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Interferon-regulatory factors during development of CD4 and CD8 thymocytes

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SUMMARY

Selection events in the thymus occur at the double-positive $CD4^+CD8^+$ (DP) developmental stage leading either to further differentiation of the $CD4^+$ and $CD8^+$ lineages or to deletion. The interferon-regulatory factor IRF-1 has been implicated in signalling for T-cell death and also in $CD8^+$ thymic differentiation. IRF-1 is an activator and IRF-2 a repressor of gene transcription regulated by type 1 interferons (IFN). To evaluate the role of IRF-1 and IRF-2 in the differentiation of CD4 and CD8 thymocytes, we analysed their DNA-binding activity before and after antigenic stimulation at different stages of thymic development and in peripheral T cells. Unseparated, double-positive and single-positive thymocytes as well as peripheral T lymphocytes from mice transgenic (tg) for a T-cell receptor (TCR), restricted either by major histocompatibility complex class I or class II, were stimulated by their nominal antigen. Our results demonstrate that the DNA-binding activity of IRF-2 and, weakly, that of IRF-1 are inducible in total thymocytes in response to antigen. There is no induction of IRF-1/IRF-2 binding activity at the double-positive stage of thymic development in the MHC class II-restricted model whereas in the MHC class I-restricted model IRF-1/IRF-2 activity is induced weakly. At the single-positive stage, antigen induces the IRF-1/IRF-2 DNA binding in both $CD4^+$ and $CD8^+$ thymocytes, but not in mature lymphocytes from the periphery. This pattern of expression suggests that IRF-1/IRF-2 binding activities resulting from antigen stimulation are developmentally regulated. No evidence for a selective role of IRF-1 in the development of the $CD8^+$ lineage was found, however.

INTRODUCTION

Elimination of self-reactive thymocytes occurs by a process in which immature thymocytes, characterized as expressing both CD4 and CD8 co-receptors [double positive (DP)], are clonally deleted through apoptosis after antigenic stimulation.[1] DP thymocytes are also subjected to positive selection and commitment to either the $CD4^+8^-$ [CD4 single-positive (SP)] T helper cell or the $CD4^-8^+$ (CD8 SP) cytolytic T-cell lineage (reviewed in ref. 2). Since unselected DP thymocytes also die in the thymus,[1] the DP population is clearly the key control point for thymic selection events. Several questions remain unanswered concerning the regulation of clonal deletion of DP thymocytes. What is the intracellular signalling involved in programmed cell death during negative selection? Which effector molecules execute the deletion? How are they regulated?

In fibroblasts, interferon regulatory factor-1 (IRF-1) has been suggested to play a role in apoptosis. IRF-1 is required for the induction of apoptosis following DNA damage or culture in low serum concentration in fibroblasts carrying an activated *c-Ha-ras* gene.[3] Furthermore, in mature T lymphocytes, mitogens induce interleukin-1β converting enzyme (ICE), a mammalian homologue of the *Caenorhabditis elegans* cell death gene *ced-3*,[4] and this induction is IRF-1 dependent.[5] Finally DNA-damage-induced apoptosis is dependent on IRF-1[5] in these cells. The reduction of $CD8^+$ SP cells in mice lacking expression of IRF-1 further suggests a role for this factor in T-lymphocyte development.

Conditions which activate the DNA binding of IRF-1 and IRF-2 in immature T cells are largely unknown. To understand the involvement of IRF-1 and IRF-2 in thymic development we analysed the binding activity of IRF-1 and IRF-2 in unseparated thymocytes, DP and SP thymocytes as well as peripheral T lymphocytes from mice transgenic (tg) for a T-cell receptor (TCR) restricted either by major histocompatibility complex (MHC) class I or II.

Transcription factors of the IRF family have a conserved DNA-binding domain that binds to the interferon (IFN)-stimulated response element (ISRE) present in the promoter regions of many genes regulated by type-1 IFN (α,β). Genes containing the ISRE include type 1 IFN genes (reviewed in ref. 6), MHC class I, interleukin-4 (IL-4), IL-5, IL-7 receptor and p53.[6] Two members of this family, IRF-1 and IRF-2, are highly homologous to one another in their N-terminal region, which confers DNA-binding specificity. Gene transfection

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studies have shown that IRF-1 can function as an activator, inducing transcription from promoters containing ISRE.[7] In contrast, IRF-2 antagonizes the function of IRF-1 by competing for the same binding site.[8,9]

Our results demonstrate that IRF-1 and IRF-2 binding activities are inducible in thymocytes when stimulated by a phorbol ester plus Ca^{++} ionophore or through the TCR with antigen. The band obtained in mobility shift assays is mainly composed of IRF-2; IRF-1 contributing only a small part of the shift. Antigen weakly induces IRF binding in DP thymocytes from the MHC class I-restricted TCR tg model, but not in DP cells from the MHC class II-restricted model. At the SP stage, antigen induces the IRF-1/IRF-2 activity in $CD4^+$ and $CD8^+$ thymocytes, whereas mature lymphocytes ($CD4^+$ and $CD8^+$) from the periphery do not show increased IRF-1/IRF-2 binding activity in response to antigen. These results document the developmental regulation of IRF-1/IRF-2 DNA binding in response to antigen in the CD8 and CD4 lineages but fail to demonstrate a selective role for IRF-1 in the CD8 lineage.

MATERIALS AND METHODS

Mice

Mice transgenic for the KB5.C20 TCR with specificity for the H-2K^b alloantigen (K^b-TCR-tg) have been described elsewhere.[10] Mice transgenic for the TCR-recognizing hen egg lysozyme (HEL-TCR-tg) were a kind gift from Dr Mark Davis (Stanford University School of Medicine, CA).[11] The HEL-TCR is specific for the HEL peptide 46–61 presented by I-A^k. Both mouse strains were on the B10.BR background.

Thymocytes

Single cell suspensions of thymocytes were obtained by gentle disruption of an intact thymus. DP and SP T cells were purified by negative selection using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously.[12] For the purification of CD4 SP thymocytes from HEL-TCR-tg mice, biotinylated anti-CD8 monoclonal antibodies (mAb) were used, followed by streptavidin–fluorescein isothiocyanate (FITC) and MACS biotin beads. The desired population was purified by negative selection using MACS. The thymic SP $CD4^+$ population was highly enriched (approximately 75% SP). As compared to the purification of $CD8^+$ SP thymocytes (approximately 85% SP), there was a slightly higher contamination of the $CD4^+$ SP population with DP thymocytes (10% DP compared to 5% DP in the $CD8^+$ population). For peripheral $CD4^+$ cells, lymph node cells were incubated with biotinylated anti-CD8 and biotinylated anti-B220 antibody, then with streptavidin–FITC and subsequently with MACS biotin beads. After sorting on the MACS by negative selection the peripheral CD4 SP population contained >90% $CD4^+$ cells and <1% B cells.

In vitro *stimulation*

In vitro stimulation of thymocytes was performed as described previously.[13] Thymocytes from K^b-TCR-tg mice were added to L-fibroblasts expressing or not the H-2K^b antigen. The HEL-TCR-tg thymocytes were added to I-A^k-expressing L-fibroblasts in the absence or presence of 100 nM HEL peptide 46–61. Stimulation with antigen was carried out at 3×10^6 thymocytes/ml for 9–12 hr. Stimulation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) and 1 μM ionomycin (Calbiochem, La Jolla, CA) was carried out for 3 hr.

Nuclear extract preparation

Nuclear extracts from thymocytes were prepared as described previously.[12] Briefly, $5 \times 10^6 - 5 \times 10^7$ thymocytes were washed with phosphate-buffered saline (PBS) and resuspended in lysis buffer (containing leupeptin). The nuclei were pelleted, and to the supernatant was added 50 μl of nuclear resuspension buffer (containing leupeptin). The nuclei were resuspended in nuclear resuspension buffer and sonicated briefly. Cytoplasmic and nuclear extracts were cleared by centrifugation. Protein concentrations in the extracts were determined by the Bradford assay (Bio-Rad protein assay; Bio-Rad laboratories, Richmond, CA). Equivalent amounts of extracts were used for *in vitro* binding assays.

Oligonucleotides

Single-stranded oligonucleotides for IRF-1 and IRF-2 were fourfold repeated hexamers of the sequence (AAGTGA) contained in the promoter of genes such as murine H-2D^d and Fas-ligand.[14] The putative binding sequence on the ICE promoter[5] is also a consensus sequence for IRF-1 and IRF-2 but less frequently used than the above[15] and differing in one nucleotide (AAGTCA). Oligonucleotides were synthesized at the facilities of the Centre d'Immunologie as shown below:

IRF-1/IRF-2
5′ TTCACTTTCACTTTCACTTTCACT
AGTGAAAGTGAAAGTGAAAGTGAA
AP-1 (Col-Tre)
5′ AGCTTAAAGCATGAGTCAGACACCT
ATTTCGTACTCAGTCTGTGGACTTAA

Annealed IRF oligonucleotide was end-labelled with [γ-^{32}P]ATP in a T4 polynucleotide kinase reaction (Biolabs, NE). Annealed AP-1 was labelled by polymerization with α-^{32}P-labelled ATP with AMV reverse transcriptase (Promega, St. Louis, WI).

Electrophoretic mobility shift assay

Binding assays were carried out by incubating the labelled DNA (20 000 c.p.m.) with 2·5–5 μg of nuclear proteins and 1 μg of poly(dI-C), a concentration found to be optimal for IRF binding in preliminary assays, in a buffer containing 10 mM Tris–HCl pH 7·5, 50 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 5% glycerol, 1 mM dithiothreitol (DTT). After 30 min at room temperature, the reaction mixture was loaded on to a 4% polyacrylamide gel in 0·25 × TBE buffer and electrophoresed. Gels were dried and exposed to a Fuji RX film at −70°. Quantification was performed with a Fuji Bio-Imaging phosphorimager BAS 1000. For supershift experiments antisera were added to the nuclear extracts 30 min prior to labelled probes. Anti-IRF-1 and anti-IRF-2 antisera were kindly provided by Drs Tadatsugu Taniguchi and Masahiko Ishihara (Institute for Molecular and Cellular Biology, Osaka).

RESULTS

The IRF complex induced in thymocytes is composed of IRF-2 and IRF-1

We first verified that we could detect DNA-binding activity of IRF-1 and IRF-2 in a murine T-cell line treated with IFN-γ in conditions known to increase the expression of MHC class I proteins. Binding activity was clearly detected in nuclear extracts and only weakly in cytoplasmic extracts (data not shown).

Next we tested whether binding activity could be detected in activated thymocytes. Thymocytes from K^b-TCR-tg mice were treated with PMA/ionomycin for 3 hr and the identity of the band detected in the nuclear extracts was assessed using anti-IRF-1 and anti-IRF-2 antibodies in supershift experiments. The band was composed of IRF-1 and IRF-2, the latter being the major component (Fig. 1). However, the DNA–protein complexes formed by either oligonucleotide + IRF-1 or oligonucleotide + IRF-2 overlapped in gel shift assays. Addition of both antisera substantially decreased the intensity of the IRF-1/IRF-2 band (data not shown). As we know, neither titre nor affinity of the antisera nor whether there is cross-reactivity between the IRF-1 and IRF-2 antisera, we cannot exclude that the remaining band after supershifting contains more than just IRF-1 and IRF–2.

IRF-1 and IRF-2 binding activity in the CD8 lineage

Considering that mice deficient in IRF-1 have an impaired development of CD8 cells, it was of interest to determine whether IRF-1 binding activity is differentially induced during the development of CD8 cells. We therefore used thymocytes from K^b-TCR-tg mice. The majority of thymocytes carrying the K^b-TCR on a positive selection background (mice are of the H-2^k haplotype) develop into MHC class I-reactive $CD8^+$ mature T cells. When tg DP thymocytes encounter their nominal antigen K^b *in vitro*, they up-regulate activation markers such as CD69 and undergo clonal deletion.

In K^b-TCR-tg thymocytes the binding activity of IRF-1/IRF-2 was increased twofold after stimulation with K^b antigen (Fig. 2). As a control of stimulation, AP-1 activity was measured in the same extracts (Fig. 2) and a sixfold increase was observed after antigenic stimulation.

To better define the developmental regulation of IRF-1 and IRF-2, thymic immature DP, mature thymic $CD8^+$ and peripheral $CD8^+$ populations were purified. DP and mature SP thymocytes showed increased binding activity for AP-1 (32-fold and sevenfold, respectively) after culture for 15 hr with K^b-fibroblasts (Fig. 2). However, IRF-1/IRF-2 binding activity was only weakly induced by antigen in DP thymocytes (three independent experiments were performed; they showed no increase, 1·7-fold and twofold increase of IRF activity whereas the AP-1 activity increase was around 30-fold in the three experiments). However, there was an increase in IRF-1/IRF-2 binding activity at the SP stage for tg $CD4^-CD8^+$ thymocytes stimulated with K^b (Fig. 2). The basal level of thymic IRF activity was higher in mature thymic $CD8^+$ as compared to immature DP thymocytes (Fig. 2). The capacity of mature thymocytes to respond to antigen by increased IRF activity was lost again at the mature SP stage in the periphery. Purified $CD8^+$ cells from the lymph nodes did not respond to their nominal antigen by increased binding activity of IRF-1 or IRF-2. In the same extracts an increase in AP-1 activity was observed. However, this increase was only twofold, significantly less than in the immature thymic populations. It is possible that a response from mature peripheral T cells is more dependent on stimulation engaging a full set of costimulatory molecules such as CD28 and CD40L. Stimulation with PMA/ionomycin or coated anti-CD3 antibody was able to induce an IRF activity in the peripheral and in the DP population (Fig. 4 and data not shown for anti-CD3). In normal mice, coated anti-CD3 antibody induced

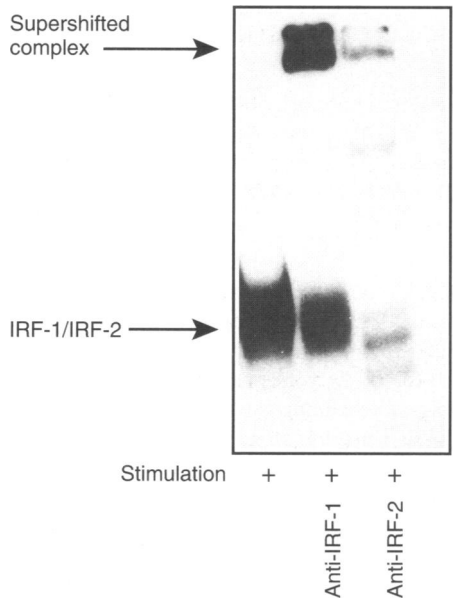

Figure 1. Thymocytes from K^b-TCR-tg mice were stimulated in the presence of PMA and ionomycin for 3 hr, nuclear extracts prepared, and analysed by gel shift assay. The same amount of labelled oligonucleotides was added in the binding assay for each lane as could be judged by the amount of free probe (not shown). Anti-IRF-1 or anti-IRF-2 antisera were added during the binding assay.

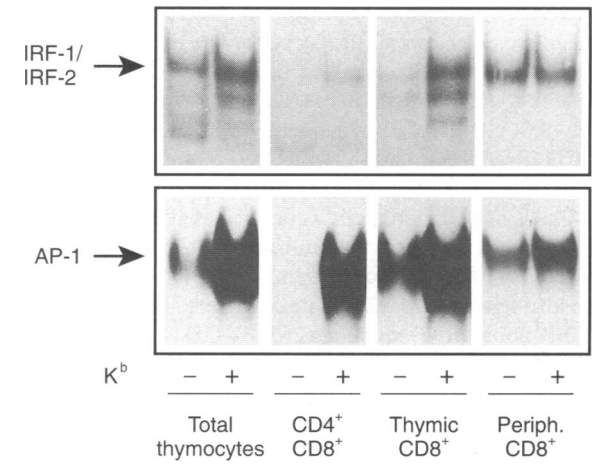

Figure 2. Unseparated, purified $CD4^+CD8^+$ and $CD8^+$ thymocytes as well as purified peripheral $CD8^+$ cells from K^b-TCR-tg mice were stimulated in the presence of L fibroblasts expressing (+) or not (−) H-$2K^b$ for 12 hr. The same nuclear extracts were analysed in gel shift assays for IRF and AP-1 oligonucleotide binding.

similar amounts of IRF activity in peripheral purified $CD4^+$ and $CD8^+$ lymphocytes.

IRF-1 and IRF-2 binding activity in the CD4 lineage

Next we asked the question whether IRF-1 and IRF-2 activity was regulated during the development of the CD4 lineage in a similar fashion to that observed in the CD8 lineage. We therefore analysed the IRF-1/IRF-2 binding activity in a transgenic TCR system restricted by MHC class II. Here, thymocytes carrying the transgenic TCR recognize a HEL peptide and develop into MHC class II-restricted $CD4^+$ T cells.

In the HEL-TCR-tg system the expression of the activation marker CD69 as tested by FACS analysis was increased on 50% of DP thymocytes cultured in the presence of L-I-A^k cells + peptide (data not shown). This up-regulation of CD69 on DP cells is significantly higher than in the K^b-TCR-tg mice. Furthermore, in a representative experiment, propidium iodide staining showed that 50% of thymocytes died when cultured in the presence of 100 nM HEL peptide as opposed to 28% in the absence of peptide. The extent of antigen-induced death of DP thymocytes in the HEL-TCR-tg system was comparable to that in the K^b-TCR-tg system.

As for the IRF activity, unseparated thymocytes from HEL-TCR-tg mice showed a twofold increase in the presence of peptide (quantified by phosphorimager), as compared to L-IA^k cells without peptide (Fig. 3). Separated DP thymocytes showed no increase in IRF activity, purified SP $CD4^+$ thymocytes increased their IRF activity 1·5-fold after culture with antigen. Finally, the peptide expressed on L-I-A^k cells was not able to induce IRF binding activity in peripheral $CD4^+$. However, in the same extracts AP-1 was induced sixfold in the total thymic population, sevenfold in the DP, fourfold in the thymic SP and twofold in the peripheral $CD4^+$ cells after stimulation with antigen (Fig. 3). A basal level of IRF-1/IRF-2 binding was observed in the SP $CD4^+$ thymocytes. Also at the DP stage, the basal level of DNA-binding was higher in the HEL-TCR-tg model than in the K^b-TCR-tg model. It is not clear whether this results from a partial contamination of the DP by the SP thymocytes or from intrinsic differences between DP thymocyte populations in which the majority are bound to differentiate to $CD4^+$ as compared to $CD8^+$ thymocytes.

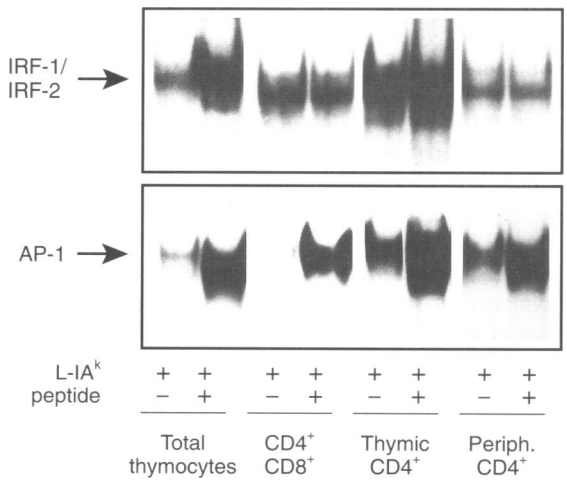

Figure 3. Unseparated, purified $CD4^+CD8^+$ and $CD4^+$ thymocytes as well as purified peripheral $CD4^+$ cells from HEL-TCR-tg mice were stimulated in the presence of I-A^k transfected fibroblasts in the presence (+) or absence (−) of HEL peptide for 12 hr. The same nuclear extracts were analysed in gel shift assays for IRF and AP-1 oligonucleotide binding.

IRF-1 only contributes weakly to the major band shifted after stimulation in thymocytes and mature lymphocytes

It was reported that the bands for IRF-1 and IRF-2 comigrate in band shift assays for a wide variey of ISRE probes.[16] To be able to differentiate between IRF-1 and IRF-2 after stimulation, supershift experiments on stimulated thymocytes and mature T lymphocytes were carried out in the presence of antisera directed against IRF-2 (Fig. 4). For experimental reasons (restricted amounts of antisera and nuclear extracts) the supershifts of the different subpopulations were carried out systematically with PMA/ionomycin stimulation and anti-IRF-2 antisera.

Total thymocytes stimulated by PMA/ionomycin showed a strong IRF-2 binding activity and a weak IRF-1 activity (Figs 1 and 4). The majority of the band obtained in thymocytes at all stages analysed after stimulation with PMA/ionomycin was composed of IRF-2, whereas IRF-1 (the remaining band after addition of anti-IRF-2) and possibly some other oligo-binding material only represents 20% to 30% of the band. (Fig. 4). Comparable results were obtained in the K^b-TCR-tg system (data not shown).

DISCUSSION

Factors binding to the IRF-1/-2 consensus motif have not previously been analysed in antigen-stimulated thymocytes or T cells. Here we study the DNA binding of IRF-1 and IRF-2 at different developmental stages of T cells restricted by MHC class I or class II before and after stimulation with antigen. We observed that the two DNA–protein complexes, including IRF-1 or IRF-2, co-migrated in gel shift assays in thymocytes and mature T cells. We found that the binding activity of IRF-1/IRF-2 is regulated during thymic development in uninduced and antigen-stimulated thymocytes. Only mature SP thymocytes consistently showed increased IRF-1 and IRF-2 binding activity in both CD4 and CD8 lineages upon stimulation with antigen whereas in DP immature thymocytes antigen did not (or only weakly in the MHC class I-restricted system) induce this activity. Moreover, peripheral T lymphocytes were not inducible for IRF activity with antigen whereas in the same extracts AP-1 activity was induced. Finally, supershift experiments indicated that the ratio IRF-1/IRF-2 is low and similar at the different thymic developmental stages and for the two lineages.

Our results confirm an earlier study showing that independently of the DNA binding probe used, there is co-migration of shifted IRF-1 and IRF-2.[16] Both factors are expressed at low constitutive levels, but the IRF-2 protein is more stable and accumulates at a higher level.[17,18] This is consistent with our finding of a high IRF-2/IRF-1 ratio observed after supershifting the bound complex with specific antibodies. More IRF-2 than IRF-1 DNA binding even after stimulation would mean that the transcription of ISRE-dependent genes is

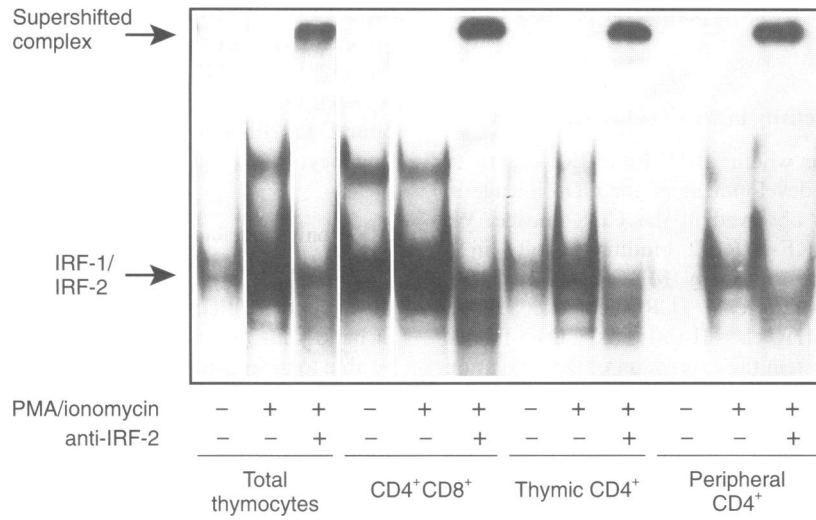

Figure 4. Unseparated, purified $CD4^+CD8^+$ and $CD4^+$ thymocytes as well as purified peripheral $CD4^+$ from HEL-TCR-tg mice were stimulated in the presence of PMA/ionomycin for 3 hr. Nuclear extracts were prepared and analysed by gel shift assay. Where indicated, anti-IRF-2 antiserum was added to the binding assay.

repressed in antigen-induced thymocytes. It has been suggested for the IFN-β promoter however, that IRF-2 may function as a repressor in uninduced cells but becomes an activator after treatment of cells with double-stranded RNA.[19,20] Moreover, in IRF2 mice the lack of the repressor IRF-2 does not cause constitutive type I IFN gene expression.[21] Whether ISRE-controlled genes in thymocytes are transcribed upon stimulation with antigen could be clarified by transfection of a reporter construct under the control of the ISRE region in these cells.

Previous data had suggested a role for IRF-1 in thymocyte development in view of the strong and selective reduction of $CD8^+$ in IRF-1 mice.[21,22] Matsuyama *et al.* stated that this asymmetrical defect was not due to a difference in MHC class I expression between the wild-type and IRF-deficient mice. Recently however, it was suggested that the reason for the paucity of $CD8^+$ in these mice is the reduction of TAP1 and LMP2 expression regulated by IRF-1.[23] TAP1 and LMP2 are central for MHC class I function and expression. Reduced MHC class I expression together with the lack of LMP2 in IRF-1 mice may lead to reduced and/or altered peptide presentation in the thymus and impaired thymic selection. Our data are consistent with the notion that the paucity of $CD8^+$ cells in IRF-1 mice is not due to a difference in IRF-binding activity between $CD4^+$ and $CD8^+$ thymocytes but rather to an altered gene expression regulated by IRF-1 and IRF-2 in thymic cells other than thymocytes.

The question of a possible role of IRF members in thymic negative selection was also approached in view of the numerous reports connecting IRF-1 with the regulation of cell death. These include the presence of an IRF binding sequence in the 5' regulatory region of the ICE[5] and the Fas-ligand[24] gene. However, mice devoid of ICE[25] or functional Fas-ligand[26] do not show any defect in thymic negative selection. Our results show that upon antigen-induced deletion of DP thymocytes *in vitro* no, or only weak IRF-1/IRF-2 binding activity was induced (in conditions in which the activation of AP-1 DNA-binding was clearly observed). This result is consistent with the absence of involvement of IRF-1/IRF-2 in thymocyte negative selection.

Recently, new members of the IFN regulatory family such as ICSBP[27] and LSIRF[28] have been cloned and shown to be lymphoid specific. ICSBP protein is undetectable in thymocytes and resting T cells but expressed in anti-CD3- or ConA-stimulated mature T cells.[29] This factor might be responsible for the control of ISRE-regulated genes in mature T cells where IRF-1 protein is only weakly expressed[29] and not activated by antigenic stimulation (this study). The same accounts for LSIRF, another member of the IRF family recently cloned. LSIRF is induced mainly by antigen-receptor-mediated stimuli rather than by IFN, and its expression is confined to the cells of the lymphoid lineage although it is not found in immature T cells.[28] Thus both factors seem to play a role in T-cell effector function rather than T-cell development.

In summary our study clearly shows that there is a regulation of IRF activity during thymic development, the mature SP thymocyte, $CD4^+$ or $CD8,^+$ being the only stage where IRF binding activity can be induced by antigen. It remains to be clarified whether the SP thymocytes represent a distinct population in their IRF binding activity from mature T lymphocytes, or whether this result merely reflects different requirements for stimulation for the two populations such as the level of antigen or costimulatory molecules.

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