B-Raf is required for positive selection and survival of DP cells, but not for negative selection of SP cells

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Abstract

The duration of signaling through the MAP kinase (or ERK pathway) cascade has been implicated in thymic development, particularly positive and negative selection. In T cells, two isoforms of the MAP kinase kinase kinase Raf function to transmit signals from the T-cell receptor to ERK: C-Raf and B-Raf. In this study, we conditionally ablated B-Raf expression within thymocytes to assess the effects on ERK activation and thymocyte development. The complete loss of B-Raf is accompanied by a dramatic loss of ERK activation in both the double positive (DP) and single positive (SP) thymocytes, as well as peripheral splenocytes. There was a significant decrease in the cellularity of KO thymi, largely due to a loss of pre-selected DP cells, a decrease in DP cells undergoing positive selection, and a defect in SP maturation. B-Raf plays significant roles in survival of DP thymocytes and function of SP cells in the periphery. Surprisingly, we saw no effect of B-Raf deficiency on negative selection of autoreactive SP thymocytes, despite the greatly reduced ERK activation in these cells.

Keywords: B-Raf, ERK, MAPK, positive selection, thymic development

Introduction

The MAP kinase (extracellular signal-regulated kinase, or ERK) pathway is involved in many aspects of T-cell development (1–5). TCR signaling is coupled to Ras and the ERK cascade via C-Raf (also called Raf-1) (6, 7). A second member of the Raf family, B-Raf, is also expressed in T cells (8). The ERK kinase MEK is the major target of both C-Raf and B-Raf, and the relative proportion of the ERK signaling cascade that is mediated by B-Raf versus C-Raf in T cells is not known.

The question of how one pathway can impart different information within the cell at different developmental checkpoints is of fundamental interest in thymocyte development. Studies in other cell types have demonstrated that the duration of ERK activation is a key component in dictating the outcome of the ERK signaling pathway, switching the outcome between that of proliferation and differentiation (9). Support for the strength of signal model has also come from examining the role of ERK signaling in thymocyte selection (3, 10, 11). Indeed, these studies indicated a role for ERK in both positive and negative selection of thymocytes. Specifically, these studies showed that positive selection required rapid, transient ERK activation, whereas negative selection required more sustained ERK activation (3, 10). Additionally, converting a sustained ERK response into a transient one, using low levels of a MEK inhibitor, turned a negatively selecting signal into a positively selecting one (11). These studies implicating the role of temporal ERK activation in regulating thymocyte selection were carried out using in vitro models evaluating the progression of double positive (DP) cells into single positive (SP) cells as evidence of positive selection and the loss (apoptosis) of DP cells as the read-out for negative selection (3, 10, 11). The requirement for ERKs in positive selection has been definitively established using ERK1 and ERK2 knockout mice (12–14). Other studies using mice expressing either dominant negative (2, 5, 15) or constitutively active (16, 17) mutants of the MAPK cascade indicate that MAPK/ERK signaling is involved in positive but not in negative selection.

Although there is evidence that DP cells undergo negative selection within the cortex (18, 19), the predominant population of thymocytes that undergo negative selection is SP cells in the thymic medulla (20–23). Indeed, the loss of negative selection in the medulla leads to autoimmunity, and it is thought that exposure of SP cells to peripheral self-antigens in the medulla deletes the self-reactive SP cells (24–26).
B-Raf is required for positive selection and survival of DP cells

In this study, we examine whether the level of ERK activity plays a role in T-cell development and function. To examine this, we created a targeted deletion of B-Raf in thymocytes using the CRE recombinase under the control of the Lck promoter. B-Raf and C-Raf are the two major Raf isoforms in thymocytes. Both have a single target the MAPK kinase, MEK. Therefore, loss of B-Raf is predicted to attenuate, but not eliminate, ERK activation. We established the conditional knockout on a transgenic TCR background, which has been shown to allow the progression of DP cells through to the SP stage in ERK knockout animals (13).

Loss of B-Raf resulted in a significant decrease in ERK activation in DP and SP thymocytes and peripheral splenocytes. This decrease in ERK activity did not have any effect on the negative selection of SP cells in the medulla. Instead, B-Raf-dependent ERK signaling was required for the survival and progression of pre-selected DP thymocytes to SP cells and affects TCR-dependent proliferation in the periphery.

Methods

Mice

RIP-mOVA (003231), OT-II (003831), MHC class II (IAβ)-deficient (003584), C57BL/6 (000664) and 129/SvJ (000691) mice were purchased from The Jackson Laboratories. Lck-CRE mice (004197) were purchased from Taconic. Dr William Snider, University of North Carolina, provided the mice with pLox sites flanking exon 10 of the B-Raf gene (27). Experiments on animals were performed according to the ethical guidelines of the IACUC committee at Oregon Health and Science University in accordance with federal regulations approved animal use and care.

Cell surface staining antibodies

Fluorochrome-conjugated antibodies were purchased from BD Biosciences: CD8α-APC, CD8α-PerCP and CD69-PE; eBioscience: CD4-eFlour450, CD8α-APC, CD8α-PE-Cy7, Vj3-8, Vj6-, Vj6-PE, Vx2-APC, Qa-2-FITC, HSA-PE; Biolegend: CD4-PE-Cy5, pan TCRβ-APC-Cy7 (H57-597). Anti-Nur77-PE was provided by Amy Moran, Earle A. Chiles Research Institute, Providence Cancer Center, Portland, OR, USA.

Intracellular staining

Intracellular staining for B-Raf was performed by fixation and permeabilization using 0.5% formaldehyde for 10 min at 37°C and 90% methanol for 30 min on ice. Cells were then incubated with anti-B-Raf (Abcam, 1:50) primary antibody in 0.5% BSA in PBS for 30 min at room temperature, washed twice and incubated with goat anti-rabbit IgG Alexa Fluor 647 for 30 min at room temperature. Nur77 intracellular staining was performed using the FoxP3 staining kit from eBioscience, according to the manufacturer’s instructions.

ERK activation

DP and SP4 cells were sorted on a FACS Vantage (BD Biosciences) and plated at 1×10⁶ cells per well in 96-well plates that had been previously coated with 10 µg ml⁻¹ anti-TCR-β antibody (Biolegend, H57-597) or 1.0 µg ml⁻¹ anti-CD3 antibody (eBioscience, 145-2C11), respectively. Cells were harvested as previously described by us (28, 29) and analysed by western blot.

Following stimulation with 1 µg ml⁻¹ anti-CD3 (Pharmingen, 145-2C11) and cross-linking with goat anti-hamster IgG2 (10 µg ml⁻¹) for the indicated times, fixation and permeabilization was performed as described above. Cells were then incubated with blocking CD16/CD32 anti-Fc receptor antibody (BD Pharmingen, 2.4G2) at 2.5 µg ml⁻¹ for 10 min at room temperature, washed once and incubated with anti-pERK (1:100, Cell Signaling 197G2) at room temperature for 30 min. Cells were washed three times and then incubated with goat anti-rabbit IgG FITC for 30 min at room temperature. Cell events were collected using an LSR-II cytometer from BD Biosciences and analysed with FCS Express software (De Novo Software Inc.).

Western blots

Cell lysis and SDS-PAGE immunoblots were performed as previously described by us (28, 29). Membranes were probed with anti-B-Raf (Santa Cruz Biotechnology), anti-pERK (Cell Signaling) or anti-ERK2 (Santa Cruz Biotechnology). Following incubation with HRP-conjugated secondary antibodies (Amersham Biosciences), proteins were detected by enhanced chemiluminescence. Western blot data analysis was performed on a Macintosh computer using the public domain NIH Image program.

Annexin staining

Thymocytes were stained using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences), according to the manufacturer’s instructions.

Superantigen deletion

Single cell suspensions of thymus and spleen from 4- to 6-week-old 129, B6, 129x129 F1, control and B-Raf KO mice were prepared. Following red blood cells lysis, cells were suspended in FACS buffer. Cells were stained and analysed by flow cytometry.

Negative selection by transgenic antigen

Nine-week-old male and female OT-II control and OT-II B-Raf KO mice were used as bone marrow (BM) donors. Following red cell lysis, BM cells flushed from the femurs and tibias were depleted of CD4+ and CD8+ cells by incubation with anti-mouse CD4-PE (L3T4; eBioscience) and anti-mouse CD8α-PE (Ly2; eBioscience) followed by anti-PE Microbeads (MiltenyiBiotec) and separated using Macs Separation Columns LS (MiltenyiBiotec). Cells were resuspended in PBS for injection into each recipient mouse through the tail vein.

Five-week-old male and female transgenic RIP-mOVA and their non-transgenic littermates (B6) mice were used as recipients. Recipient mice were lethally irradiated with 1300 cGy and reconstituted with 2×10⁶ OT-II control or OT-II B-Raf KO T-cell-depleted BM cells. Blood was collected 5–6 weeks and thymus and spleen were collected 6–8 weeks after transplant. Cells were stained and analysed by flow cytometry.
Class II-deficient BM chimeras

Mice deficient in MHC class II genes have a dramatic decrease in the number of CD4+ T cells due to the lack of positive selection in these animals. OT-II control and OT-II B-Raf KO mice were used as BM donors and transferred into MHC class II-deficient mice or control C57BL/6 mice, as described above. Thymocytes were collected 8 weeks after transplant. Cells were stained and analysed by flow cytometry.

Proliferation

Proliferation was measured by [3H] thymidine incorporation as previously described (28, 29).

Results

Expression of B-Raf in thymocytes

B-Raf was expressed in all wild-type thymic subsets, as examined by western blot and intracellular staining (Fig. 1A, B and C). To examine the role of B-Raf in thymocyte development we generated a conditional knockout of B-Raf in thymocytes by crossing mice with pLox sites flanking exon 10 of the B-Raf gene with mice expressing CRE recombinase under the control of the Lck promoter. This results in the excision of exon 10 in T lymphocytes, leading to an unstable B-Raf protein, which is degraded (27). The F1 progeny that were Lck-CRE negative/homozygous pLox B-Raf are referred to as control (C), and sibling mice that were Lck-CRE positive/homozygous pLox B-Raf are referred to as KO. All the main thymocyte subsets [DN (CD4–/CD8–), DP (CD4+/CD8+), SP4 (CD4+/CD8–) and SP8 (CD4–/CD8+)] were present in the conditional KO, which showed complete loss of B-Raf expression in DP, SP4 and SP8 subsets (Fig. 1A). There was a partial loss of B-Raf in the DN subset (Fig. 1A). Lck expression occurs in late DN (30, 31), consistent with the loss of B-Raf at DN3 (Fig. 1B and C).

B-Raf KO thymocytes show a deficiency in ERK activation

B-Raf is a robust activator of ERK in many cell types (8, 32–34), so we examined whether the loss of B-Raf significantly

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**Fig. 1.** B-Raf expression in control and KO thymocytes. (A) Control (C) and KO thymocytes were sorted into double negative (DN), double positive (DP), CD4 single positive (SP4) and CD8 single positive (SP8) populations and examined for B-Raf expression by western blot (top panel); ERK2 is shown as control (bottom panel). (B) Intracellular staining for B-Raf in the major thymocyte populations. Isotype control antibody (black line), B-Raf expression in control (dark gray shading), KO (light gray shading) thymocytes. (C) Intracellular staining for B-Raf in DN thymocyte subsets. Isotype controls and B-Raf staining as are indicated for B.
decreased TCR-induced ERK activation in the two most abundant thymic subsets, DP and SP4, purified from control and KO thymi. DP cells from both control and KO thymi showed transient ERK activation following TCR stimulation. This was more rapidly terminated in KO cells, seen as a decrease in magnitude of ERK activation at 5 and 10 min (Fig. 2A and B). ERK activation by TCR in SP4 thymocytes was robust and maximal at 2–20 min and declining by 40 min (Fig. 2C and D). However, in KO SP4 cells ERK activation was significantly reduced at 2–10 min and was back to basal by 20 min (Fig. 2C and D), demonstrating that B-Raf contributed to both the magnitude and duration of ERK activation in SP4 thymocytes. These data show that B-Raf contributes to ERK activation in both DP and SP4 thymocyte populations.

B-Raf contributes to the development of DP and SP thymocytes

We examined the consequence of the loss of B-Raf using two models, polyclonal TCR thymocytes that express a variety of TCR-α and -β chains and thymocytes expressing the transgenic OT-II TCR. The percentages of all major thymic subsets (DN, DP, SP4 and SP8) were the same between control and KO thymi on both a polyclonal (Fig. 3A) and the OT-II TCR (Fig. 3B) background. However, in both models the total cellularity of the KO thymus was significantly lower than control (Fig. 3C and D) and there were significantly fewer total, DP and SP cells (Fig. 3C and D).

For both the polyclonal and OT-II TCR models, the total number of DN cells was not significantly different between control and KO mice (Fig. 3C and D). Also the percentage and number of cells in DN subsets (DN1-DN4) were the same. The development of DN into DP cells requires ERK-dependent proliferation (13). Therefore, we measured DN proliferation with 5-bromo-2-deoxyuridine pulse-chase assay and found no significant differences between C and KO thymocytes (Supplementary Fig. 1A–D, available at International Immunology Online). These data suggest that the decrease in DP cells was not due to a defect with DN to DP transition in the KO.

Non-conventional thymocyte populations were also examined (35). We found that the percentage of non-conventional thymocyte populations (NK T cells, γδ T cells and Tregs) were not significantly affected by the loss of B-Raf (Supplementary Fig. 2A and B, available at International Immunology Online).

Positive selection is impaired in the B-Raf KO

To assess the role of B-Raf in positive selection, we examined the expression of Vβ TCR chains and CD69 on DP and SP thymocytes, as these markers are up-regulated during positive selection (19, 36). In thymocytes deficient in B-Raf, the percentage of CD69⁺Vβ⁺ DP cells was significantly reduced on both polyclonal (Fig. 4A) and OT-II (Fig. 4B) DP cells, suggesting that positive selection was perturbed. Indeed, in KO thymi from both models, the number of CD69⁺Vβ⁺ DP cells was also reduced compared with their control littermates (Fig. 4C and D).

Polyclonal SP4 cells from both control and KO thymi were all Vβ⁺ reflecting normal up-regulation of TCR Vβ chains in both
groups (Fig. 4E, bottom left panel). Polyclonal SP4 cells from KO thymi, but not control thymi, showed reduced expression of CD69 (Fig. 4E, top left panel), which is likely attributable to the decrease in ERK signaling in the KO (37, 38). In the OT-II model there was also reduced expression of CD69 on the cell surface of OT-II SP4 cells from KO thymi (Fig. 4E, top right panel). Expression of V\(\beta\)5 was also reduced on these cells (Fig. 4E, bottom right panel). We further examined the OT-II SP4 cells to determine if the KO cells were TCR\(^{hi}\), by comparing the staining of pan V\(\beta\) and V\(\beta\)5 antibodies. We demonstrated that all OT-II SP4 cells were TCR\(^{hi}\) irrespective of whether they were from control or KO thymi. But OT-II SP4 cells from KO thymi expressed more endogenous V\(\beta\) chains than controls (Fig. 4F). This suggests that positive selection of the OT-II TCR is inefficient in the KO thymocytes.

We examined CD69\(^{+}\)/V\(\beta\)\(^{hi}\) DP cells for Nur77 expression, a marker for the strength of TCR stimulation. In DP cells, Nur77 expression identifies those cells stimulated to undergo positive selection (39). In control DP cells, there was an increase in Nur77 expression in CD69\(^{+}\)/V\(\beta\)\(^{hi}\) population compared with pre-selected cells (CD69\(^{-}\)/V\(\beta\)\(^{lo}\) DP cells that have not yet undergone positive selection) (Fig. 4G, left
Fig. 4. B-Raf expression contributes to the positive selection of DP thymocytes. (A, B) Representative flow cytometry plots showing expression of (A) Vβ/CD69 expression on polyclonal DP cells and (B) Vβ5/CD69 expression on OT-II DP cells. (C, D) Absolute pre-selection (CD69+/Vβlo) and post-selection (CD69+/Vβhi) DP cell numbers in (C) polyclonal TCR C and KO thymi and (D) OT-II C and KO thymi were calculated by multiplying the percentage of live cells of each subset by the total cell number of the thymus. Data expressed as mean ± SE, n = 3 and the differences between C and KO were significant at **P < 0.01 and ***P < 0.001. (E) Representative flow cytometry histogram showing expression of CD69 on polyclonal and OT-II C and KO SP4 cells (top row) and TCR Vβ expression on polyclonal and OT-II C and KO SP4 cells (bottom row). Histogram gates define the CD69, pan Vβ and Vβ5 positive cells, respectively. (F) Representative flow cytometry plot showing expression of pan Vβ/Vα2 and Vβ5/Vα2 on OT-II C and KO SP4 cells. (G) Representative flow cytometry histogram showing expression of Nur77 in polyclonal CD69+/Vβlo pre-selection DP cells (pre) and CD69+/Vβhi post-selection DP cells (post). Histogram gate defines the Nur77 positive cells.
CD69+Vβ^hi KO DP cells also showed an increase in Nur77 expression compared with CD69-/Vβ^lo KO DP cells (Fig. 4G, right panel). However, Nur77 expression in the KO CD69+/Vβ^hi population was less than that seen in the control cells (Fig. 4G). Taken together, these data suggest that KO DP cells receive a reduced signal through the TCR as they undergo positive selection.

**DP cell survival is decreased in the B-Raf KO**

Although we showed inefficient positive selection in the KO thymi, the significant decrease in total cellularity of the KO thymi (Fig. 3C) was greater than could be accounted for solely by a decrease in positive selection. There was also a decrease in the numbers of CD69-/Vβ^lo DP cells in the polyclonal (Fig. 4C) and OT-II (Fig. 4D) KO thymi consistent with a loss of cells prior to positive selection. Therefore, we looked at an early marker of apoptosis, Annexin V.

Annexin V staining was increased on total polyclonal KO DP cells (Fig. 5A), suggesting that apoptosis of DP cells from the KO also contributed to the decrease in thymic cellularity. To confirm that there was a loss of DP cells, independent of positive selection, we compared the development of OT-II control and KO thymocytes from BM chimeras in class II-deficient recipients that lack positive selection (Fig. 5B and C). As expected, there was a nearly complete loss of Vβ5-expressing control and KO thymocytes from class II-deficient recipients (Fig. 5B, left panel). We also examined Vβ5 expression on OT-II control and KO thymocytes from B6 recipients. Similar to the non-transferred OT-II thymocytes, there was a reduction of KO cells isolated from B6 recipients compared with control cells (Fig. 5B, right panel) confirming that there was inefficient positive selection of the OT-II TCR in KO thymocytes.

To test the hypothesis that there was a loss of KO DP cells in the absence of positive selection, we compared the subsets of OT-II control and KO thymocytes from BM chimeras in B6 recipients to class II-deficient recipients that lack positive selection. When positive selection was blocked in class II-deficient recipients, both control and KO thymocytes showed a significant loss of SP cells (Fig. 5C) compared with B6 recipients (Fig. 5D). Importantly, both in the presence (Fig. 5D) and absence (Fig. 5C) of positive selection there was a significant loss of KO DP cells compared with controls. These data strongly suggest that there is a loss of pre-selected DP cells in B-Raf KO thymi.

**Fig. 5. B-Raf contributes to the survival of DP cells.** (A) Representative flow cytometry histogram showing Annexin V staining on polyclonal C and KO DP cells. Histogram gate defines the Annexin V positive cells. (B) Representative flow cytometry plots showing expression of Vβ5 in DP thymocytes. Left panel: OT-II C and KO cells into MHC class II-deficient (class II–/–) recipients; Right panel: OT-II C and KO cells into B6 recipients. Histogram gate defines percentage of Vβ5-positive populations. (C) Thymocyte subset cell numbers in OT-II C into class II–/– recipient (n = 3) and KO cells into class II–/– recipient (n = 3). (D) Thymocyte subset cell numbers from OT-II C into B6 recipient (n = 3) and KO cells into B6 recipient (n = 3). Data expressed as mean ± SE and the difference between control and KO mice was significant at *P < 0.05 and **P < 0.01.
B-Raf is not required for negative selection of SP cells

A role for ERKs in negative selection has been proposed (3, 10, 11). The presence of SP cells in the B-Raf conditional KO described here allowed us to examine the consequence of decreased ERK signaling on negative selection in the medulla. We examined two classical models of negative selection: deletion by endogenous superantigens (40) and deletion by thymic expression of transgenic peptide antigen (26).

To examine superantigen-mediated deletion, we generated mice on a mixed background. To do this, we crossed 129/SV (129) mice expressing pLox sites flanking B-Raf exon 10 with C57BL/6 (B6) mice expressing Lck-CRE, which produced control and KO littermates on a mixed B6/129 background. This allowed us to evaluate the deletion of specific Vβ-expressing thymocytes mediated by endogenous superantigens encoded in the 129 genome (41). When examined on 129 and 129xB6 F1 backgrounds, Vβ3- and Vβ5-expressing control thymocytes were deleted, but Vβ6-expressing cells were not, which was readily apparent when comparing the percentage of Vβ-expressing cells in the SP4 population (Fig. 6A). As a control, we showed that B6 did not delete any of the Vβ3-, Vβ5- or Vβ6-expressing control or KO cells. Comparison of the absolute cell numbers demonstrated the same loss of SP4 cells in these populations within the KO, independent of negative selection (Supplementary Figure. S3A, available at International Immunology Online). These results demonstrate that negative selection of thymocytes responding to endogenous superantigens is not impaired by the loss of B-Raf.

Thymic deletion was also evaluated using a model system expressing peptide antigen within the thymic medulla (26). BM chimeras were made by transplanting OT-II control or KO BM into RIP-mOVA recipients, where expression of mOVA in the thymic medulla deletes OT-II thymocytes (26). Negative selection of OT-II thymocytes was monitored by the expression of Vβ5/Vα2 in the CD4 gate. As expected, control and KO SP4 cells were found in the thymus of B6 recipients (Fig. 6B). Both the percentage (Fig. 6B) and number (Supplementary Figure. S3B, available at International Immunology Online) of control and KO SP4 cells were significantly reduced in RIP-mOVA recipients, again demonstrating that loss of B-Raf did not impair negative selection of SP4 cells.

Fig. 6. SP negative selection is not affected by the loss of B-Raf. (A) Thymocytes isolated from 129, B6, mixed 129xB6, control (C, mixed 129/B6) and KO (mixed 129/B6) mice were stained with CD4 and Vβ3, Vβ5 and Vβ6 antibodies and analysed for expression of Vβ3, Vβ5 and Vβ6 TCR chains. Values from individual mice are plotted as percentage of live SP4 thymocytes with the mean indicated as a black bar. The dotted line indicates the mean of the two control groups of 129 and B6 mice. (B) Analysis of Vβ5/Vα2 expression as a marker of the OT-II TCR transgenic SP4 T cells isolated from BM chimeras. Black, OT-II control BM into B6 recipient (n = 6); dark gray, OT-II control BM into RIP-mOVA recipient (n = 9); gray, OT-II KO BM into B6 recipient (n = 6); white, OT-II KO BM into RIP-mOVA recipient (n = 9). Data expressed as mean ± SE, and the difference between C and KO mice was significant at *P < 0.01 and **P < 0.001.
Fig. 7. Loss of B-Raf affects the maturation and function of SP cells. (A) Representative flow cytometry plot showing expression of HSA and Qa-2 expression on polyclonal C and KO DP (top row) and SP4 (bottom row) thymocytes. (B) Graph of the percentage of live DP (HSA\(^{hi}/\)Qa-2\(^{lo}\)) and SP4 (HSA\(^{lo}/\)Qa-2\(^{hi}\)) cells from control and KO polyclonal thymi. Data expressed as mean ± SE, \(n = 3\) and the difference between C and KO mice was significant at **\(P < 0.01\). (C) Representative flow cytometry histogram showing pERK staining in polyclonal SP4 C and KO splenocytes stimulated with anti-CD3 for the indicated times. Histogram gate defines the pERK-positive cells. (D) Representative flow cytometry plots showing CD8 and CD4 staining of polyclonal C and KO splenocytes. (E) Absolute CD4 and CD8 splenocyte cell numbers in C and KO mice were calculated by multiplying the percentage of live cells of each subset by the total cell number of the spleen. Data expressed as mean ± SE, \(n = 18\) and the difference between C and KO mice was significant at *\(P < 0.05\) and **\(P < 0.01\). (F) Proliferation of KO SP4 splenocytes is reduced compared with control cells. Polyclonal C and KO SP4 cells were stimulated with increasing dose of plate bound anti-CD3 as indicated and proliferation was measured by incorporation of \(^{3}H\)-thymidine at 72h. Data expressed as mean ± SE, \(n = 3\).
Maturation and proliferation of SP4 cells are deficient in B-Raf KO

We examined whether there was a defect in SP differentiation in the KO thymocytes using the maturation markers Qa-2 and HSA (CD24) (42). Early SP cells are HSA/Qa-2<sup>+</sup> and mature into SP cells that are HSA/Qa-2<sup>+</sup> SP4 cells in the KO thymus (Fig. 6A and B), reflecting a perturbation in SP thymocyte maturation.

Mature, proliferation-competent SP cells emigrate from the thymus to populate the periphery (42). Therefore, we examined peripheral SP splenocytes for ERK activation and proliferation. Following stimulation with anti-CD3, SP4 splenocytes from KO mice showed decreased ERK activation as measured by phospho-ERK (pERK) intracellular staining (Fig. 7C) compared with control littermates. We showed a decrease in both the percentage (Fig. 7D) and number (Fig. 7E) of KO peripheral SP T cells compared with control. Additionally, SP4 cells from spleen showed decreased proliferation following stimulation with anti-CD3 (Fig. 7F). Taken together these data show that loss of B-Raf affects peripheral T-cell numbers and function.

Discussion

We demonstrate that B-Raf is expressed in all thymocyte subsets. Conditional ablation of B-Raf in thymocytes after the DN3 stage provides a model suitable for examining the role of B-Raf in all developmental stages subsequent to DN3. This is in contrast to a previous study using B-Raf null chimeras (43), where B-Raf was ablated in all cells and T-cell development examined in mice reconstituted using T-cell precursor cells derived from the B-Raf<sup>−/−</sup> fetal liver. This model showed a complete block in development beyond DP. In this study, B-Raf was completely lost in thymocytes prior to differentiation to DP cells but development did proceed to the SP stage.

B-Raf ablation had a significant impact on TCR-dependent ERK activation in both DP and SP subsets. In DP cells the loss of B-Raf reduced the duration of ERK activation. In SP thymocytes the loss of B-Raf reduced both the magnitude and duration of ERK activity. The greatest difference in ERK activation was seen at later time points. This may be due to C-Raf being involved in early ERK activation and B-Raf playing a role in later activation, as has been proposed in other models (9, 33). The consequence of decreased ERK activation was seen most prominently in DP cells. We showed that B-Raf contributes to the percentage and number of DP cells undergoing positive selection as measured by the up-regulation of Vβ chains and CD69. Decreased expression of CD69 was expected, as it is an ERK-dependent marker of early positive selection (37, 38, 44). SP cells from KO thymi also showed decreased expression of CD69. In addition, SP cells from the OT-II KO showed decreased expression of transgenic Vβ5, which demonstrated inefficient positive selection of the transgenic TCR. DP cells undergoing positive selection from the KO also showed decreased Nur77 expression compared with controls. This may be due to the decreased signaling following TCR stimulation during positive selection of this population. The presence of SP cells demonstrates that positive selection could occur in the KO, although SP cells are present at lower numbers.

Our conditional model allowed us to evaluate the role of B-Raf in the function of SP cells. Surprisingly, despite showing a significant loss of ERK activation, SP4 cells from B-Raf KO mice did not show a defect in negative selection of SP. We examined two models of medullary negative selection and found no differences between control and KO thymocytes, despite a significant decrease in the magnitude and duration of ERK activation in KO SP cells. This contrasts with what was found for negative selection of DP cells in vitro, which are sensitive to changes in ERK activation (4, 11). Since the current consensus is that most negative selection on self occurs in SP thymocytes in the medulla (20–22), our data suggest that B-Raf signaling to ERKs does not play a role in medullary negative selection. Moreover, negative selection in the medulla was not sensitive to ERK threshold effects in vivo, in contrast to what has been shown for DP selection in vitro (3, 10, 11).

A consequence of decreased SP thymocyte maturation in the KO is a decrease in peripheral T cells. SP splenocytes from KO mice show decreased ERK activation, and reduced proliferation in response to TCR stimulation. However, these effects may reflect loss of ERK signaling in peripheral T cells as well as the indirect consequences of defects in thymocyte development.

The loss of B-Raf results in decreased thymic cellularity with significant reductions in numbers of DP, SP4 and SP8 cells. Decreased thymic cellularity is often due to the loss of DP cells as they are the predominant cell type. However, DN to DP transition, which is ERK dependent, was normal, suggesting that ERK signaling through C-Raf is sufficient to maintain this transition. We did identify a developmental defect intrinsic to DP cells. In KO thymi we found decreased pre-selected DP cell numbers and increased apoptosis of DP cells. Our studies using BM chimeras also demonstrated a significant loss of DP cells in both the presence and absence of positive selection. Therefore, we propose that the loss of B-Raf reduces the level of a tonic survival signal in pre-selected DP cells.

The loss of B-Raf reduces but did not eliminate ERK activation, allowing us to test the consequence of reduced ERK signaling during thymocyte differentiation. We propose that the threshold of ERK signaling influences the fate of pre-selected DP cells leading to increased apoptosis with further selective loss of the small population of DP cells that are undergoing positive selection.

Supplementary data

Supplementary data are available at International Immunology Online.

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References


