Thymic progenitors of TCRαβ+ CD8αα intestinal intraepithelial lymphocytes require RasGRP1 for development

Dominic P. Golec,1 Romy E. Hoeppli,2 Laura M. Henao Caviedes,1 Jillian McCann,1 Megan K. Levings,2 and Troy A. Baldwin1

1Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada
2Department of Surgery, University of British Columbia, Vancouver, BC, Canada

Strong T cell receptor (TCR) signaling largely induces cell death during thymocyte development, whereas weak TCR signals induce positive selection. However, some T cell lineages require strong TCR signals for differentiation through a process termed agonist selection. The signaling relationships that underlie these three fates are unknown. RasGRP1 is a Ras activator required for agonist selection of TCR, to transmit weak TCR signals leading to positive selection. Here, we report that, despite being dispensable for thymocyte clonal deletion and removal of thymocytes with self-reactive TCRs, RasGRP1 is critical for agonist selection of TCRαβ+CD8αα intraepithelial lymphocyte (IEL) progenitors (IELps), even though both outcomes require strong TCR signaling. Bim deficiency rescued IELp development in RasGRP1−/− mice, suggesting that RasGRP1 functions to promote survival during IELp generation. Additionally, expression of CD122 and the adhesion molecules αβ, and CD103 define distinct IELp subsets with differing abilities to generate TCRαβ+CD8αα IEL in vivo. These findings demonstrate that RasGRP1-dependent signaling underpins thymic selection processes induced by both weak and strong TCR signals and is differentially required for fate decisions derived from a strong TCR stimulus.

INTRODUCTION

The affinity of TCR:self-peptide-MHC interactions is a key determinant of thymocyte fate (Klein et al., 2014). Relatively low-affinity self-peptide-MHC stimulation through the TCR drives thymocyte positive selection, resulting in the development of conventional CD4+ and CD8+ T cells. Generally, high-affinity TCR stimulation results in clonal deletion and removal of thymocytes with self-reactive TCRs from the T cell repertoire. However, more recently, it has been appreciated that high-affinity, or agonist, stimulation through the TCR is required for positive selection of several alternatively selected thymocyte lineages. This process has been termed “agonist selection” and instructs the development of numerous unconventional T cell lineages such as invariant NKT cells (iNKT), thymic regulatory T cells (tTreg), and natural Tαβ7 cells (nαβ7) within the thymus (Stritesky et al., 2012). In addition, agonist selection guides the development of progenitor cells that give rise to a subset of intestinal TCRαβ+ intraepithelial lymphocytes (IELs) characterized by their unique expression of unconventional CD8αα homodimers (TCRαβ+CD8αα IELs). Although the requirement for high-affinity TCR signaling to drive agonist selection and clonal deletion has been well defined, the nature of the signals that differentiate these two opposing processes remain unclear.

The intestinal epithelium contains distinct populations of IELs that are thought to regulate intestinal homeostasis and whose dysregulation has been implicated in numerous disease models (Cheroutre et al., 2011). Intestinal IELs are heterogeneous, reflecting their distinct developmental origins. Broadly, the intestinal IEL pool includes both γδ- and αβ−T cell lineages. TCRαβ+IEL can be further classified into “natural” and “induced” subsets (Cheroutre et al., 2011). Induced IELs arise from mature naive CD4+ or CD8+ T cells after peripheral activation. Natural IELs are generated from thymic precursors that migrate to the intestine where they complete their development. Furthermore, natural IELs are characterized by expression of CD8αα homodimers but not CD8αβ heterodimers. Local IL-15 signals induce expression of the transcription factor T-bet and the CD8αα homodimer (Lai et al., 2008; Ma et al., 2009; Klose et al., 2014; Reis et al., 2014). Historically, the identity of the thymic precursor of TCRαβ+CD8αα IELs has been contentious. Although early work suggested preselection double-negative (DN) thymocytes as the direct IEL precursor, more recent studies have conclusively shown IEL progenitors (IELps) arise from double-positive (DP) thymocytes after a high-affinity TCR signal (Leishman et al., 2002; Eberl and Littman, 2004; Ganghadaran et al., 2006). Part of the confusion likely stemmed from the fact that after a high-affinity TCR signal, DP thymocytes...
down-regulate expression of CD4 and CD8 and IELp cells reside within the DP\(^{a,b}\) and DN thymocyte fractions (Gangadharan et al., 2006; McDonald et al., 2014). Within this DN + DP\(^{a,b}\) population, IELp cells display high expression of TCR\(\beta\) and CD5, but only a fraction of this population expresses markers of high-affinity Ag encounter, such as CD122, PD-1, and Helios (Hanke et al., 1994; Gangadharan et al., 2006; Klose et al., 2014; McDonald et al., 2014). Whether the entire TCR\(\beta\)CD5\(^{+}\) population, or only a subfraction thereof, is truly an IELp is unclear. IELp cells are also thought to up-regulate expression of gut-homing molecules like the chemokine receptor CCR9 (Zabel et al., 1999; Mora et al., 2003) and the integrins \(\alpha\beta\); and CD103 within the thymus (Andrew et al., 1996; Guo et al., 2015). Although we are beginning to learn more about the phenotypic characteristics of IELp, the signaling events controlling IELp generation in the thymus are not well understood.

Perhaps the best-defined feature of the IELp population is their expression of self-reactive TCRs. Early work demonstrated an enrichment of “forbidden” superantigen reactive V\(\beta\) clones within the TCR\(\alpha\beta\)CD8\(^{a,a}\) IEL compartment, indicating that signals that would normally result in thymic clonal deletion promoted the development of this population (Rocha et al., 1991; Poussiar et al., 1992). Consistent with high-affinity signals driving both clonal deletion and agonist selection, thymocytes impaired in cell death pathways show dramatically increased numbers of IEL cells and TCR\(\alpha\beta\) CD8\(^{a,a}\) IELs (Pobeizinsky et al., 2012; McDonald et al., 2014). This increase likely occurs as a result of impaired thymocyte clonal deletion. TGF\(\beta\) signaling also promotes agonist selection as TGF\(\beta^{1/-}\)and TGF\(\beta^{R1/-}\) mice lack IELp cells and intestinal TCR\(\alpha\beta\)CD8\(^{a,a}\) IELs (Konkel et al., 2011), possibly because of curbing clonal deletion rather than providing an inductive signal (Ouyang et al., 2010). Finally, thymocytes lacking CD28 costimulatory signals show large increases in numbers of IELp cells and TCR\(\alpha\beta\)CD8\(^{a,a}\) IELs, as the result of clonal diversion of cells normally destined for clonal deletion into the IEL lineage (Pobeizinsky et al., 2012). Collectively, these data support an intimate relationship between the signals that induce clonal deletion and those that promote agonist selection.

Although there is relatively abundant information regarding the molecular mechanisms that regulate positive and negative selection, there is limited information regarding the pathways that differentiate clonal deletion from agonist selection. Furthermore, whether signals that drive conventional positive selection are also required for agonist selection is unclear. One pathway that has long been shown to be differentially involved in positive selection and clonal deletion is the Ras–extracellular signal-regulated kinase (Erk) pathway. Although Erk is not required for clonal deletion (McGargill et al., 2009), high-affinity TCR signaling induces a transient burst of high-intensity Erk activation (Welgen et al., 2003; Daniels et al., 2006). In contrast, positive selection requires sustained Erk activation (McNeil et al., 2005; Daniels et al., 2006). This paradigm is reinforced through analysis of mice lacking RasGRP1, a positive regulator of Ras–Erk signaling in DP thymocytes (Dower et al., 2000; Prielat et al., 2002). Thymocytes from RasGRP1-deficient mice display a block in positive selection, but appear to undergo clonal deletion normally, suggesting that RasGRP1 specifically regulates the duration of Erk activation (Prielat et al., 2002; Kortum et al., 2012). With respect to the relationship between positive selection, clonal deletion and agonist selection, the connecting peptide motif within the TCR\(\alpha\) chain (\(\alpha\)-CPM) was previously shown to be required for positive selection by regulating Erk activation (Bäckström et al., 1998; Welgen et al., 2000). The \(\alpha\)-CPM is also required for agonist selection, but mutation of the \(\alpha\)-CPM does not impact clonal deletion (Leishman et al., 2002). In contrast, it was recently shown that Themis, a molecule important for regulating TCR signal strength and downstream Erk activation leading to positive selection, is not required for either clonal deletion or agonist selection (Lesourne et al., 2012; Fu et al., 2013). Of note, the role of RasGRP1 in the generation of TCR\(\alpha\beta\)CD8\(^{a,a}\) gut IELs has not been investigated. Therefore, although the Ras–Erk pathway is a central discriminator of thymocyte fate during positive selection and clonal deletion, its role and regulation in agonist selection is less clear.

To gain insight into the relationship between positive selection, clonal deletion and agonist selection and the potential role of the Ras–Erk pathway in regulating these fates, we examined thymocyte selection in mice lacking RasGRP1. We observed markedly reduced numbers of both TCR\(\alpha\beta\) CD8\(^{a,a}\) IEL and IELp cells in RasGRP1-deficient mice. Furthermore, of the RasGRP1-deficient IELps that were present, most showed impaired expression of markers of high-affinity antigen encounter. Genetic deletion of the pro-apoptotic protein Bim on the RasGRP1-deficient background rescued the development of IELps, including the percentage of cells that had received a strong TCR signal, suggesting that RasGRP1 is required to transmit survival signals during IELp selection. In addition, we found distinct IELp subsets that varied with respect to cell surface phenotype, age after selection, and ability to generate TCR\(\alpha\beta\)CD8\(^{a,a}\) IELs in vivo. These results provide novel insight into the TCR signaling events that guide agonist selection of IELp cells and highlight the functional heterogeneity of this subset.

**RESULTS**

**RasGRP1 regulates TCR\(\alpha\beta\)CD8\(^{a,a}\) IEL development through control of IELp generation**

To investigate a potential role for RasGRP1 in TCR\(\alpha\beta\) CD8\(^{a,a}\) IEL generation, we examined IEL populations present in small intestines from WT and RasGRP1 KO (1KO) mice. Within the CD45\(^{+}\)CD4\(^{-}\) compartment, 1KO mice showed significantly reduced frequencies and numbers of TCR\(\alpha\beta\)CD8\(^{a,a}\) IELs relative to WT mice (Fig. 1, A and B). Additionally, greater frequencies and numbers of TCR\(\gamma\delta\)CD8\(^{a,a}\) IELs were isolated from 1KO mice relative to WT, suggesting that
RasGRP1 is not required for TCRγδ⁺CD8αα IEL development (Fig. 1, A and B).

Because thymic progenitors are known to give rise to TCRαβ⁺CD8αα IELs, we examined thymocytes from WT and 1KO mice for the presence of IELps. Based on several previous studies (Gangadharan et al., 2006; Klose et al., 2014; McDonald et al., 2014), we identified IELp cells as having a CD4⁻/dullCD8⁻/dullNK1.1⁻TCRβ⁺CD5⁺ phenotype (Fig. 1 C). Compared with WT mice, 1KO mice contained higher frequencies of CD4⁻/dullCD8⁻/dull thymocytes, but fewer of these cells were NK1.1⁻TCRβ⁺CD5⁺. Similar to the pattern observed in TCRαβ⁺CD8αα IELs, 1KO thymi showed significantly reduced numbers of IELps compared with WT (Fig. 1 D). Collectively, these data suggest that RasGRP1 regulates TCRαβ⁺CD8αα IEL generation through promoting the development of thymic IELp cells.

RasGRP1-deficient IELp cells show signs of impaired TCR signaling

Signaling through CD122, expressed on the cell surface of IELp cells that have reached the small intestine, is required for the IL-15-dependent generation of TCRαβ⁺CD8αα IELs within the gut. Furthermore, CD122 has been shown to be up-regulated after high-affinity antigen encounter in the thymus and is expressed on the surface of a fraction of IELp cells within the thymus (Hanke et al., 1994). Therefore, we wished to determine whether RasGRP1 influenced the frequency of IELps that express CD122. Despite finding markedly reduced numbers of IELps in 1KO thymi, we observed similar frequencies of CD122⁺ and CD122⁻ IELs within WT and 1KO animals (Fig. 2 A and Fig. S1 A). Given that we observed two distinct populations of IELps based on expression of CD122, we divided the IELp population into CD122⁻ and CD122⁺ fractions for subsequent analysis of IELp development.

Because IELp's are generated after strong TCR signaling (McDonald et al., 2014) and RasGRP1 is a key regulator of TCR signaling at various stages of thymocyte development (Prietel et al., 2002; Shen et al., 2011; Golec et al., 2013), we examined the expression of proteins regulated by strong TCR signals. Both the co-inhibitory molecule PD-1 and transcription factor Helios are induced after high-affinity TCR signaling (Baldwin and Hogquist, 2007; Daley et al., 2013). In both WT and 1KO IELps, we observed significantly increased frequencies of PD-1⁺Helios⁺ cells within CD122⁺ IELs compared with CD122⁻ IELps, suggesting that high-affinity signaled IELps were enriched within the CD122⁺ fraction. However, there was a significant reduction in the frequency of PD-1⁺Helios⁺ cells in 1KO mice compared with WT mice in both the CD122⁻ and CD122⁺ IELp fractions (Fig. 2, B and C). Collectively, these data suggest that RasGRP1 deficiency impairs the generation of high-affinity signaled IELps.
RasGRP1 is dispensable for thymocyte clonal deletion but is required for positive selection and transmission of high-affinity TCR signals

To further dissect the involvement of RasGRP1 during thymic selection events, we crossed RasGRP1-deficient mice to the physiological HYcd4 TCR transgenic mouse model to allow examination of thymocytes with a fixed TCR specificity (Baldwin et al., 2005). HYcd4 mice express an MHCII-restricted, male Ag–specific TCR that is recognized by the mAb T3.70. Female HYcd4 mice are a model of positive selection because of a lack of high-affinity Ag expression, whereas male mice provide a model to examine responses to high affinity peptide. As expected from a previous study (Priatel et al., 2002), HYcd4 female mice showed a robust Ag-specific (T3.70+) CD8SP thymocyte population, which was severely reduced in the absence of RasGRP1 (Fig. 3 A). Thymocytes from HYcd4 RasGRP1−/− male mice showed a similar reduction of Ag-specific CD8SP thymocytes compared with their RasGRP1–sufficient counterparts, suggesting that clonal deletion remains intact in the absence of RasGRP1. To directly evaluate clonal deletion, we examined caspase 3 activation within male HYcd4 and HYcd4 RasGRP1−/− T3.70+ thymocytes. Although T3.70+ DPbright thymocytes showed little caspase 3 activation in both HYcd4 and HYcd4 RasGRP1−/− male mice, T3.70+ DP dull thymocytes from both strains showed similar, elevated percentages of active caspase 3+ cells (Fig. 3 B). These data confirm that although RasGRP1−/− is not required for positive selection, it is dispensable for thymocyte clonal deletion, as has been reported previously using conventional TCR transgenic models (Priatel et al., 2002).

We next evaluated the impact of RasGRP1 deficiency on high-affinity TCR signaling in vivo using the HYcd4 TCR transgenic model. As conventional TCR transgenic mice expressing high-affinity Ag largely lack a DP thymocyte population, the use of the HYcd4 model allows the examination of RasGRP1-dependent signaling in DP thymocytes in vivo. As expected, we observed higher frequencies of PD-1+Helios+ thymocytes in T3.70+ DP dull compared with DPbright populations in HYcd4 male mice (Fig. 3 C; Hu and Baldwin, 2015). However, T3.70+ DPbright and DP dull fractions from HYcd4 RasGRP1−/− male mice showed lower PD-1+Helios+ cells (Fig. 3 C). Additionally, we examined the CD4−/dullCD8−/dull compartment of HYcd4 male mice to examine the expression of other proteins induced by strong TCR signaling. We found a predominant population of T3.70+CD5+ thymocytes within the CD4−/dullCD8−/dull population that was substantially reduced in HYcd4 RasGRP1−/− male mice (Fig. 3 D). Furthermore, within the CD4−/dullCD8−/dullT3.70+CD5+ thymocyte compartment, HYcd4 RasGRP1−/− male mice showed reduced frequencies of CD122+ cells relative to their WT counterparts (Fig. 3 D and Fig. S1 B), which was different than what was observed with polyclonal mice. Altogether, these results demonstrate that RasGRP1−/− thymocytes show reduced expression of multiple markers of strong TCR signaling, suggesting that RasGRP1 regulates TCR signaling strength after high-affinity antigen encounter.

Bim deletion rescues the generation of IELps in RasGRP1–deficient mice

Either impairment in the transmission of differentiation signals required for selection and/or an inability of IELps to survive could underlie the defect in IELp generation in 1KO mice. To determine whether increased apoptosis caused the defect in IELp generation, we intercrossed RasGRP1−/− and Bim−/− (BKO) mice to create RasGRP1−/− Bim−/− double KO (DKO) mice. As previously reported, BKO thymocytes were impaired in apoptosis induction and display enhanced selection of IELps (McDonald et al., 2014) compared with WT (Fig. 4, A and B). Deletion of Bim in the 1KO background resulted in the generation of IELps to levels above WT, but...
below BKO mice (Fig. 4, A and B). There was no change in the frequency of CD122− IELps in DKO compared with WT mice, but a rescue in the frequency of CD122+ IELps was observed in BKO mice (Fig. 4, A and B). There was no change in the frequency of CD122+ IELps in DKO compared with WT mice, but a rescue in the frequency of PD-1 +Helios+ cells within the CD122+ fraction (Fig. 4, C and D; and Fig. S1 A). DKO mice contained a similar number of TCRαβ+CD8αα IELs as WT, indicating that 1KO IELps rescued from apoptosis by Bim deficiency could give rise to TCRαβ+CD8αα IELs (Fig. S2). Examination of apoptosis directly ex vivo through active caspase 3 staining revealed consistently higher levels of apoptosis in 1KO IELps in both CD122− and CD122+ fractions compared with WT, but this was not statistically significant (Fig. 4 E). However, it is important to note that the transition from CD122− to CD122+ resulted in a statistically significant decrease in the frequency of active caspase 3+ cells in both WT and 1KO mice, suggesting that CD122− IELps are protected from apoptosis (Fig. 4 E).

**Heterogeneity within the IELp population is highlighted by differences in expression of adhesion molecules and proteins regulated by TCR signal strength**

Given the impact of RasGRP1 deficiency on the number of intestinal TCRαβ+CD8αα IELs (Table 1), we hypothesized that RasGRP1 may further regulate specific subsets of thymic IELps that could give rise to TCRαβ+CD8αα IELs. Therefore, we examined IELp cells for the expression of adhesion molecules α4β7 and CD103 because both integrins have been shown to be induced on IELps within the thymus and may regulate progenitor migration to the gut (Andrew et al., 1996; Guo et al., 2015). Most CD122− IELp cells did not express either α4β7 or CD103 (Fig. 5 A). In contrast, CD122+ IELps contained four distinct populations of α4β7−CD103−, α4β7+CD103−, and α4β7−CD103+ populations being most prominent and α4β7−CD103+ cells constituting a minor fraction (Fig. 5 A). To determine whether the absence of RasGRP1 and/or Bim regulated these IELp subsets, we examined adhesion molecule expression on CD122+ IELps from 1KO and BKO mice. RasGRP1-deficient CD122+ IELps showed increased frequencies, but reduced numbers of the α4β7−CD103− subset, and reduced frequencies and numbers of all other subsets, relative to WT (Fig. 5, A and B). In contrast, BKO IELps showed significantly increased frequencies and numbers of α4β7−CD103− cells relative to WT and reduced frequencies, but similar numbers, of α4β7+CD103+ IELps (Fig. 5, A and B). Collectively, these results suggest the composition of the CD122+ IELp pool is altered in 1KO and BKO thymi, which may account for altered TCRαβ+CD8αα IEL development in these animals.
Because we observed significant differences in \( \alpha \beta \) and CD103 expression between CD122\(^{-} \) and CD122\(^{+} \) IEL\(^{p} \)s and within the different strains of mice, we further analyzed these distinct fractions to gain insight into the relationship between TCR signaling and adhesion molecule expression in these cells. To examine the strength of TCR signaling in CD122\(^{-} \) and CD122\(^{+} \) IEL\(^{p} \)s, we used the Nur77\(^{GFP} \) reporter mouse (Moran et al., 2011). Levels of Nur77 expression downstream...
of TCR stimulation are proportional to the strength of TCR signaling experienced by thymocytes, making this a useful model for studying TCR signal strength. Similar to previously published data using Nur77GFP mice (Moran et al., 2011), we saw that both mature CD4SP and CD8SP thymocytes expressed increased levels of GFP relative to DP thymocytes, with CD4SP thymocytes displaying elevated levels of GFP compared with CD8SP thymocytes (Fig. 5 C). CD122⁺ IELp cells showed a relatively uniform pattern of GFP expression that overlapped both CD4SP and CD8SP thymocytes. The CD122⁺ IELp pool displayed a bimodal pattern of GFP expression, such that one fraction showed similar levels of GFP as DP thymocytes, whereas the other showed higher levels of GFP than CD4SP thymocytes (Fig. 5 C). Given that most
CD122⁺ IELps expressed GFP to levels similar to conventionally selected single-positive (SP) thymocytes, it appears this IELp fraction is enriched in cells that have not received agonist signals. However, CD122⁺ IELps showed a prominent population of cells with greater GFP expression than SP thymocytes, indicating that only the CD122⁺ IELp pool contained cells that have recently received agonist signals.

Given that we observed two distinct populations of CD122⁺ IELps in Nur77GFP mice based on expression GFP, we wanted to examine these fractions for other phenotypic differences, namely expression of αβ, and CD103. CD122⁺GFPhi IELp cells had prominent populations of αβ⁺CD103⁺ cells and αβ⁺CD103⁻ cells with few αβ⁺CD103⁻ cells (Fig. 5 D). The CD122⁺GFPlo IELp fraction also contained a prominent population of αβ⁺CD103⁺, but few αβ⁺CD103⁻ cells. Rather, many CD122⁺GFPlo IELps were αβ⁺CD103⁻. Therefore, CD122⁺GFPlo and CD122⁺GFPhi IELps from Nur77GFP mice show unique expression patterns of αβ and CD103 that may impact their ability to generate TCRαβ⁺CD8αα IELs. This heterogeneity was confirmed using PD-1 expression as an additional marker of high-affinity Ag signaling (Fig. 5 E).

CD122−, αβ⁻, and CD103-expressing subpopulations of IELps display distinct temporal patterns of development

To gain further insight into the development of the heterogeneous IELp pool, we made use of RAG2p-GFP reporter mice (Yu et al., 1999). Because developing thymocytes distinguish RAG expression at the DP stage, GFP expression in mature thymocytes identifies cells that were recently derived from DP. Furthermore, we can measure the time elapsed from precursor DP thymocytes to the progeny cell of interest based on decay of the GFP signal (∼54-h half-life; McCaughtry et al., 2007). Similar to a previous study (McCaughtry et al., 2007), most CD4SP and CD8SP thymocytes were GFP⁺, with CD8SP thymocytes expressing less GFP (mean fluorescence intensity [MFI]; Fig. 6 A). For agonist selected lineages, ∼50% of Treg and 20% of inNKT cells were GFP⁺, and nascent tTreg and inNKT were similar in age to CD4SP thymocytes (Fig. 6 A). Similar to tTreg, ∼50% of CD122⁺ IELps were GFP⁺, again suggesting heterogeneity within this population (Fig. 6 A, top). Furthermore, nascent CD122⁺ IELps displayed similar MFIs of GFP expression as CD8SP thymocytes, suggesting similar developmental kinetics (Fig. 6 A, middle and bottom). In contrast, CD122⁺ IELps showed similar frequencies of GFP⁺ cells and similar GFP MFIs as DP thymocytes. These data suggest that the CD122⁺ IELp fraction is very recently derived from DP and appears to be immature relative to other post-selection populations.

Given the presence of two distinct CD122⁺ IELps based on Rag2p-GFP expression, we next examined αβ and CD103 expression in these subsets (Fig. 5, A and B). The majority of αβ⁺CD103⁺ and αβ⁺CD103⁻ cells were GFP⁺, ∼50% of αβ⁺CD103⁻ cells were GFP⁺, and only ∼10% of αβ⁺CD103⁺ cells were GFP⁺ (Fig. 6 B, top). These results indicated that the majority of αβ⁺CD103⁻ and αβ⁺CD103⁺ CD122⁺ IELps are recently derived from DP thymocytes, whereas only half of αβ⁺CD103⁻ cells are nascent and the majority of αβ⁺CD103⁺ cells are aged. Within the GFP⁺ populations, we further analyzed GFP MFIs to determine temporal patterns of development. GFP⁺ αβ⁺CD103⁻ cells were the youngest in the CD122⁺ IELp pool, followed by αβ⁺CD103⁻ cells, αβ⁺CD103⁻ cells, and finally αβ⁺CD103⁻ cells (Fig. 6 B, middle and bottom). These results suggest that the CD122⁺ IELp pool contains distinct subpopulations of cells with unique temporal patterns of development.

Using the RAG2p-GFP model, we defined distinct subpopulations of IELp cells with unique temporal signatures that may represent cells at various stages of maturity. Ultimately, for IELp cells to traffic to intestinal tissues, they must first exit the thymus. Before export, conventional thymocytes up-regulate the S1P receptor, S1P1, to promote thymocyte egress (Matloubian et al., 2004; Xing et al., 2016). Therefore, we measured the expression of S1P1 on the surface of IELp subsets by flow cytometry (Fig. 6 C). We observed significantly increased levels of S1P1 expression on CD122⁺ IELp cells relative to CD122⁻ IELps. Furthermore, CD122⁺ IELps had a significantly lower MFI of S1P1 expression relative to DP thymocytes, which themselves do not exit the thymus under physiological conditions. We next examined S1P1 expression on αβ and CD103-expressing subpopulations of CD122⁺ IELps. We found that αβ⁺CD103⁻ and αβ⁺CD103⁺ cells both showed significantly higher S1P1 MFIs compared with either αβ⁺CD103⁻ or αβ⁺CD103⁺ counterparts. However, all of the CD122⁺ IELp subpopulations examined showed substantially higher S1P1 expression than DP thymocytes or CD122⁺ IELps, suggesting that all of these populations may have the potential to egress from the thymus.

αβ⁻ and CD103-expressing subpopulations of IELps show distinct patterns of IEL development in vivo

Given that the IELp pool is heterogeneous in nature, we sought to determine whether different IELp subpopulations had differing abilities to generate TCRαβ⁺CD8αα IELs. Because the CD122⁺ IELp population showed protection from apoptosis (Fig. 4 D), expressed proteins induced by high-affinity TCR signaling (Fig. 2, B and C; and Fig. 5, C–E), contained subsets that expressed the adhesion molecules αβ, and CD103 (Fig. 5 A), and expressed higher levels of egress receptor S1P1 than CD122⁻ IELps (Fig. 6 C), we focused our analysis on prominent subpopulations within the CD122⁺ IELp fraction. We chose to examine CD122⁺PD-1⁺αβ⁺CD103⁻ IELps and CD122⁺PD-1⁻αβ⁺CD103⁻ IELps because they represented two of the major subsets of IELs, were mostly homogeneous from an “age” perspective, and were polar opposites with respect to age and cell surface phenotype. We sorted these IELp populations from CD45.1⁺ or CD45.1/2⁺ donors, mixed these populations at a 1:1 ratio, co-adoptively transferred them into RAG1−/− recipient mice (CD45.2⁺), and examined
the IEL compartment 6 wk after transfer. At the time of harvest, TCRαβ+ IELs from both CD122+PD-1+α4β7−CD103+ and CD122+PD-1−α4β7−CD103+ IELp donors were clearly detectable in recipient mice (Fig. 7 A, left). However, the PD-1−α4β7−CD103+ subset showed almost complete skewing toward TCRαβ+CD8αα IEL development, whereas PD-1−α4β7−CD103+ cells showed nearly exclusive development of TCRαβ+CD8αα IEL (Fig. 7 A, middle, right). Therefore, it appears that despite both residing within the CD122+ IELp fraction, PD-1−α4β7−CD103+ and PD-1−α4β7−CD103+ cells showed distinct patterns of IEL development.

Because we found that CD122+PD-1−α4β7−CD103+ IELp cells did not give rise to TCRαβ+CD8αα IELs, we tested the ability of another CD122+ IELp subset to give rise to intestinal IEL populations in vivo. We chose PD-1−α4β7−CD103+ cells to compete against the PD-1−α4β7+CD103− fraction because they constituted a relatively major fraction of IELp cells (Fig. 4) and were “younger” than α4β7−CD103+ cells.
(Fig. 6 B). Both PD−1αβ−CD103− and PD−1αβ−CD103− donor cells predominantly gave rise to TCRαβ−CD8αα IELs (Fig. 7 B, middle, right), suggesting that TCRαβ−CD8αα IEL lineage potential is not limited to the CD122+PD−1αβ−CD103− IELp fraction.

**Human thymi contain IELp phenotype cells**

Although TCRαβ−CD8αα IEL cells have been identified in human intestine (Latthe et al., 1994), it is unclear whether human thymi contain IELp cells. We examined human thymi for the presence of thymocytes with an “IELp” phenotype. Similar to IELps in mice, within the CD4−/dullCD8−/dull fraction, a robust population of TCRβ+CD5+ cells was found (Fig. 8, A and C). However, in contrast to mouse, human CD4−/dullCD8−/dull TCRβ+CD5+ thymocytes showed a near absence of CD122-expressing cells (Fig. 8, B and C). Despite showing a lack of CD122-expressing cells, human CD4−/dullCD8−/dull TCRβ+CD5+ thymocytes showed other attributes consistent with mouse IELps, including a small fraction of PD−1−Helios+ cells (Fig. 8, B and C). Furthermore, within the CD4−/dullCD8−/dull TCRβ+CD5+ population, we observed a near absence of CD103-expressing cells (Fig. 8, B and C), but a prominent population of β7−CD103− cells. These cells resemble the mouse αβ−CD103− IELp population that gave rise to TCRαβ−CD8αα IELs.

**DISCUSSION**

We have identified RasGRP1 as a critical regulator of natural TCRαβ−CD8αα IEL development through its control of agonist selection and IELp generation in the thymus. Given that RasGRP1 is required for conventional thymocyte positive selection in response to low-affinity TCR signaling and is also required for iNKT (Shen et al., 2011) and TCRαβ−CD8αα IEL development in response to high-affinity TCR stimuli, there is clearly an overlap between the signal transduction molecules that regulate positive selection and agonist selection. These data suggest a global requirement for RasGRP1 in clonal deletion versus IELp generation, both of which require strong TCR signals in the thymus. Whether the genetic programs induced by RasGRP1 from either weak or strong TCR selection signals in the thymus suggests that RasGRP1 signaling is pro-survival rather than fate specifying in this context. As such, these data provide a possible explanation for the differential requirement of RasGRP1 in clonal deletion versus IELp generation, both of which require strong TCR signals. For clonal deletion, an active apoptotic program is initiated, and therefore pro-survival signaling mediated by RasGRP1 would be detrimental to this outcome. In contrast, IELp generation requires survival of the cells that receive a strong TCR signal, and under these conditions, RasGRP1 appears to be required to deliver these signals. However, it should be noted that RasGRP1−/− Bim−/− mice show reduced numbers of IELPs compared with Bim−/− mice, suggesting a role for RasGRP1 signaling outside of survival.

The primary function of RasGRP1 is to activate the highly conserved signal transduction protein Ras. The activation kinetics of the Ras–ERK cascade are differentially regulated during thymocyte positive and negative selection.
During positive selection, thymocytes show a sustained increase in ERK activation whereas thymocytes undergoing negative selection show a strong, but transient, burst of ERK activation (Mariathasan et al., 2001; McNeil et al., 2005; Daniels et al., 2006). Importantly, numerous studies have shown that ERK activity is dispensable during thymocyte negative selection but is required for efficient thymocyte positive selection (Werlen et al., 2000; McNeil et al., 2005; McGargill et al., 2009). Given the requirement for RasGRP1 in agonist selection of both iNKT (Shen et al., 2011) and IELPs, ERK activity is likely important for thymocyte agonist selection generally.

Consistent with the above-described pattern of Erk activation, thymocytes undergoing positive selection show numerous, brief serial interactions with stromal cells over the course of days, whereas thymocytes undergoing negative selection show relatively stable interactions with stromal cells over the course of hours (Melichar et al., 2013). Because the strength of signal that drives clonal deletion and agonist selection are similar, one might predict that those cells undergoing agonist selection also engage in stable interactions with APC. However, given that IELPs have a relative age similar to CD8SP, there must be mechanisms in place to regulate the outcome of the interactions with APC, that being differentiation or death. One such regulatory mechanism could be the CD28-B7 signaling pathway (Pobezinsky et al., 2012). Alternatively, or in addition to, it is possible that it is the nature of the APC that regulates the outcome of these interactions. For example, in the HYαβ model, thymocytes undergoing apoptosis are positioned closely to DCs in the thymus, and restricting male Ag expression to cTEC or radio-resistant APC impaired apoptosis induction (McCaughtry et al., 2008). Given that agonist-selected cells do not undergo apoptosis during development, it is likely that thymocyte agonist selection does not involve stable interactions with apoptosis-inducing DCs, but rather involves interactions with cTEC cells that are less potent inducers of clonal deletion. Whether the nature of the APC presenting high-affinity Ag regulates IELP development requires investigation.

Distinct CD122+ and CD122− populations make up the “IELp” fraction and highlight the diversity of cells present within this pool. In polyclonal mice, RasGRP1 did not appear to regulate the fraction of IELPs that expressed CD122, whereas in the HYαβ model, RasGRP1-deficient males displayed reduced frequencies of CD122+ IELPs. The reason for this difference is currently unclear. CD122+ IELPs lacked expression of proteins induced by strong TCR signaling and showed high frequencies of cell death, whereas CD122− IELPs were enriched in cells expressing markers of high-affinity antigen encounter and were largely protected from apoptosis. Furthermore, using RAG2p-GFP animals, we found that CD122+ IELPs were similar in age to immature DP cells, whereas CD122− IELPs showed a similar age as post-selection CD8SP thymocytes. In addition, CD122+ IELPs lacked expression of egress receptor S1P1, whereas CD122− IELPs showed robust expression of S1P1, consistent with the known phenotype of mature post-selection cells (Xing et al., 2016). Of note, McDonald et al. (2014) recently determined that in TCR transgenic mice that express a TCR cloned from TCRαβ CD8αα IELs, almost all recent thymic emigrants expressed CD122 and most were αβ+. Collectively, these data suggest that CD122 expression marks an important transition in the development and maturation of IELPs. Of note, CD122 expression per se may not be important for IELp thymic development as IL-15−deficient thymi contain similar numbers of IELPs (Klose et al., 2014) and are able to reconstitute the IEL compartment of nude mice (Lai et al., 2008).

Although CD122 expression appears to separate distinct IELP fractions, expression of αβ and CD103 revealed an additional layer of diversity within the IELPs. Notably, CD122− IELP cells lacked αβ− and CD103-expressing
cells altogether, whereas CD122+ IELps showed four distinct populations of αβ- and CD103-expressing progenitors. Although RasGRP1-deficient IELps largely lacked expression of αβ- and CD103, it is unclear whether this is caused by RasGRP1-dependent signal transduction or increased apoptosis of the progenitor. Using PD-1 and Nur77-GFP as readouts of TCR signaling, we found that αβ- expression was associated with CD122+ cells showing recent high-affinity TCR signaling, whereas CD103 expression was enriched on cells lacking signs of recent TCR signaling (Fig. 5, D and E). Additionally, analysis of Rag2p-GFP animals revealed that nascent αβ-CD103+ were the youngest of the CD122+ IELp pool, whereas αβ-CD103- and αβ-CD103+ were slightly older and αβ-CD103+ cells were especially aged. Testing the significance of αβ- and CD103 expression in vivo revealed that both PD-1αβ-CD103- and PD-1αβ-CD103+ developed into TCRαβ+CD8αα IELs almost exclusively. However, PD-1αβ-CD103+ gave rise to TCRαβ+CD8αβ IELs. These data suggest that PD-1 expression correlates well with the ability to differentiate into TCRαβ+CD8αα IELs. Importantly, however, S1P1 expression analysis showed that αβ-CD103+ cells expressed significantly higher levels of S1P1 than αβ-CD103- cells, making it tempting to speculate that the CD122+PD-1αβ-CD103+ IELp subset is the population that emigrates out of the thymus under physiological conditions. Data from McDonald et al. (2014) support this finding.

Recently, a study from Ruscher et al. (2017) also examined the thymic IELp compartment using a T-bet reporter mouse. The findings of our study reported here and the Ruscher study are largely congruent. Using the T-bet reporter mouse. The findings of our study reported here and the Ruscher et al. (2014) support this finding. Under physiological conditions. Data from McDonald et al. (2017) when compared with the data described here. We found that sorted CD122+PD-1αβ-CD103+ IELps adoptively transferred to Rag-/- recipients gave rise to TCRαβ+CD8αβ IELs, whereas purified CD122+PD-1- (type B) IELps generated TCRαβ+CD8αα IELs. We think it is likely that different sorting strategies resulted in different outcomes for these seemingly similar populations of IELps. In particular, our sorting strategy involved isolating NK1.1+ IELps, whereas Ruscher et al. (2017) used CD11d-tetramer to specifically exclude INKT cells. Furthermore, we positively sorted on CD303-expressing IELps, whereas Ruscher et al. (2017) did not discriminate between CD103+ or CD103- fractions, both of which are present in the type B IELps. Therefore, it appears that the composition of the adaptively transferred cells was substantially different in the two studies and resulted in the disparate findings. Finally, the other major difference was Ruscher et al. (2017) used a mixture of DN thymocyte competitors, whereas we directly competed different IELp populations in the adoptive transfer.

Previous work has identified the presence of CD8αα-expressing αβ T cells within the human intestine (Latthe et al., 1994). However, whether these cells resemble TCRαβ+CD8αα IELs in either their development or functionality remains unclear. We found a human thymocyte population that resembled murine IELp cells. This included expression of PD-1 and Helios and expression of β7 without CD103. Human IELps lacked CD103+ cells, which made up a significant portion of the mouse IELp compartment. Furthermore, human IELps did not appear to express CD122. Although the developmental origin of TCRαβ+CD8αα IELs in humans has not been strictly examined, these results suggest that human thymi contain cells that largely recapitulate the phenotype of murine IELps. In fact, it was recently reported that human thymus contains a distinct population of PD-1- thymocytes that appear to undergo agonist selection (Verstichel et al., 2017). Further studies are required to determine whether human TCRαβ+CD8αα IELs originate from a thymic progenitor similar to mice.

The functional heterogeneity of the IELp population is defined by the TCR signaling events experienced by developing thymocytes. RasGRP1-mediated signals are clearly required for efficient generation of IELps, and RasGRP1-deficient IELps show signs of impaired high-affinity TCR signaling. Furthermore, the expression of markers of TCR signaling strength by IELp cells was associated with their thymic expression of CD122 and of adhesion molecules αβ- and CD103. IELp cells showing signs of recent high-affinity TCR signaling are skewed toward expression of αβ- and were high fidelity progenitors of TCRαβ+CD8αα IELs. The requirement of RasGRP1 activity during agonist selection separates the signaling requirements during agonist selection and clonal deletion. These results provide critical insight into the signaling events that drive TCRαβ+CD8αα IEL development, but the precise outcome of RasGRP1-mediated signals in agonist-selected thymocytes that specify the development of IELps is unclear. One possibility is the induction of a lineage-specifying transcription factors. Future work examining the transcriptional landscape of the specific IELp that gives rise to TCRαβ+CD8αα IELs will be required.

MATERIALS AND METHODS

Mice

The generation of RasGRP1 KO (Dower et al., 2000), Bim KO (Bouillet et al., 1999), HYα4 (Baldwin et al., 2005), Nur77GFP (Moran et al., 2011), RAG2p-GFP (Yu et al.,
Antibodies and flow cytometry

Fluorochrome-conjugated and biotinylated Ab were purchased from eBioscience, BioLegend, or BD PharMingen. Active caspase 3 Ab was purchased from Cell Signaling Technologies. Cells were stained with Ab cocktails in FACS buffer (PBS, 1% FCS, 0.02% sodium azide) for 30 min on ice. Cells were washed twice with FACS buffer after primary and secondary Ab staining. For intracellular Ag staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Foxp3 Staining Buffer Set (eBioscience). S1P1 antibody was purchased from R&D Systems, and surface staining was performed using the Fix/Perm method. For intracellular staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Fix/Perm method. For intracellular staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Fix/Perm method. For intracellular staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Fix/Perm method.

IEL preparations

Small intestines were removed from mice and cleaned, and Peyers' patches were excised. Intestinal pieces were placed in Ca²⁺/Mg²⁺-free HBSS supplemented with 5% FCS, 5 mM EDTA, and 2 mM DTT and shaken at 37°C for 30 min. After incubation, cell-containing media was passed through 70-µm cell strainers, and cells were pelleted. Cell pellets were resuspended in 40% Percoll, layered on top of an 80% Percoll solution, and the gradient was centrifuged at 900 g for 20 min. IELs were extracted from the 40%/80% interface, washed in PBS, and used for analysis. Flow cytometry and CountBright beads (Life Technologies) were used for quantification.

Adoptive transfer experiments

Thymocytes were obtained from congenic CD45.1⁺ and CD45.1/2⁺ donor mice and stained as described in Antibodies and flow cytometry. Cells were stained with Ab cocktails in FACS buffer (PBS, 1% FCS, 0.02% sodium azide) for 30 min on ice. Cells were washed twice with FACS buffer after primary and secondary Ab staining. For intracellular Ag staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Foxp3 Staining Buffer Set (eBioscience). S1P1 antibody was purchased from R&D Systems, and surface staining was performed using the Fix/Perm method. For intracellular staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Fix/Perm method. For intracellular staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Fix/Perm method. For intracellular staining, cells were treated with BD Cytofix/Cytoperm (BD Bioscience) or the Fix/Perm method.

Human thymus

Human thymus were collected from patients (age 10.8 mo ± 17.5 mo, range 0.2–42 mo) during pediatric surgery at British Columbia Children’s Hospital, Vancouver. Thymic tissue was manually dissociated in RPMI complete media (RPMI 1640; Gibco) containing 1% GlutaMAX (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 10% heat-inactivated fetal bovine serum (Gibco). After filtering and washing, cells were stained for flow cytometry. Collection of human tissue was approved by the University of British Columbia Clinical Research Ethics Board (Vancouver, Canada). Informed consent was obtained in accordance with local regulatory guidelines.

Online supplemental material

Fig. S1 is a compilation of the frequency of CD122⁺ IELs in the indicated mice. Fig. S2 shows that Bim deletion rescues the generation TCRαβ⁺ CD8αα⁺ IELs in RasGR1⁺/− deficient mice.

ACKNOWLEDGMENTS

The authors thank the Faculty of Medicine and Dentistry flow cytometry core and Health Sciences Lab Animal Services at the University of Alberta for support. Mr. Bing Zhang for technical assistance, Drs. Qian Nancy Hu and Sylvie Lesage for critical reviews, and Drs. Roland Ruscher and Kristin Hogquist for feedback and discussions. We are grateful to Dr. Colin Anderson for providing RAG2–GFP and RAG1⁻/⁻ mice for our experiments. In addition, we would like to thank Dr. Andrew Campbell and the surgical staff at the University of British Columbia Children’s Hospital for collection of pediatric thymus.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (418161 to T.A. Baldwin), the Canadian Institutes of Health Research (CIHR; MOP 86595 to T.A. Baldwin), and the Canadian National Transplant Research Program (ITU 127880 to M.K. Levings). D.P. Golec holds an Alberta Innovation Grant (TFU 127880 to M.K. Levings). D.P. Golec and T.A. Baldwin analyzed the data and wrote the manuscript. The authors declare no competing financial interests.

Author contributions: D.P. Golec, R.E. Hoeppli, M.K. Levings, and T.A. Baldwin designed the experiments. D.P. Golec, R.E. Hoeppli, M.K. Levings, and T.A. Baldwin performed the experiments. D.P. Golec and T.A. Baldwin analyzed the data and wrote the manuscript.

Submitted: 12 May 2017
Revised: 16 June 2017
Accepted: 20 June 2017

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Regulation of thymic IELp development by RasGRP1 | Golec et al.


