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Egr2 Is Required for Bcl-2 Induction during Positive Selection

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The repertoire of TCR specificities is established by a selection process in the thymus, during which precursor survival and maturation is dictated by the nature of the TCR signals. The differences in signals that determine whether precursors will survive and mature or be induced to die remain poorly understood. Among the molecular effectors involved in executing the differentiation process initiated by TCR-ligand interactions is a family of Zn-finger transcription factors termed early growth response genes (Egr). Indeed, ablation of the Egr1 gene impairs ligand-induced maturation (positive selection) but not ligand-induced deletion (negative selection). The partial impairment of positive selection by Egr1 deficiency is not enhanced by simultaneous deletion of another Egr family member, Egr3. Accordingly, we asked whether results from compensation by another family member, Egr2. In this manuscript, we demonstrate that deletion of Egr2 impairs positive selection of both CD4 and CD8 single-positive thymocytes. Interestingly, many of the genes involved in positive selection and T cell differentiation are up-regulated normally in the Egr2-deficient thymocytes. However, Bcl-2 up-regulation is not sustained during late stages of positive selection. This defect is at least partially responsible for the developmental blockade in Egr2-deficient thymocytes, as enforced expression of Bcl-2 rescues T cell development in Egr2−/− thymocytes. Taken together, these data suggest that Egr2 plays a central role in the up-regulation of the survival molecule Bcl-2 during positive selection. The Journal of Immunology, 2008, 181: 7778–7785.

During T cell development, the organism generates a T cell population with an extended repertoire of Ag specificities. This repertoire is molded in the thymus through signals derived from the interaction of TCRs on thymocytes with their ligands, MHC molecules with bound peptides. The majority of thymocytes bear TCRs that do not recognize the MHC molecules present in the thymus, and these cells die relatively rapidly (3–4 days). Those cells bearing a TCR able to interact with self-MHC can receive signals that induce their differentiation into mature T cells (positive selection) or apoptosis (negative selection). Furthermore, those cells that are positively selected develop into two different lineages, CD4 or CD8, depending on the ability of their TCRs to bind MHC class II or I, respectively. Most of what we understand about these processes has been learned using genetically modified mice, especially mice engineered to express a rearranged transgenic (Tg) TCR. The majority of double positive (DP) thymocytes in these mice express the same TCR and, therefore, behave as a relatively homogeneous population. Breeding TCR Tg mice to mice with alterations in different signaling molecules has provided most of our current knowledge regarding the role of different signal transduction pathways in the regulation of the different cell fate decisions during T cell development.

One of the first pathways identified as important for positive selection during T cell development was the Ras/Erk cascade (1). The immediate/early gene Egr1 has been proposed as one downstream effector of Erk during positive selection. Egr-1 is one of the earliest transcription factors expressed after TCR stimulation on DP thymocytes, and its induction is Erk dependent (2). Furthermore, mice overexpressing Egr1 have more efficient positive selection of mature CD4 and CD8 thymocytes even in genetic backgrounds that are very weakly selecting (3). Although the initial analyses of Egr-1 null mice did not reveal apparent defects in T cell development (4), a more recent study examined thymocyte development in Egr1-deficient mice in more detail using three different Tg TCR backgrounds (5). This study suggests that Egr-1 is important for positive selection of both CD4 and CD8 cells. The lack of an observable effect on positive selection in the original Egr1−/− report may have been due to partial compensation by other members of the Egr family.

There are four Egr genes (Egr1–4) expressed during thymocyte differentiation (2) (D. L. Wiest, unpublished observations). Egr proteins contain highly conserved zinc-finger DNA-binding domains that can bind a number of common target gene promoters and potentially cooperate in regulating their expression (6). In thymocytes, Egr1, Egr2, and Egr3 are induced by pre-TCR signaling and overexpression of these proteins in pre-TCR signaling-deficient thymocytes can partially facilitate progression through the β-selection checkpoint (7) Conversely, dominant-negative Egr proteins inhibit progression from DN3 to DN4 in vitro (7). Similarly, Egr1, Egr2 and Egr3 can be induced by TCR signals in a DP thymoma-derived cell line (2). In vivo, Egr1-deficient thymocytes have a small but significant defect in positive selection but have no apparent defect in negative selection (5). In contrast, Egr3-deficient thymocytes have a partial block at the DN3 stage that is...
largely explained by reduced proliferation of DNA thymocytes (8, 9). Simultaneous abrogation of Egr1 and Egr3 function in developing thymocytes results in a severe thymic atrophy not seen in mice lacking only Egr1 or Egr3. In contrast, the defects in positive selection were not more pronounced in Egr1/3 double knockout (KO) mice than in Egr1 single KO(9). This suggested to us that Egr2 could be more involved at this stage of T cell development.

We have explored this possibility using conditional Egr2 KO, and our results indicate that deletion of Egr2 in DP thymocytes results in a significant reduction in the generation of both CD4 and CD8 single-positive (SP) thymocytes without affecting negative selection. Interestingly, many of the genes known to be involved in positive selection and T cell differentiation are up-regulated normally in the Egr2 null thymocytes. However, Bcl-2 up-regulation is not sustained late in this process, and, using rescue experiments, we show that forced expression of Bcl-2 can rescue the defect in positive selection in Egr-2 thymocytes, suggesting that a central role of this transcription factor during positive selection is the up-regulation of the survival molecule Bcl-2.

Materials and Methods

Mice

All mice were maintained in the Association for Assessment and Accreditation of Laboratory Care-accredited animal colonies of the Fox Chase Cancer Center and Oklahoma Medical Research Foundation and were handled in compliance with guidelines established by the Institutional Animal Care and Use Committees. Both conventional Egr2-deficient (Krox20-deficient; Krox20+/-) mice (10) (provided by T. Gridley, Jackson ImmunoResearch Laboratories, West Grove, PA) and floxed conditional Egr2-deficient (Egr2fl/fl) mice (11) (a gift of Patrick Charnay, Institut National de la Santé et de la Recherche Médicale, Paris, France) were imported into our laboratory animal facility by embryo rederivation. Deletion of floxed Egr2 alleles was accomplished through crossing to lck-Cre Tg mice (purchased from Jackson ImmunoResearch Laboratories) either alone or in conjunction with AND (12) or H-Y TCR Tg mice (13) (provided by Dietmar la Sante and de la Recherche Médicale, Paris, France) were imported into our laboratory animal facility by embryo rederivation. Deletion of floxed Egr2 alleles was accomplished through crossing to lck-Cre Tg mice (purchased from Jackson ImmunoResearch Laboratories) either alone or in conjunction with AND (12) or H-Y TCR Tg mice (13) (provided by Dietmar Kappe, Fox Chase Cancer Center, Philadelphia, PA). B6-LYS.2/Cr congeneric mice were obtained from the National Cancer Institutes Frederick animal production program.

Flow cytometry

Thymic lobes were gently ground with a syringe plunger in PBA (PBS, 1% BSA, and 0.02% NaN3) to produce a single-cell suspension, which was filtered through mesh, dispensed into a round-bottom microtiter plate (1 × 107–1 × 108 cells per well), and stained and analyzed as described previously (14). Fluorochrome-coupled Ab were purchased from BD Pharmingen and eBioscience. Cells were stained with the indicated commercially prepared Ab, and samples were analyzed using a BD LSR cytometer (BD Pharmingen) and FlowJo Software (Treestar).

Bcl-2 up-regulation

For Bcl-2 staining, thymocytes were isolated from Egr2fl/fl-Cre or Egr2fl/fl mice and cultured for 20 h in plates coated with or without 1 μg/ml anti-CD3 (145.2C11). Cells were harvested and surface stained for CD69 expression followed by intracellular Bcl-2 staining using the BD Cytofix/ Cytoperm kit. Subsequently, the cells were analyzed for CD69 and Bcl-2 expression by flow cytometry, as described above.

Fetal thymic organ culture

At 13.5 dpf, fetuses were harvested from pregnant Krox20+/- females and cultured on top of filter discs on gelfoam in a conventional fetal thymic organ culture (FTOC) system described previously (15) and cultured for the indicated time before analysis by flow cytometry as described above.

In vitro Ab stimulation

For negative selection studies, CD4+8+ DP thymocytes from AND Tg Egr2fl/fl and Egr2fl/fl-Cre mice were purified by cell sorting and plated at 1 × 106/ml on control-coated plates or plates coated with anti-CD3 (10 μg/ml) plus anti-CD28 (50 μg/ml). After incubation for 20 h at 37°C, the fraction of cells surviving was determined by flow cytometry and staining with annexin. Survival cells were defined as those that were negative for both annexin and propidium iodide staining.

Knockdown of Egr2 delays the generation of both CD4 and CD8 T cells. However, Bcl-2 up-regulation is not sustained late in this process, and, using rescue experiments, we show that forced expression of Bcl-2 can rescue the defect in positive selection in Egr-2 thymocytes, suggesting that a central role of this transcription factor during positive selection is the up-regulation of the survival molecule Bcl-2.

FIGURE 1. Egr2 deficiency impairs development of fetal TCRhi SP thymocytes. A, A schematic of the intermediates of positive selection defined by changes in expression of TCR and CD69 is depicted on the left. The expression level of Egr2 mRNA in TCRhiCD69int and TCRhiCD69low intermediates from C57BL/6 and in TCRhiCD69hi cells from MHC-I/II double-deficient mice was measured in triplicate by real-time PCR and normalized to β-actin. The fold induction of CD69 mRNA expression in C57BL/6 intermediates is depicted graphically on the right side of the panel. B, Fetal thymic lobes explanted at day 14 of gestation were cultured on filters at the air:medium interface for 14 days following which thymocyte development was assessed by flow cytometry using the indicated Abs. Analysis was conducted on a minimum of three lobes per genotype. Gate frequencies are listed on the histograms. Results are representative of two experiments performed.

Sorting

The cell populations used in Fig. 1A were isolated from the indicated mice after staining with fluorescently labeled Abs against CD4, CD8, CD69, and TCR (BD Pharmingen, and eBioscience). Thymocytes were stained with Abs against CD69 (FITC), TCR β (PE), CD4 (PE-Cy5, CyC), and CD8 (allophycocyanin) and sorted for the populations indicated in the figure. Three- and four-color sorts were performed on a FACSDiva (BD Biosciences). The purity of all sorted populations was above 95%, except that the CD69-PE Ab became photobleached during the sort giving an apparent CD69 down-regulation on reanalysis.

Isolation of RNA

RNA was isolated from cells using Qiagen RNeasy mini kit (cat no. 74104) according to the manufacturer’s protocol with minor modifications. Briefly, the cells were homogenized and lysed using QIAshredder columns (Qiagen cat no. 79654) in 1 ml/l × 106 cells of RLT lysis buffer provided with the RNeasy mini kit and stored at −80°C until ready for RNA isolation. Samples were analyzed for quantity and quality by spectrophotometry (A260/280 ratio), agrose gel electrophoresis, and RNA bioanalysis (Agilent technologies 2100 Bioanalyzer). RNA samples exhibiting very high purity with...
no detected degradation of 18S or 28S rRNAs were used for preparation of cDNA for real-time PCR.

Relative quantitation of gene expression by real-time PCR
cDNA was synthesized from 2 µg total RNA purified as described above using TaqMan reverse transcription reagents (Applied Biosystems, cat no. n808-0234). Gene expression was analyzed by TaqMan real-time PCR using commercially prepared primers and probes (Applied Biosystems). Reactions were performed in triplicate for each gene, and expression was normalized to β-actin expression. Quantitation was done using the comparative C<sub>T</sub> method as described by ABI 7500 system SDS software.

Retroviral production and hematopoietic cell infection
Retroviral constructs in the MIG vector (16) were cotransfected along with the pCL/Eco plasmid into 293 cells as described (17). For viral transduction, 1–2 × 10<sup>6</sup> cells were mixed with 2 ml of retroviral supernatant plus 20 µg/ml Polybrene per well in 24-well culture plates and centrifuged for 1 h, at 210 × g, 21°C, as described (18). Supernatant was replaced with fresh medium. Cells were cultured overnight before using them for adoptive transfer experiments.

Bone marrow chimeras
Hematopoietic stem cells (HSC) were enriched from bone marrows using the mouse hematopoietic progenitor cell enrichment kit (BD Biosciences). The cells thus isolated were rested overnight in the presence of stem cell factor, IL-3, and IL-6 (10 ng/ml) in 24-well culture plates. Next day the HSC-enriched cells were infected with MIG or MIG-Bcl2 virus as described above, and incubated at 37°C overnight. Infection efficiency was monitored by flow cytometry. The HSC were suspended at 2 × 10<sup>6</sup>/ml, and 100 µl of the suspension was retro-orbitally injected into lethally radiated B6-Ly5.2 mice (National Cancer Institute, Frederick, MD). The mice were placed on Baytril and Doxycyclin treated antibiotic water for 6 wk. Analysis was performed by flow cytometry, as described above.

Results

Egr-2 is up-regulated during positive selection
The process of thymocyte positive selection can be minimally subdivided into stages based on changes in expression of the CD69 activation marker and TCRβ. They are: 1) TCRβ<sup>int</sup>CD69<sup>high</sup>; 2) TCRβ<sup>high</sup>CD69<sup>high</sup>; and 3) TCRβ<sup>high</sup>CD69<sup>low</sup> (Fig. 1A). To determine whether Egr2 mRNA levels are increased during positive selection, TCRβ<sup>high</sup>CD69<sup>high</sup> and TCRβ<sup>high</sup>CD69<sup>low</sup> intermediates were isolated by flow cytometry. Real-time PCR analysis on mRNA samples from these populations revealed that Egr2 was substantially induced in both the TCRβ<sup>high</sup>CD69<sup>high</sup> and
Egr2 deficiency disrupts the development of αβ but not γδ thymocytes in FTOC

To assess the role of Egr2 in positive selection, we analyzed thymocyte development in Egr2-deficient (Krox20−/−) mice. Since Egr2 deficiency results in perinatal lethality due to defective myelination (10), we analyzed explanted d14 fetal lobes cultured for 10 days in FTOC (15). Flow cytometric analysis revealed no significant differences in the number of γδ lineage cells, DN subsets, or the number of DP thymocytes in Krox20+/+ thymocytes (Fig. 1B and data not shown); however, there was a reduction in both the CD4SP and CD8SP subsets as well as in TCRβhigh cells (Fig. 1B). Development of Krox20−/− thymocytes was not impaired relative to that of littermate controls. Taken together, these data demonstrate that Egr2 plays an important role in the late stages of T cell differentiation.

Conditional deletion of Egr-2 in T lineage precursors partially blocks development of mature αβ T cells

To address the role of Egr-2 in T cell development during adult hematopoiesis we used floxed-Egr2 (Egr2fl/fl) mice in which Egr2 was conditionally ablated in T cells by Cre recombinase expressed under the control of the lck proximal promoter (lck-Cre) (19). Deletion of Egr2 in DN thymocytes results in thymi with slightly decreased cellularity (Fig. 2A). The percentage and absolute numbers of mature CD4 and CD8 SP thymocytes are also decreased, indicating that the lack of Egr-2 impairs maturation of αβ thymocytes. This block is already evident in early stages of the positive selection process as the TCRβhighCD69high and TCRβhighCD69low subpopulations are also somewhat reduced (Fig. 2B). Interestingly, Egr2 deficiency appears to skew the CD4:8 ratio among the TCRβhighCD69high early intermediates, increasing CD4SP and decreasing CD8SP; however, this differential effect on the CD4 and CD8 lineages is minimized upon differentiation to the TCRβhighCD69low stage (Fig. 2B). Consequently, Egr2 deficiency impairs development of both fetal and adult mature thymocytes, with the inhibition being slightly more pronounced for CD8 lineage cells. It should be noted that the number of CD4 T cells is preferentially decreased in the spleen raising the possibility that Egr2 deficiency may impair growth or survival of CD4 T cells after exit from the thymus (Fig. 2C).

As observed in the FTOC with conventional Krox20−/− mice, no effect on γδ T cell development was observed in conditional Egr2-deficient mice; however, the number of αβTCRβhighCD4−
CD8− cells was diminished somewhat, suggesting that development of NKT cells might also be impaired by Egr2 deficiency (data not shown).

**Egr-2 deficiency interferes with positive selection**

The effects of a gene product during positive selection can sometimes be obscured by a heterogeneous TCR repertoire, where TCR specificities of differing affinity may compensate for the absence of an important molecular effect. To assess the effect of Egr2 deficiency on development of thymocytes with a monoclonal TCR repertoire, we crossed Egr2f/f/lck-cre mice with mice bearing the Tg AND TCR (12). AND Tg thymocytes are positively selected on I-Ak and I-Ek molecules but not I-Ak and I-Ek (20), and this model was previously used to reveal the importance of many signal transduction pathways in positive selection and CD4/CD8 lineage commitment (21–23). As shown in Fig. 3A, Egr-2 deficiency clearly decreased both the percentage and absolute number of CD4SP thymocytes. Indeed, Egr-2 deficiency reduced the absolute number of CD4 SP from 27.9(±3.5) × 10⁶ to 10.8(±3.2) × 10⁶ cells. This decrease is even more evident when the thymocytes were resolved into positive selection intermediates based on expression of TCR and CD69. As discussed above (Fig. 2), changes in expression of TCR and CD69 distinguish cellsearly in the process of positive selection (TCRhighCD69high) from those in the more advanced phases (TCRhighCD69low). Egr2 deficiency caused a substantial reduction of the TCRhighCD69low subset, indicating that Egr-2 function may be important primarily during the later stages of positive selection. This reduction was still evident in the periphery, where the numbers of splenic AND CD4 cells was 24.22(±6.7) × 10⁶ in Egr2f/f mice vs 13.5(±4.1) × 10⁶ cells in Egr2f/f-cre mice. Since Egr2 deficiency interfered with selection of CD4SP thymocytes, we asked whether selection of CD8SP was similarly impaired. For this, we used the H-Y TCR Tg model (Fig. 3B). The H-Y TCR is reactive with the male Ag presented by H-2k/Db and paired. For this, we used the H-Y TCR Tg model (Fig. 3).

To elucidate the mechanism responsible for the blockade in positive selection is mediated by the Ras/MAPK pathway during positive selection, Egr2 deficiency clearly decreased both the percentage and absolute number of CD4SP thymocytes. Indeed, Egr-2 deficiency reduced the absolute number of CD4 SP from 27.9(±3.5) × 10⁶ to 10.8(±3.2) × 10⁶ cells. This decrease is even more evident when the thymocytes were resolved into positive selection intermediates based on expression of TCR and CD69. As discussed above (Fig. 2), changes in expression of TCR and CD69 distinguish cells early in the process of positive selection (TCRhighCD69high) from those in the more advanced phases (TCRhighCD69low). Egr2 deficiency caused a substantial reduction of the TCRhighCD69low subset, indicating that Egr-2 function may be important primarily during the later stages of positive selection. This reduction was still evident in the periphery, where the numbers of splenic AND CD4 cells was 24.22(±6.7) × 10⁶ in Egr2f/f mice vs 13.5(±4.1) × 10⁶ cells in Egr2f/f-cre mice. Since Egr2 deficiency interfered with selection of CD4SP thymocytes, we asked whether selection of CD8SP was similarly impaired. For this, we used the H-Y TCR Tg model (Fig. 3B). The H-Y TCR is reactive with the male Ag presented by H-2k/Db and paired. For this, we used the H-Y TCR Tg model (Fig. 3).

To elucidate the mechanism responsible for the blockade in positive selection (TCRhighCD69low) from those in the more advanced phases (TCRhighCD69low). Egr2 deficiency caused a substantial reduction of the TCRhighCD69low subset, indicating that Egr-2 function may be important primarily during the later stages of positive selection. This reduction was still evident in the periphery, where the numbers of splenic AND CD4 cells was 24.22(±6.7) × 10⁶ in Egr2f/f mice vs 13.5(±4.1) × 10⁶ cells in Egr2f/f-cre mice. Since Egr2 deficiency interfered with selection of CD4SP thymocytes, we asked whether selection of CD8SP was similarly impaired. For this, we used the H-Y TCR Tg model (Fig. 3B). The H-Y TCR is reactive with the male Ag presented by H-2k/Db and paired. For this, we used the H-Y TCR Tg model (Fig. 3).

To elucidate the mechanism responsible for the blockade in positive selection (TCRhighCD69low) from those in the more advanced phases (TCRhighCD69low). Egr2 deficiency caused a substantial reduction of the TCRhighCD69low subset, indicating that Egr-2 function may be important primarily during the later stages of positive selection. This reduction was still evident in the periphery, where the numbers of splenic AND CD4 cells was 24.22(±6.7) × 10⁶ in Egr2f/f mice vs 13.5(±4.1) × 10⁶ cells in Egr2f/f-cre mice. Since Egr2 deficiency interfered with selection of CD4SP thymocytes, we asked whether selection of CD8SP was similarly impaired. For this, we used the H-Y TCR Tg model (Fig. 3B). The H-Y TCR is reactive with the male Ag presented by H-2k/Db and paired. For this, we used the H-Y TCR Tg model (Fig. 3).

To elucidate the mechanism responsible for the blockade in positive selection (TCRhighCD69low) from those in the more advanced phases (TCRhighCD69low). Egr2 deficiency caused a substantial reduction of the TCRhighCD69low subset, indicating that Egr-2 function may be important primarily during the later stages of positive selection. This reduction was still evident in the periphery, where the numbers of splenic AND CD4 cells was 24.22(±6.7) × 10⁶ in Egr2f/f mice vs 13.5(±4.1) × 10⁶ cells in Egr2f/f-cre mice. Since Egr2 deficiency interfered with selection of CD4SP thymocytes, we asked whether selection of CD8SP was similarly impaired. For this, we used the H-Y TCR Tg model (Fig. 3B). The H-Y TCR is reactive with the male Ag presented by H-2k/Db and paired. For this, we used the H-Y TCR Tg model (Fig. 3).
TCR<sup>hi</sup>CD69<sup>lo</sup> population, suggesting that the role of Egr2 is more important during late stages of positive selection. In contrast, overexpression of Bcl-2 in Egr2-sufficient thymocytes did not result in an increase in positive selection (Fig. 6B).

**Discussion**

Egr proteins play a role during positive selection. Overexpression of Egr-1 improved the efficiency of positive selection (3), while its deletion resulted in a partial blockade in positive selection, both in class I- and class II-restricted T cells (5). The partial phenotype suggests that other Egr family members may also play a role in this process, and expression of a dominant negative pan-Egr construct in rFTOCs results in a much more dramatic block in mature T cell development (7) (J. Alberola-Ila, unpublished results). Since the defects in positive selection were not more pronounced in Egr1/3 double KO mice than in Egr1 single KO (9), we suspected that Egr-2 could be the family member involved in this process. In this manuscript, we provide evidence that demonstrates a role for Egr-2 in positive selection. As with Egr1-deficient thymocytes, the block in positive selection is partial, and seems equally profound for class I- and class II-restricted T cells. The prediction from our experiments is that a double Egr1-Egr2 KO would result in a much more profound blockade in positive selection, and the experiments to test this hypothesis are currently in progress.

Besides positive selection, interactions of the TCR with MHC plus peptide in immature DP thymocytes may result in deletion. Thymocytes defective in Egr-2 seem to undergo normal deletion, as do Egr-1-deficient thymocytes, both using an in vitro model with α-CD3γ-α-CD28 Abs and in vivo using the HY Tg model (13). Neither of these approaches perfectly mimics the physiological process of negative selection (i.e., a caveat of the in vivo results is that HY deletion could occur before Cre-mediated deletion of the Egr-2 gene), so additional experiments will be required to assess the contribution of Egr-2 to negative selection. However, these results are consistent with the lack of effect on negative selection of many alterations in the Ras/MAPK pathway, one of the principal regulators of Egr expression (1). Likewise, Egr2 induction is also dependent upon Ca<sup>2+</sup> signaling and perturbations in Ca<sup>2+</sup> signaling caused by ablation of the calcineurin B1 block positive but not negative selection (37).
and CD69. This is intriguing because Egr-2 is induced very early during positive selection, and suggests that Egr-2 controls the expression of other genes that are necessary during late stages in the process of positive selection. Surprisingly, its effects are very selective among the genes required for positive selection and whose expression is altered during this process (Schnurri2, Id3, Tox, Gfi1 and Gfi1b, GATA-3, runx3, TH-POK, and Bcl-2) (18, 24–34, 38), only Id3 and Bcl-2 expression were significantly altered in Egr2-deficient intermediates.

Id3 is a negative regulator of E-box transcription factors, and, in its absence, positive selection is partially blocked. Interestingly, it has been reported that Id3 up-regulation in DP thymocytes is regulated by the Ras/MAPK pathway and Egr1 (35), although these results have been controversial (39). However, the phenotype of Id3−/− mice is less profound than that of Egr2-deficient mice, especially in regards to CD8 development (25), suggesting that it is probably not the primary Egr2-modulated gene responsible for the defects in Egr2-deficient mice. It is, however, possible that it may contribute to the phenotype, and genetic rescue experiments will be necessary to address this possibility.

Up-regulation of Bcl-2 is important for the survival of positively selected thymocytes (38), but not much is known about the factors that regulate its expression during this process. Because Egr2 deficiency both attenuated induction of Bcl-2 after in vitro stimulation and lowered its expression in purified intermediate populations, diminished Bcl-2 was very attractive as an explanation for the positive selection defect. It must be pointed out that the Bcl-2 expression defect is not very profound. This is probably due in part to Egr-1 compensation. This is likely to be compounded by selective pressure, which enables only those Egr2-deficient precursors whose Bcl-2 induction exceeds a minimum threshold to mature to the TCRhighCD69low stage. Accordingly, the requirement of Egr transcription factors in controlling Bcl-2 expression during positive selection is probably more profound than the subtle defect noted here suggests. Analysis of double Egr1/Egr2-deficient thymocytes will probably make this more evident.

Our genetic rescue experiments of Egr2-deficient thymocytes with a Bcl-2-encoding retrovirus clearly demonstrate that positive selection is enhanced in the presence of Bcl-2, both for CD4 and CD8 SP thymocytes. Therefore, while quantitatively modest, the effect in Bcl-2 induction during positive selection in Egr2-deficient thymocytes is biologically significant and plays a predominant role in impairing positive selection in Egr2-deficient mice. It remains unclear at present whether Bcl-2 is a direct target of Egr2. In that regard, Egr2 induction is most pronounced late in positive selection (TCRhighCD69low) where Bcl-2 expression is the likely mechanism that underlies this effect. These results are in contrast with a recent published observation (40) where no alterations in peripheral T cell populations were observed in Egr2 deficient bone marrow chimeras. However, in this report, only peripheral populations were analyzed, and the defect in positive selection in the mice may be

One interesting aspect of the phenotype we observed in Egr2-deficient thymocytes is that the defect is more pronounced in late stages of positive selection, as defined by the expression of TCR

**FIGURE 6.** Forced expression of Bcl-2 rescues the generation of TCRhigh cells in Egr2-deficient mice. Lin− bone marrow cells from Egr2fl/fl-Cre mice were infected with MIG-Bcl-2 and injected i.v. into lethally irradiated Ly5.2 recipients. After 6 wk, the mice were euthanized, and T cell development of the transferred cells was assessed by gating on Ly5.1+ cells. The effect of Bcl-2 is determined by comparing development in Ly5.1+/GFP− vs Ly5.1+/GFP+ cells. A, We show a representative histogram of thymocytes stained with TCR or CD4/CD8 comparing GFP+ cells (top) to GFP− (bottom) and a quantification of the TCRhigh cells (n = 8). Expression of Bcl-2 rescues the development of mature T cells in an Egr2fl/fl-Cre background. The same thymocytes were stained with TCR and CD69 to determine the effect of Bcl-2 at the different stages of T cell differentiation after positive selection. We show a representative histogram and the quantification (n = 8). As can be clearly observed, the effect of Bcl-2 is more prominent at the latest stages of T cell differentiation. B, Similar experiments were performed on Lin− bone marrow cells from wild-type mice (n = 7).
masked by homeostatic expansion of the selected populations in the periphery.

Our findings also raise the interesting possibility of differential involvement of particular Egr family members in positive selection. Indeed, while Egr1 and Egr2 deficiency partially impair positive selection, mice doubly deficient for both Egr1 and Egr3 do not exhibit a more profound defect than do Egr1-deficient mice (9). This suggests that among Egr family members, the function of Egr1 and 2 is most important during positive selection, whereas the function of Egr1 and 3 are most important during early thymocyte development and β-selection. Analysis of multiply deficient mice lacking combinations of Egr1, Egr2, and Egr3 will be necessary to address this possibility and establish its molecular basis.

Disclosures

The authors have no financial conflict of interest.

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