6 h after 2C11 injection of ANDtg RAG-$2^{-/-}$ H-2^d mice from either a single cell suspension of thymocytes or the remaining stroma (Figure 6). While the cell suspension used to prepare thymocyte RNA is consistently >95% Thy-1^+ (data not shown), the stromal 'remnant' may contain some contaminating lymphoid cells. Among the seven differentially expressed transcripts that were tested, five (Mig, Spi-2, RV-2, IL-1β and B94) showed predominant expression within the stromal compartment. The cAMP phosphodiesterase isoform fragment cloned in this study appears to be expressed in both stromal and thymocyte compartments. CD69 was induced primarily in thymocytes.

Evidence for activation of thymic stroma following TCR triggering of the $CD4^+CD8^+$ thymocytes was also obtained by immunohistologic analysis. At 20 h after 2C11 treatment, the ANDtg RAG-$2^{-/-}$ H-2^d thymuses showed increased epithelial expression of both class II MHC molecules and IL-2R (Figure 7). In the untreated animals, class II expression was predominant in the medullary regions, with a faint dendritic staining pattern in the cortex (Figure 7A). Following anti-CD3ε treatment, intense staining for class II was widespread, a result of cortical collapse, with an increased density of class II positive stromal cells and/or increased expression of these proteins (Figure 7B). Similarly, in untreated animals, thymic IL-2Rα expression was limited to scattered cells with a 'dendritic' pattern (Figure 7C), while following treatment, the number of cells expressing this receptor increased markedly (Figure 7D). A panel of anti-macrophage antibodies resulted only in faint diffuse staining in the untreated thymus (Figure 7E). Following treatment, there was an increase in the number of thymic macrophages and they tended to be arranged in clumps, often surrounding cystic structures (Figure 7F and inset). In agreement with this result, 2C11 treatment induced an alteration in the number and distribution of macrophages expressing CD11c, a molecule ordinarily expressed in the thymus only on medullary dendritic cells (Figure 7G and H) (Metlay *et al.*, 1990).

TCR-regulated cytokine production among $CD4^+$ $CD8^+$ TCR^{low} thymocytes

Activation of stromal cells by a stimulus targeted to the $CD4^+CD8^+$ thymocyte population suggested that a signalling mechanism existed between these two compartments early after TCR cross-linking. Since one of the two up-regulated cytokines identified, Mig, was known to be selectively activated by IFN-γ, we wished to determine whether this cytokine was induced in DP thymocytes by 2C11 treatment. Reverse PCR of total thymic RNA documented strong induction of IFN-γ mRNA at 2 h and 4 h after 2C11 injections (Figure 8A). To verify that this transcript was made by the dominant DP TCR^{low} thymocyte population, we triple-color sorted for these cells prior to and following 2C11 treatment (Figure 8B). IFN-γ was clearly markedly up-regulated in the purified DP TCR^{low} cells following 2C11 treatment (Figure 8C).

Up-regulation of IFN-γ following TCR cross-linking suggested that one unexpected outcome of activation of the DP TCR^{low} population was the activation of stromal cells by a cytokine network. We confirmed that the steady-state level of Mig mRNA is regulated by IFN-γ stimulation by performing Northern analysis of IFN-γ mutant mice (Dalton *et al.*, 1993). As shown in Figure 9, 2C11 stimulation of the IFN-γ mutant mice predictably failed to induce two dominant 1.7 and 3.5 kb Mig transcripts. In contrast, the induction of two other differentially induced RNAs identified in this study, the 1.7 kb IL-1β and 2.7 kb Spi-2 species, remained normal, with expression equivalent to that seen in ANDtg RAG-$2^{-/-}$ H-2^d animals.

As IFN-γ secreted by the DP TCR^{low} thymocytes

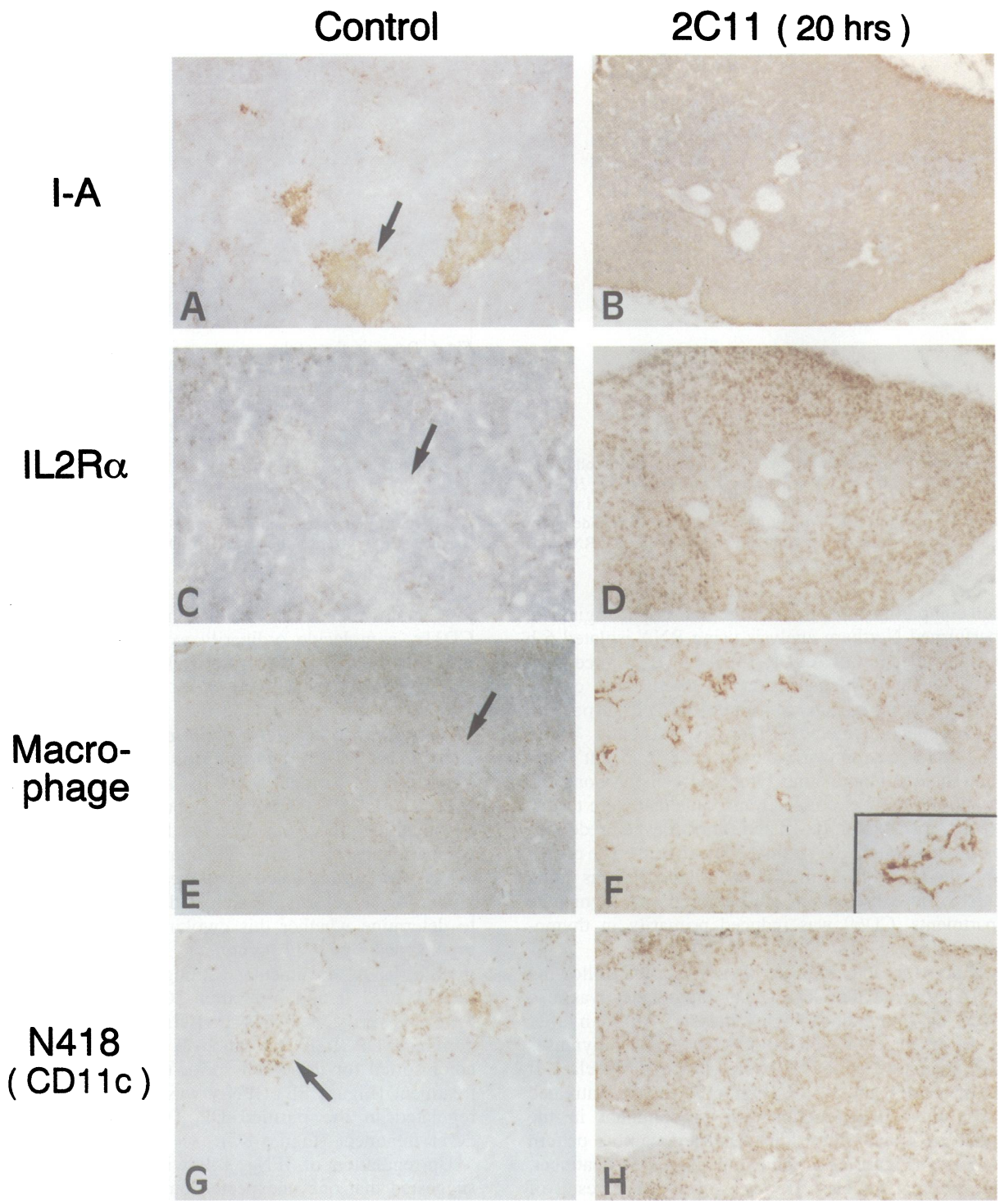

Fig. 7. Activation of thymic stromal cells following TCR cross-linking of DP TCR^{low} thymocytes in ANDtg RAG-2$^{-/-}$ H-2^{d} mice. Immunohistologic analyses of cryostat sections of thymuses prior to and 20 h after 2C11 injection with mAbs specific for I-A, IL-2Rα, macrophage antigens and CD11c. Representative medullary regions are indicated by arrows. The insert in panel (F) is taken from the upper left region of the field and demonstrates intense staining of macrophage markers around a cystic structure in the 2C11-treated animals. A 10× objective was used for all panels except the inset in panel (F), which used a 20× objective.

was clearly responsible for up-regulating stromal expression of Mig, we were interested in determining whether IFN-γ was in fact necessary for the efficient removal of apoptotic thymocytes. We performed histologic analyses of IFN-γ knockout mice and their heterozygous littermates prior to and 20 h following 2C11 treatment and found no evidence of any significant alterations in either the induction of apoptosis or the removal of dead cells (data not shown). Hence, IFN-γ production alone is not sufficient to explain the activation of macrophages we have identified through the isolation of differentially expressed macrophage mRNAs and histologic analysis.

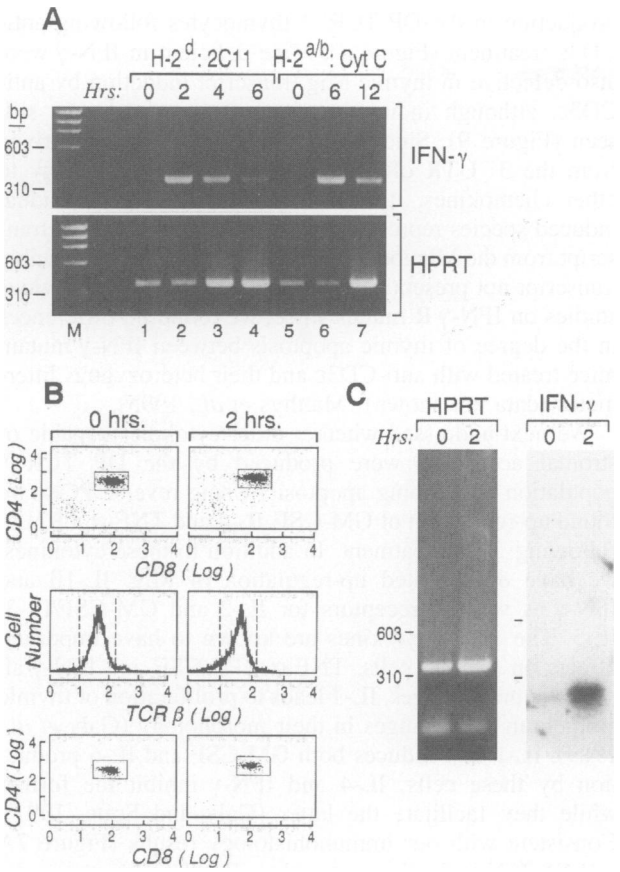

Fig. 8. TCR cross-linking induces IFN-γ in DP TCR^{low} thymocytes. (**A**) Reverse PCR of IFN-γ using 1 μg of total thymic RNA in either ANDtg RAG-2$^{-/-}$ H-2^{d} mice treated with anti-CD3ε for 0, 2, 4 or 6 h or ANDtg RAG-2$^{-/-}$ H-2$^{a/b}$ mice injected with cytochrome *c* for 0, 6 or 12 h. The integrity of the oligo(dT)-primed cDNA was verified by amplification of HPRT cDNA. (**B**) Three-color FACS purification of 1×10^{6} DP TCR^{low} thymocytes from uninjected (0 h) and 2C11-injected (2 h) ANDtg RAG-2$^{-/-}$ H-2^{d} mice. The top panels show the CD4/CD8 gate and the middle panels the TCRβ gate used when sorting thymocytes from the two animal groups. The bottom panels show a re-analysis of CD4 and CD8 expression on the sorted cells. Anti-TCRβ was FITC conjugated H57, anti-CD4 was PE conjugated GK1.5 and anti-CD8 was Red-613 conjugated 53.6. (**C**) Reverse PCR of total thymic RNA from the sorted DP TCR^{low} thymocytes. The HPRT PCR products were detected by EtBr staining of an agarose gel. The IFN-γ PCR products were blotted and probed with a ^{32}P-labelled full-length IFN-γ cDNA clone.

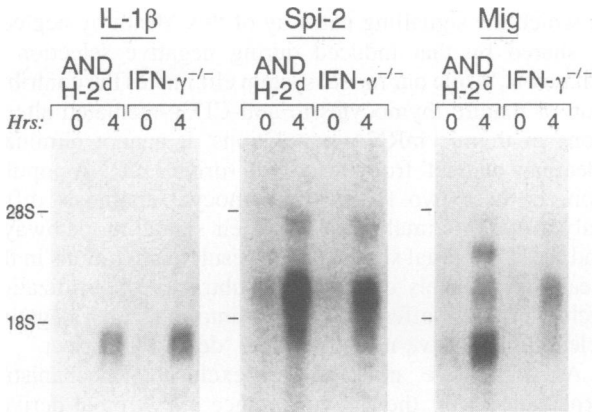

Fig. 9. Mig, but not IL-1β or Spi-2 induction by anti-CD3ε is altered in IFN-γ$^{-/-}$ mutant mice. 5 μg of total thymic RNA from ANDtg RAG-2$^{-/-}$ H-2^{d} or IFN-γ$^{-/-}$ mutant mice prior to or 4 h after 2C11 injection was Northern blotted and hybridized with the indicated probes.

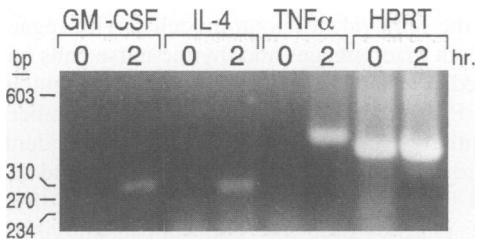

Fig. 10. Cytokines known to activate stromal cells are induced by TCR cross-linking in DP TCR^{low} thymocytes. RNA from the sorted thymocytes described in Figure 8 was analyzed for expression of the indicated cytokines by 40 cycles of reverse PCR. HPRT amplification confirms cDNA integrity.

We therefore sought evidence for other cytokines derived from the DP TCR^{low} thymocytes that might contribute independently to macrophage activation. PCR analysis of cDNAs derived from the control and TCR cross-linked DP TCR^{low} thymocytes revealed that anti-CD3ε treatment induced the synthesis of GM-CSF, IL-4 and TNF-α in this population within 2 h of 2C11 administration *in vivo* (Figure 10).

Discussion

We have identified 14 mRNAs whose steady-state levels are increased upon induction of apoptosis in immature DP thymocytes by CD3ε cross-linking *in vivo*. At least two of the transcripts are derived from the targeted thymocyte compartment; a cAMP phosphodiesterase and CD69. TCR-mediated apoptosis in thymocytes is blocked by agents which raise cAMP, consistent with cAMP's known ability to interfere with TCR-mediated signalling (Lerner *et al.*, 1988; Hoshi *et al.*, 1994). Thus, the up-regulated cAMP PDE transcript levels we observe in cells undergoing TCR-mediated apoptosis may increase the sensitivity of the thymocyte to apoptotic signals. CD69 is a calcium-dependent lectin recently noted to be up-regulated on, but not restricted to, dissociated apoptotic DP thymocytes (Kishimoto *et al.*, 1995).

To our surprise, however, the majority of the transcripts identified are derived from thymic stroma rather than from the targeted thymocyte population. There are several, non-mutually exclusive explanations for this finding which can be categorized as either 'technical' or 'mechanistic'. The first explanation is a technical one: the cellular complexity of the thymic stroma, consisting of macrophages, dendritic cells, epithelial cells, fibroblasts and vascular cells, among others, is high relative to the thymocyte compartment, which is relatively homogenous, particularly in this H-2^{d} (i.e. non-selecting) ANDtg Rag-2$^{-/-}$ model. Thus, we may have cloned anti-CD3ε treatment-induced stromal transcripts simply because there are more of them. The second technical explanation is that the control mRNA (driver) may already contain all the mRNAs necessary for the induction of thymocyte death following strong cross-linking through the T-cell receptor, since the predominant form of cell death within the thymus is that of thymocytes which undergo neither positive nor negative selection (Egerton *et al.*, 1990; Surh and Sprent, 1994). The extent

to which the signalling pathway of this 'death by neglect' is shared by that induced during negative selection is unknown. While our model system eliminates the contribution of mature thymocytes to anti-CD3ε-mediated alterations in thymic mRNA populations, it cannot eliminate 'death by neglect' from the control (driver) mRNA population. If these two forms of thymocyte apoptosis differ only in the proximal portion of their signalling pathways, and these proximal signals fail to result in alterations in the steady-state levels of mRNAs, subtractive hybridization technology will offer little insight into models of negative selection that have not eliminated 'death by neglect.'

An alternative, non-mutually exclusive 'mechanistic' explanation for the preponderance of stromal-derived transcripts induced by anti-CD3ε treatment is that strong TCR cross-linking of the DP TCR^{low} thymocyte population results in thymocyte cell surface receptor up-regulation and cytokine production which activate the surrounding thymic stroma. This activation of stromal cells may play a pivotal role both in the induction of thymocyte apoptosis and in the removal of apoptotic cells. With regard to the latter, both macrophages and thymic nurse cells have been proposed to clear apoptotic thymocytes (Aguilar *et al.*, 1994). Previous studies suggest that a number of the differentially expressed transcripts we have identified in this study (Mig, Spi2, LRG-21, TPA-S1 and IL-1) are derived from tissue macrophages. Apoptotic thymocytes can be phagocytosed by both phosphatidylserine and vitronectin-receptor-mediated mechanisms, depending on the source of the macrophage population studied (Fadok *et al.*, 1992; Verhoven *et al.*, 1995). The degree to which thymocyte-derived cytokines play a role in facilitating this process, both by attracting and activating macrophages, is unclear from these studies.

The appearance of a large number of CD11c-positive cells in the thymic cortex 20 h following anti-CD3ε treatment (Figure 7G and H) is most likely the result of the up-regulation of this integrin α subunit on peripheral blood monocytes by inflammatory mediators such as TNF-α, with subsequent adherence to endothelium and diapedesis (Miller *et al.*, 1987). A number of the other macrophage-derived differentially expressed mRNAs identified in this study are known to be regulated by cytokines which we herein show are up-regulated in the DP population upon 2C11 treatment. IL-1, which can be secreted by macrophages, lymphocytes, dendritic cells and epithelial cells, among others, can be induced by cell–cell contact, IFN-γ, GM-CSF and TNF-α (di Giovine and Duff, 1990). LRG-21, a leucine-zipper-containing transcription factor isolated from a murine macrophage cell line, is induced by IFN-γ and IL-4 (Drysdale *et al.*, 1996). TPA-S1 may represent the murine homologue of TIMP, a tissue inhibitor of metalloproteases whose synthesis is known to be induced by IL-1 (Murphy *et al.*, 1985). Finally, the murine Spi-2 protease inhibitor locus, a multigene family with a hypervariable reactive site domain, is induced by inflammatory stimuli (Hill *et al.*, 1985).

Two cDNA fragments of the chemokine Mig were cloned in this study and Northern analysis demonstrates marked up-regulation of this transcript by 2 h after anti-CD3ε treatment. IFN-γ is the only physiologic stimulus known to induce this mRNA (Farber, 1990). Given this background, we sought and found evidence for IFN-γ production in the DP TCR^{low} thymocytes following anti-CD3ε treatment (Figure 8). Mice deficient in IFN-γ were also defective in thymic Mig transcript induction by anti-CD3ε, although induction of a minor species was still seen (Figure 9). Since the Mig probe used was derived from the 3′ UTR of Mig-1 and shows no homology to other chemokines, it is not clear whether this residual induced species represents an alternatively regulated transcript from the Mig locus or a highly homologous inducible transcript not present in GenBank. In contrast to previous studies on IFN-γ R mutant mice, we found no differences in the degree of thymic apoptosis between IFN-γ mutant mice treated with anti-CD3ε and their heterozygous littermates (data not shown) (Matthys *et al.*, 1995).

We next addressed whether other cytokines capable of stromal activation were produced by the DP TCR^{low} population undergoing apoptosis. Using reverse PCR, we found up-regulation of GM-CSF, IL-4 and TNF-α mRNAs following 2C11 treatment. In addition to these cytokines, we have documented up-regulation of Mig, IL-1β and IFN-γ as well as receptors for IL-2 and GM-CSF/IL-3/IL-5. The above cytokines are known to have important effects on stromal cells. TNF-α, GM-CSF and IFN-γ all activate macrophages. IL-1 leads to proliferation of thymic epithelium and changes in their morphology (Galy *et al.*, 1989). IL-1 also induces both GM-CSF and IL-6 production by these cells; IL-4 and IFN-γ inhibit the former while they facilitate the latter (Galy and Spits, 1991). Consistent with our immunohistology results (Figure 7A and B), IFN-γ also increases class II MHC expression on these cells (Galy *et al.*, 1991). Finally, both IL-1 and GM-CSF are potent activators of dendritic cells (Kampgen *et al.*, 1994).

Thus, cytokines induced in DP TCR^{low} thymocytes studied here are capable of widespread activation of thymic stroma, both within bone-marrow-derived macrophages and dendritic cells, the putative mediators of negative selection, and within thymic epithelium (Miller and Heath, 1993). In addition, the stromal cytokines IL-1β and Mig are likely to 'talk back' to the thymocyte compartment. Are these cytokines an epiphenomenon or could they play a role in thymocyte apoptosis? Several considerations argue in favor of the latter. Thymocytes may require more than TCR ligation by peptide–MHC complexes to undergo apoptosis. A putative second signal derived from thymic stroma in conjunction with a corresponding TCR-triggered alteration in thymocyte cell surface signalling molecules would maintain the antigen/MHC specificity of the negative selection process. Although a variety of cell surface receptor–ligand pairs have been studied such as Fas/FasL, CD28/B7, CD30/CD30L and CD40/CD40L, the role of these molecules in negative selection remains incompletely understood (Punt *et al.*, 1994; Singer and Abbas, 1994; Foy *et al.*, 1995; Amakawa *et al.*, 1996). The induction of signal transduction molecules, cytokines and cytokine receptors we have documented in this study by RDA, immunohistology and reverse PCR suggests a variant model in which thymocyte apoptosis results from a combination of cytokine and cell surface receptor 'cross-talk' between the thymocyte and stromal compartments. Although the specificity of negative selection is likely to be satisfied only by cell surface receptor–ligand interactions, stromal cytokines may modu-

late or conceivably be required for the delivery of the complete stromal 'second signal' inducing thymocyte apoptosis. For example, transient elevations in $[Ca]_i$ levels in thymocytes induced by Mig could synergize with a cell death signal (McConkey *et al.*, 1989; Liao *et al.*, 1995). Analysis of the role of cytokine networks in the regulation of thymocyte selection will be complicated by the likely redundancy of signalling pathways. Nonetheless, this work demonstrates that the impact of cytokine signalling in both the stromal and thymocyte compartments will have to be taken into account in future analysis of the role of second signals in negative selection. In addition, our findings extend the phenomenon of thymocyte–stroma cross-talk (van Ewijk *et al.*, 1994) to include DP thymocytes.

Materials and methods

Animals

A breeding pair of heterozygous AND transgenic mice in an H-2^b background (Kaye *et al.*, 1989) was kindly provided by S.Hedrick (UCSD, La Jolla, CA). These mice were crossed to either B10.A (H-2^a) or B10.D2 (H-2^d) mice (Jackson Laboratories, Bar Harbor, ME). H-$2^{a/b}$ and H-2^d AND transgene positive mice were then crossed to RAG-$2^{-/-}$ H-2^b mice (Shinkai *et al.*, 1992; S.Koyasu and E.L.Reinherz, unpublished results). H-$2^{a/b}$ and H-$2^{b/d}$ AND transgene-positive RAG-$2^{+/-}$ progeny were crossed to RAG-$2^{-/-}$ H-2^b or H-2^d mice (S.Koyasu and E.L.Reinherz, unpublished), respectively, to generate H-$2^{a/b}$ or H-2^d AND transgene positive RAG-$2^{-/-}$ mice. The AND transgene was detected by Vα11 expression in peripheral blood (see below) except in H-2^d RAG-$2^{-/-}$ mice, in which the low numbers of peripheral T cells required PCR of genomic tail DNA (see below). Haplotype and RAG status were determined by staining peripheral blood with haplotype-specific mAbs and anti-mouse Ig, respectively. The H-2^a and H-2^d haplotypes were recognized with 34-2-12S and the H-2^b haplotype with 5F1 (see below). Purified 2C11 was administered as 200 μg of intraperitoneal antibody in 250 μl of PBS. Pigeon cytochrome *c* (500 μg; Sigma) was administered i.v. A full description of the generation of the N15 TCR transgenic H-2^b RAG-$2^{-/-}$ mice will be forthcoming (Y.Ghendler *et al.*, in preparation). The latter TCR is restricted to H-$2K^b$ and is specific for a VSV octapeptide (Shibata *et al.*, 1992). IFN-γ knockout mice were kindly provided by R.Mitchell and P.Libby with permission from T.Stewart (Dalton *et al.*, 1993).

Antibodies and flow cytometric analysis

145.2C11 (anti-CD3ε) was obtained from J.Bluestone; RR8–1 (anti-Vα11.1.2) from O.Kanagawa; H57–597 (anti-Cβ) from R.Kubo; 5F1 (anti-H-$2K^b$) from D.Sachs. 34-2-12S (anti-H-$2D^d$) and GK1.5 (anti-CD4) were from ATCC. J11d (anti-HSA); 53.6 (anti-CD8α); 25-9-17 (anti-I-$A^{b,d}$) and 7D4 (anti-IL-2Rα) were from Pharmingen. ER-TR5 (anti-medullary epithelium), NLDC-145 (anti-cortical epithelium), N418 (anti-medullary dendritic cells; CD11c) and a macrophage 'cocktail' (F4/80, ER-TR9, ER-HR3 and MOMA-1) were all supernatants. Staining was performed in the presence of 10 μg/ml unconjugated 2.4G2 mAb (anti-FcγRII/III) to block Fc receptor-mediated binding of the mAbs. All the antibodies used for FACS analysis were used at a concentration of 10 μg/ml. Antibodies were directly coupled to fluorochromes (see figure legends) or were detected with FITC anti-rat (Caltag) or FITC anti-mouse (Boehringer Mannheim) secondary reagents. Flow cytometry was performed with a FACScan and cell-sorting with a FACS Vantage (Becton-Dickinson). Data were analyzed on a computer with the LYSYS II program.

Immunohistologic analysis and TUNEL assay

Thymuses were frozen in O.C.T. compound (Miles, Elkhart, IN) on dry ice. Immunohistochemical studies were performed as previously described (Mombaerts *et al.*, 1993). For the TUNEL assay, frozen tissue sections were fixed in 3% buffered formalin for 10 min at room temperature. After washing with PBS, sections were fixed again in ethanol/acetic acid (2:1) for 10 min at –20°C. Endogenous peroxidase activity was blocked by covering the sections with 0.5% H_2O_2 for 20 min at room temperature. The sections were rinsed with PBS and immersed in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). The sections were incubated with 2–4 mM biotinylated dUTP (Boehringer Mannheim) and 5–10 U TdT (Promega) in 25 μl TdT buffer in a humidified atmosphere at 37°C for 2 h. The reaction was terminated by transferring the slides to TB buffer (300 mM NaCl, 30 mM Na citrate) for 15 min. After a PBS rinse, sections were incubated with 2% BSA for 10 min, rinsed in PBS for 5 min, incubated with avidin–biotinylated-peroxidase complexes (Dako) diluted 1:100 in PBS for 30 min, washed in PBS and developed with 3-amino-9-ethylcarbazole (Aldrich). Sections were lightly counterstained with Meyer's hematoxylin (Gavrieli *et al.*, 1992).

RNA purification and cDNA synthesis

Immediately after harvesting, thymuses were frozen in liquid nitrogen and then homogenized with a PT 10/35 Brinkmann homogenizer in 4 M guanidium isothiocyanate, 0.5% Sarkosyl, 25 mM Na Citrate, 0.1 M 2-mercaptoethanol. RNA was then pelleted through a CsCl step gradient. Poly(A) RNA was isolated from total RNA by adhesion to oligo(dT)-polystyrene latex particles [Oligotex-(dT)30; Qiagen, Chatsworth, CA]. Double-stranded cDNA synthesis utilized the Gubler–Hoffman modification of Okayama and Berg's technique (i.e. DNA ligase was omitted) (Okayama and Berg, 1982; Gubler and Hoffman, 1983). For RDA, a total of 12 μg of either control poly(A) or RNA from thymuses of mice injected 2, 4 or 6 h earlier with 2C11 (1:1:1 mix) was used as template. The yield, judged by an EtBr plate assay, was 12.5%. The cDNA for RDA was not radioactively labelled; labelled side reactions were utilized to judge cDNA size.

To determine whether the source of cDNA fragments was thymocyte or stromal, thymuses were first ground between the frosted edges of two glass slides. The dissociated cells were used for thymocyte RNA and were 95% Thy-1 positive. The remaining tissue, which is predominantly stromal but may contain residual lymphoid cells, was used for 'stromal' RNA. For RNA from sorted cells, 1×10^6 cells were pelleted, transferred in PBS–2%FCS to an Eppendorf tube, washed in PBS and lysed in Tri-Reagent LS (Molecular Research Center, Cincinnati, OH). 10 μg of glycogen was added to precipitate RNA.

Representational difference analysis (RDA)

A detailed protocol was kindly provided by M.Hubank (Hubank and Schatz, 1993). R-, J- and N-Bgl oligonucleotide pairs were purified on C20 Sep-Pak columns (Waters, Milford, MA). *Dpn*II cut driver and gel-purified tester had an average size of 600 bp. The DP2 product was digested with *Dpn*II, gel purified and ligated to the *Bam* site of pGEM-3 (Promega). The resulting plasmid library was electroporated into XL-1 Blue (Stratagene). Plasmids with inserts were identified by IPTG/X-Gal screening. Sequencing was performed with a Sequenase kit (United States Biochemical, Cleveland, OH). Sequence analysis utilized Intelligenetics software.

Polymerase chain reaction (PCR)

First strand cDNA synthesis was performed (using a kit from Boehringer Mannheim) and was primed with oligo(dT). 1 μg of total thymic RNA or one-half of the RNA derived from 1×10^6 sorted cells was used as template. Assays for cytokine and control mRNAs were designed as follows with oligonucleotide sequences given 5′→3′: IFN-γ was 267 bp (1st base 147; sense = CATTGAAAGCCTAGAAAGTCTG, antisense = CTCATGAATCATCCTTTTTCG) (Gray and Goeddel, 1983). IL-4 was 240 bp (1st base 154; sense = CATCGGCATTTTGAACGAGGTCA, antisense = CTTATCGATGAATCCAGG CATCG) (Lee *et al.*, 1986). HPRT was 352 bp (1st base 575; sense = GTTGGATACAGGCCAG ACTTTGTTG, antisense = GAGGGTAGGCTGGCCTATAGGCT) (Johnson *et al.*, 1988). TNF-α was 383 bp (1st base 383; sense = GTTCTATGGCCCAGACCCTCACA, antisense = TCCCAGGTAATG GGCTCATACC) (Caput *et al.*, 1986). GM-CSF was 254 bp (1st base 82; sense = AATGAAGAG GTAGAAGTCGTCTC, antisense = CATTCAAAGGGGATATCAGTCAG) (Gough *et al.*, 1984). All PCRs were performed for 40 cycles with 1 min at 94°C, 1 min at 60°C and 1 min at 72°C.

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