ROLE OF RASGRP1 IN T CELL DEVELOPMENT AND FUNCTION

by

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ABSTRACT

The unique feature of the thymus is to establish the discrimination between self and non-self and providing immunity to foreign peptides, particularly those derived from pathogens, that are presented by self-major histocompatibility complex (MHC) molecules. The development and activation of T lymphocytes require many critical decisions that profoundly affect their differentiation and ability to mount an effective immune response. A central mediator of this developmental program is the small GTPase Ras, emitting cytoplasmic signals through downstream MAPK pathways and eventually affecting gene expression. TCR signal transduction orchestrates the activation of Ras by integrating at least two Ras-guanyl nucleotide exchange factors, RasGRP1 and Sos. RasGRP1 is especially critical for the survival and differentiation of developing thymocytes whereas negative selection of thymocytes bearing an autoreactive TCR appears to be RasGRP1 independent. In this thesis, by using RasGRP1 null mutant mice, we characterized the role of RasGRP1 in conventional and regulatory T cell (Treg) development, as well as the consequential effects that result from its deficiency in peripheral tolerance, T cell activation, proliferation and immune response against infections. Investigations on RasGRP1$^{-/-}$ mice expressing a transgenic (Tg) MHC class II-restricted TCR revealed that RasGRP1 transmits differentiation signaling critically required for conventional CD4 T cell development. As a result of the impaired developmental program, RasGRP1$^{-/-}$ mice spontaneously acquire an acutely activated and proliferating CD4 T cell population that exhibits characteristics of T cell exhaustion. Interestingly, studies using MHC class I-restricted TCR Tg mice lacking RasGRP1 suggest that RasGRP1 is essential for lowering activation threshold and achieving optimal cytokine receptor expression on conventional CD8 T cells upon activation. We also investigated the consequence of RasGRP1 deficiency on the development and function of regulatory T cells. We found that RasGRP1 differentially affected the development and function of CD4 and CD8 regulatory T cells. Overall, our studies provide novel insights to the significance of RasGRP1 signaling pathway in T cell development and function, along with how its deficiency affects the development and function of regulatory T cells.

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LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
Ag	antigen
AIRE	autoimmune regulator gene
APC	antigen-presenting cell
BCR	B cell receptor
CCR	CC-chemokine receptor
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
CXCR	CXC-chemokine receptor
DAG	diacylglycerol
DN	double-negative (CD4$^-$CD8$^-$) thymocyte
DP	double-positive (CD4$^+$CD8$^+$) thymocyte
ERK	extracellular-regulated kinase
GTP	guanosine-5'-triphosphate
HSA	heat stable antigen
IFN-γ	interferon gamma
IL-2	interleukin 2
JNK	jun-N-terminal kinase
LAT	linker of activated T cells
LCMV	lymphocytic choriomeningitis virus
LLO	listeriolysin-O
LM	Listeria monocytogenes
mAbs	monoclonal antibodies
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
NK	natural killer
OVA	ovalbumin
PD-1	programmed cell death

PD-L1	PD-1 ligand 1
PLCγ1	phospholipase Cγ1
PM	plasma membrane
PMA	phorbol 12-myristate 13-acetate
RAG	recombinase activating gene
Ras GAPs	Ras GTPase-activating proteins
Ras GEFs	Ras-guanyl nucleotide exchange factors
SLE	systemic lupus erythromatosus
Sos	son-of-sevenless
SP	single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocyte
TCR	T cell receptor
TECs	thymic epithelial cells
Tg	transgenic
TGF-β	tumor growth factor-β
TNF	tumor necrosis factor
Treg	T regulatory cell
ZAP-70	zeta-associated protein of 70 kDa

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CO-AUTHORSHIP STATEMENT

For the co-authored chapter 2, I contributed 70% of the experimental design, 80% of the research and data analysis, 30% of the manuscript preparation. For the co-authored chapter 3, I contributed to 90% of the experimental design, 70% of the research and data analysis, 50% of the manuscript preparation. For the co-authored chapter 4, I contributed to 60% of the experimental design, 40% of the research and data analysis, 30% of the manuscript preparation. For the co-authored chapter 5, I contributed all of the experimental design, 95% of the research, all the data analysis, and wrote the manuscript.

CHAPTER 1   INTRODUCTION

1.1   Preface

This thesis represents a culmination of work and learning that has taken place over a period of almost five years (2004-2009) at Department of Microbiology and Immunology, University of British Columbia. The initiative of this study came from our preliminary data published in Immunity (2002) by the senior scientist Dr. John Priatel in our lab. The foundation for this thesis is the strength of signal hypothesis, which argues that weak signals lead to the selective activation of ERK/MAPK and positive selection whereas strong signals lead to the activation of full range of MAPK and negative selection. The Immunity paper suggests that the Ras-guanyl nucleotide exchange factor, RasGRP1, serving as a critical TCR signaling module, plays a relatively minor role in CD8 T cell development. Based on the strength of TCR signals model, we followed the logical trend of investigating the role of RasGRP1 in CD4 T cell development (chapter 2). Due to the importance of T cell development in establishment of central tolerance and maintenance of functional T cell population in the periphery, studies were performed to understand how the impaired T cell development resulted from RasGRP1 deficiency would affect peripheral T cell homeostasis and function (chapter 3). Furthermore, the relatively normal positive selection of thymocytes expressing a strongly selecting TCR (2C) lacking RasGRP1 offered us the unique opportunity to investigate the role of RasGRP1 in peripheral naïve CD8 T cell activation (chapter 4). Finally, since accumulating evidence suggest that T regulatory cells are an essential force in suppressing autoreactive T lymphocyte and acting as a safeguard for peripheral tolerance, we characterize the role of RasGRP1 in the development, homeostasis and function of different T regulatory subsets (chapter 5). In this first chapter I provide a general introduction to T cell biology, as well as a survey of the latest scientific discovery.

1.2   T cell development

1.2.1   Generation of CD4$^+$CD8$^+$ immature thymocytes

The thymus is an organ that supports the differentiation and selection of T cells. Mammalian T cells of diverse functional types share a complex developmental history. They originate from pluripotent precursors in the bone marrow or fetal liver and migrate to the thymus to begin a protracted differentiation process in which they gradually acquire T cell characteristics (1). Relatively few T cell progenitors migrate into the thymus per day, but they respond to this new environment by proliferating extensively, while initiating the T cell differentiation transcriptional program (2-4) and gradually turning off genes that allow differentiation to non-T-cell lineages. They then undergo TCR gene rearrangements and assemble TCR complexes, upon which their future survival and functions will depend (1). These cells can then mature into different T cell lineages, including γδ T cells and αβ T cells. The αβ T cells further diverge into different sub-lineages, such as CD4$^+$ T cells, CD8$^+$ T cells, natural killer T (NKT) cells and regulatory T cells (Treg cells), each of which have greatly differing functions once they emigrate from the thymus to the periphery.

The thymic development of T cells consists of several processes that require that dynamic relocation of developing lymphocytes into, within and out of the multiple environments of the thymus (Fig. 1.1) (5). It all begins when the lymphoid progenitor cells make their way into the thymus through blood vessels at the cortico-medullary junction (2). In the thymus, these cells lose the potential to become B cells and natural killer (NK) cells (6). The result is a CD4$^-$CD8$^-$ double-negative (DN), committed-T cell precursor. DN thymocytes then go through a sequential differentiation process characterized by the acquisition and loss of their surface expression of CD44 and CD25: DN1, CD44$^+$CD25$^-$; DN2, CD44$^+$CD25$^+$; DN3, CD44$^-$CD25$^+$; and DN4, CD44$^-$CD25$^-$ (7). This stepwise progress is accompanied with the crosstalk between developing thymocytes and thymic stromal cells, as well as the thymocyte trafficking including the outward migration of DN thymocytes to the capsule regulated by chemokine signals through CXC-chemokine receptor 4 (CXCR4) and CC-chemokine

receptor 7 (CCR7) followed by the further migration to subcapsular region mediated by CCR9 signals (5).

Double-negative T cells can give rise to either γδ or αβ TCR-expressing cells (8). For cells that proceed along the αβ TCR pathway, DN3 stage is thought to be a developmental checkpoint (7, 9-11). Development through the first "pro-T-cell" stages – that is, from the early T-cell-lineage progenitor (ETP) stage to DN3 stage – is independent of the TCR (12). ETPs and DN2 cells proliferate extensively while acquiring their first T cell characteristics. As the cells reach the DN3 stage, they first express pre-TCR-α (pTα), which is encoded by a non-rearranging locus and used as a surrogate α chain (7, 9, 10). pTα then pairs with the TCR β-chain, which is the product of a set of somatic DNA rearrangements that require expression of recombination activating gene 1 (RAG1) (13) and RAG2 (14) protein. Defects in *Rag* gene expression prevents β-selection and hence, pre-TCR assembly, leads to developmental arrest at the DN3 stage (6). At the cell surface, the pre-TCR is then associated with a collection of proteins (the CD3ζ complex) that is involved in signal transduction (15). Alternatively, DN3 T cells that successfully rearrange TCR γ- and δ-chains instead of β-chains develop into γδ T cells (Fig. 1.2).

T cells that emerge from β-selection (late DN3 and DN4) undergo 6-8 cell divisions, after which gene rearrangement at the TCR-α locus produces the second component chain of the mature αβ antigen receptor. The expression of pTα is lost during this stage, which results in the cell-surface display of a low level of αβ TCR assembled with CD3/ζ proteins. The thymocytes also begin to turn on the expression of both CD4 and CD8 coreceptor proteins – most often CD8 first, followed by CD4 (8) – and eventually, form a large population of CD4^{+}CD8^{+} double-positive (DP) αβ-TCR-expressing immature cells that constitute 90% of the lymphoid compartment in the thymus of young individuals (6, 7). At this time, the DP thymocytes are prepared for the next stage of developmental program: TCR-mediated selection.

1.2.2 Fate decision of DP thymocytes

DP thymocytes are unique among T-lineage cells in that they express both CD4 and CD8 coreceptors. CD4 and CD8 coreceptors are transmembrane proteins with extracellular domains that promote TCR engagement of MHC ligands and intracellular domains that enhance TCR signal transduction. As a result, CD4 and CD8 are molecules that promote signaling by MHC-restricted TCRs (16). DP thymocytes that are the product of β-selection undergo a series of TCR-mediated selection to test if their newly rearranged receptor has some reactivity to self-peptide in the context of self-MHC molecule, so that the subset best suited to function in the host environment is permitted to mature and migrate to peripheral lymphoid tissues. Four distinguishable processes characterize this selection – death by neglect, negative selection, positive selection and lineage-specific development (also referred as lineage commitment) (8, 17). Most DP thymocytes express TCRs that interact so poorly with the available self-peptide-MHC ligands, so that the intracellular signals that are required to sustain viability are not generated, die as a result of death by neglect (18, 19). By contrast, a small proportion of thymocytes that bear TCRs that bind very well to self-ligands could cause autoimmune pathology if they were permitted to develop and leave the thymus. Thus, signaling on engagement of these TCRs with self-peptide-MHC ligands promotes rapid apoptotic death, a process referred to as negative selection. Only a small fraction of DP thymocytes, which express TCRs of low to intermediate avidity for self-peptide-MHC complexes, initiate a multi-step process known as positive selection that ultimately results in lineage-specific differentiation in either $CD4^+$ or $CD8^+$ single-positive (SP) mature T cells (Fig. 1.2) (20, 21).

For developing thymocytes to experience the selecting forces at the right places and in the right order, the developing thymocytes and thymic stromal cells have to communicate with each other both in close proximity and remotely (2). In thymus, the cortex contains DP $CD4^+CD8^+$ thymocytes that are subject to T cell selection events, whereas in the thymic medulla, accumulating SP $CD4^+$ and $CD8^+$ T cells are screened further for self reactivity before their exit from the thymus (5). Stromal cells consisting of various cell types are present in the thymus, including thymic epithelial cells (TECs) and bone marrow-derived

cells such as dendritic cells (DCs) and macrophages. The TECs are present in both cortical and medullary thymic areas, both of which can express MHC class I and MHC class II molecules. The cortical TECs (cTECs) are efficient mediators of positive selection (22). By contrast, abnormal medullary TECs (mTECs) development and organization are often associated with autoimmunity, which might be due to the impaired development of Treg cells (23, 24) or failed expression of autoimmune regulator gene (AIRE) (2, 25). Cells of bone marrow origin are involved in shaping the developing T cell repertoire through efficient clonal deletion (negative selection) of self-reactive thymocytes by dendritic cells and elimination of apoptotic thymocytes by macrophages (5).

1.2.3 Models of CD4/CD8-lineage choice

DP thymocytes that have been positively selected ultimately develop into either $CD4^+$ or $CD8^+$ SP T cells, with their precise lineage fate being determined by the MHC-restriction specificity of their TCR. In the peripheral lymphoid tissues, mature $CD4^+$ T cells function as T helper cells when activated, assisting effector components of the immune system (such as B cells) through cytokine secretion and up-regulation of expression of specific membrane ligands, whereas mature $CD8^+$ T cells are can acquire cytotoxic properties after activation, allowing them to directly kill target cells expressing their cognate antigen. As $CD4^+$ and $CD8^+$ T cells are derived from a common precursor pool of DP thymocytes, the question arises as to how the concordance between MHC specificity and CD4/CD8-lineage choice is established.

The CD4/CD8-lineage choice was classically thought to result in the transcriptional termination of one or the other coreceptor gene as a consequence of the same TCR signaling event that mediates positive selection (6, 16). All classical models of CD4/CD8-lineage choice incorporate this perspective and fall into two main categories, 'stochastic' or 'instructive', which differ depending on whether termination of coreceptor transcription is random or instructed (16). The stochastic selection model proposes that lineage choice is independent from MHC/TCR signals. According to this model, the termination of coreceptor

gene expression during positive selection of DP thymocytes occurs randomly and that a second TCR-dependent rescue step occurs after positive selection, so that only SP thymocytes with matching TCRs and coreceptor survive and differentiate into mature T cells (26-28) (Fig. 1.3A). Alternatively, the instructive models postulate that during positive selection, TCR signals direct DP thymocytes to specifically terminate the expression of mismatching coreceptor molecule (29, 30). Consequently, instructive models require that MCH class I- and class II-restricted TCR signals be distinct from one another, that is, MHC class II-induced signals promote positive selection and differentiation into $CD4^+$ T cells, whereas MHC class-I-induced signals promote positive selection and differentiation into $CD8^+$ T cells. Attempts to validate the selective or instructive models have generated a large amount of evidence that cannot be explained by either model as originally formulated (6, 16, 31, 32).

In the original instructional model, CD4 and CD8 coreceptors were thought to transduce qualitatively different instructional signals (33). However, this idea was subsequently replaced by the proposal that DP thymocytes are instructed by quantitative differences in the intensity of signaling transduced by TCR and coreceptor co-engagement during positive selection (34) (Fig. 1.3B). The strength of signal model proposes that lineage choice is dictated by the overall strength of signals that are transduced by co-engaged TCR and coreceptor molecules, with strong signals promoting CD4-lineage choice and weak signals promoting CD8-lineage choice (34). This concept is supported by evidence that the cytosolic tail of CD4 binds significantly more intracellular Lck than the cytosolic tail of CD8 (35, 36). TCR and CD4 co-engagement generates strong signals, whereas TCR and CD8 co-engagement generates weak signals. It is the relative strength of these signals that induces thymocytes to specifically terminate either *cd8* or *cd4* gene expression (16). Supporting this view, transgenic expression of CD8 molecules with CD4 cytoplasmic tails allows MHC class I-restricted thymocytes to differentiate into CD4 lineage (33, 34), whereas CD4 disruption causes MHC class II-restricted thymocytes to differentiate into $CD8^+$ T cells (37, 38).

The strength of signal model provided a straightforward explanation for the experiments that manipulate the activity of intracellular kinases, such as Lck, Csk (C-terminal SRC kinase),

TEC-family kinases and ERKs in DP thymocytes (16). However, the crucial experiments assessing its core concept by directly altering the signaling intensity of TCR or coreceptor molecules themselves suggested the signal intensity did not determine CD4/CD8-lineage choice (6). The results indicate that, rather than determining lineage choice, the coreceptor tails control the number of thymocytes being selected, thereby ensuring the numerical predominance of MHC class II-restricted T cells (signaled through CD4) over MHC I-restricted T cells (signaled through CD8) (16).

An updated version of the original strength of signal model is the duration of signal instructional model, with the important twist that TCR signal duration, perhaps in addition to signal strength, determines the CD4/CD8-lineage choice (39, 40) (Fig. 1.3C). According to this model, TCR signals of long duration instruct DP thymocytes to terminate *cd8* gene expression and to differentiate into CD4$^+$ T cells, whereas TCR signals of short duration instruct DP thymocytes to terminate *cd4* gene expression and to differentiate into CD8$^+$ T cells (40). Experiments that restrict the expression of ZAP70, a tyrosine kinase required for TCR-signal transduction to DP thymocytes, thereby genetically limiting the duration of TCR signaling during positive selection, favor this hypothesis (41). When TCR signals are confined to DP thymocytes, only CD8-lineage cells are permitted to develop. Furthermore, thymocytes expressing MHC class II-restricted TCRs were redirected to the CD8 lineage, suggesting the duration of signal directly affects lineage choice.

Although it was originally unclear why MHC class I- and MHC class II-restricted TCR signals would be of different duration (40), an explanation adopted from the kinetic signaling model (39, 42) provided a solution: all TCR-signaled DP thymocytes selectively decrease the surface expression of CD8, which disrupts MHC class I-restricted TCR signaling but does not affect MHC class II-restricted TCR signaling. The kinetic signaling model relies on three postulates. First, that TCR signaling in DP thymocytes, regardless of its MHC specificity, down-regulates *cd8* gene expression (39, 43). Second, that CD4-lineage commitment does not occur at DP stage, but rather after TCR signaling has transcriptionally down-regulated CD8, as in CD4$^+$CD8^{low} thymocytes. Finally, that in CD4$^+$CD8^{low} cells, the persistence of TCR signaling blocks interleukin-7 (IL-7)-mediated signaling and induces differentiation

into mature CD4$^+$ T cells. By contrast, cessation or disruption of TCR signaling allows IL-7-mediated signaling, which induces CD4$^+$CD8^{low} intermediate thymocytes to undergo coreceptor reversal and differentiate into CD8$^+$ T cells (16) (Fig. 1.4). Conclusively, the kinetic signaling model proposes that CD4/CD8-lineage choice is determined by TCR-signal duration and that cytokines, such as IL-7, serves as sensors to detect the duration of the TCR signal (39, 42, 44). This model is based on a different set of fundamental principles than those that underlie classical models, as it was promoted by experimental observations that could not be reconciled with the concepts on which classical models are based (16).

1.2.4   Mechanisms of CD4/CD8-lineage decision

Instead of analyzing the function of TCR signaling (the "top-down" approach), an alternative approach for determining lineage-choice signals stems from the fact that lineage differentiation signals eventually lead to the transcriptional regulation of *Cd4* and *Cd8*. By defining first the *cis*-acting elements required for lineage-specific control of *Cd4* and *Cd8* and then the functionally critical *trans*-acting factors that bind to these elements, this approach aims to define the control of lineage commitment from the "bottom-up" (45). Important insights have emerged from this approach, including fundamental distinctions between the lineage-specific regulation of *Cd4* and *Cd8* and the identification of a crucial factor controlling lineage-specific *Cd4* expression (46).

Cd4 gene transcription is regulated differently from *Cd8* gene transcription (16). Cell-specific expression of *Cd4* results from the activity of a silencer element that abrogates *Cd4* gene transcription in CD4$^-$ cells (47, 48). The absolute requirement for this element in initiation of *Cd4* silencing was demonstrated by the fact that germline deletion results in *Cd4* derepression in all T cells (49). By contrast, cell-specific expression of *Cd8* is the result of stage-specific enhancer elements that actively induce its expression in CD8$^+$ T cells (50, 51). Five enhancer elements that regulate the expression of *Cd8a* gene have been identified (known as E8$_I$-E8$_V$) (50-53), and two of these enhancer elements might be particularly relevant to our understanding of CD4/CD8-lineage choice, as the E8$_{III}$ enhancer is active only

in DP thymocytes and the E8$_I$ enhancer is active in CD8 SP thymocytes and CD8$^+$ T cells (16). No *trans*-acting factors have yet been identified that control lineage-specific expression of *Cd8*.

RUNX (runt-related transcription factor) proteins are members of the runt-domain family of transcription factors, which have similar structural organization and conserved DNA-binding sites (54). In the thymus, RUNX1 is mainly expressed by DN thymocytes and RUNX3 is expressed by post-selection CD8$^+$ thymocytes. RUNX1 and RUNX3 both bind to the *Cd4* silencer and silence *Cd4* gene transcription, indicating a role for RUNX proteins in CD4/CD8-lineage choice (54). Importantly, RUNX3 expression is up-regulated during the differentiation of CD4$^+$CD8^{low} thymocytes into CD8$^+$ T cells, when it provides two crucial functions: first, RUNX3 binds to the *Cd4* silencer element and silences *Cd4* gene transcription (54); and second, RUNX3 binds to the E8$_I$ *Cd8* enhancer element and reinitiates *Cd8* gene transcription (55). So, RUNX3 may be the transcriptional mediator of coreceptor reversal by silencing *Cd4* and reinitiating *Cd8* gene expression during the differentiation of CD4$^+$CD8^{low} intermediate thymocytes into CD8$^+$ mature T cells (16).

GATA3 is an enhancer-binding zinc-finger protein that functions as a lineage-specific transcription factor in T cells at various stages of development (56, 57). GATA3 is expressed in the earliest progenitor T cells and is required for thymocytes to differentiate beyond the DN stage (56). GATA3 may also have an important role in CD4-lineage choice, as suggested by observations that GATA3 is preferentially expressed by CD4$^+$ T cells (57), that GATA3 expression is up-regulated by TCR signaling in DP thymocytes and that sustained expression of GATA3 blocks the generation of CD8$^+$ T cells (58). In addition, conditional deletion of *Gata3* in DP thymocytes markedly decreases CD4$^+$ T cell number without affecting CD8$^+$ T cell generation, indicating a crucial role for GATA3 in the survival and/or differentiation of positively selected thymocytes into CD4-lineage T cells (16). However, enforced expression of GATA3 fails to redirect MHC class I-restricted thymocytes to differentiate into CD4$^+$ T cells (57), suggesting that GATA3 might not be a CD4-lineage regulator. Since it is expressed in the early stage of thymocyte development, GATA3 might function upstream of other lineage-specific factors and play a crucial role in CD4-lineage choice.

A recently identified transcription factor has provided novel insight on the mechanisms of CD4/CD8-lineage choice. Th-POK (T helper-inducing POZ-Krüppel factor, also called cKrox) is a zinc-finger protein that is encoded by the *Zbtb7b* (zinc-finger-and-BTB-domain-containing 7B) gene, which is responsible for the phenotype of the "helper-deficient" (HD) mice (59, 60). In an exciting series of experiments, two laboratories discovered that Th-POK was important for CD4-lineage choice and CD4$^+$ T cell differentiation (59, 60). The HD mice were originally identified by virtue of their specific lack of mature SP thymocytes and peripheral T cells (61). Notably, in those mice, MHC class II-signaled thymocytes failed to differentiate into CD4$^+$ cells and instead differentiated into mature CD8$^+$ T cells. In one of the studies, the HD mice were found to have a point mutation in the second zinc-finger domain of Th-POK that presumably disrupts DNA binding (59). Therefore, the phenotype of HD mice suggested that a functional Th-POK molecule is necessary for CD4 T cell differentiation. The reciprocal experiments done by the other group (60) revealed that the expression of transgene-encoded Th-POK proteins throughout thymocyte development forced virtually all positively selected thymoyctes to differentiate into CD4$^+$ T cells, regardless of their MHC specificity. Hence, Th-POK seemed to be the "master switch" of CD4$^+$ T cell development, as it seemed to be both necessary and sufficient to impose the CD4$^+$ fate on TCR-signaled DP thymocytes (46, 62). It seems reasonable to conclude that Th-POK serves both as a lineage-specification factor, turning on CD4$^+$ lineage-specific genes, as well as a commitment factor, preventing the expression of CD8$^+$ lineage-specific genes. Consistent with this perspective, retroviral transduction of mature CD8$^+$ T cells with Th-POK led to decreased cytotoxic T cell lineage function and induced some CD4$^+$ T-helper-cell characteristics, which indicates that even mature CD8$^+$ T cells are susceptible to the CD4-lineage-promoting effects of Th-POK (63).

However, surprisingly, latest data from the groups of Taniuchi (64), Bosselut (65) and Littman (66) now collectively demonstrate that Th-POK is not the sole "master regulator" of CD4$^+$ lineage and does not reside at the apex of the molecular circuitry controlling CD4$^+$-versus-CD8$^+$ lineage choice (62, 64-66). Instead, they proposed that GATA3 acts upstream of Th-POK to initiate CD4$^+$ specification, whereas Th-POK acts later as a commitment factor and serves to "lock down" the CD4$^+$ fate (62). Wang *et al.* made the unexpected finding that

Th-POK is not expressed when GATA3 is absent (65). They further showed that GATA3 binds in the proximal regulatory element of Th-POK encoding gene *zbtb7b*, suggesting that GATA3 is necessary, although probably not sufficient, for Th-POK expression in DP thymocytes (62). However, the converse is not true, as thymocytes lacking Th-POK still can express GATA3. Furthermore, transgenic expression of Th-POK could not rescue CD4 T cell development in the absence of GATA3, indicating that Th-POK is not sufficient to impose the CD4$^+$ T cell lineage when GATA3 is absent. Together, their findings suggest Th-POK is required at the late phase of CD4$^+$ T cell differentiation, downstream of GATA3 (62). Studies by Egawa and Littman (66), using *Runx3d* reporter mice, successfully showed the derepression of Runx3 expression in MHC class II-selected Th-POK deficient mice contributes to the redirection of DP thymocytes to CD8$^+$ T cell lineage. Strikingly, in the absence of both Th-POK and Runx, this redirection was prevented and cells potentially belonging to the CD4$^+$ lineage, presumably specified independently of Th-POK, were generated. Their results suggest that MHC class II-selected thymocytes are directed toward the CD4$^+$ lineage independently of Th-POK but require Th-POK to prevent Runx-dependent differentiation toward the CD8$^+$ lineage (66). Muroi *et al.*, however, provide insight into the mechanisms of action of Th-POK late in CD4$^+$ T cell differentiation. Their data indicate that Th-POK controls a positive feedback loop, locking down its own expression and consequently the CD4$^+$ T cell fate by binding to and antagonizing the activity of both the *zbtb7b* and *Cd4* silencers (64). With these new data, it is becoming apparent how persistent TCR signals communicate with the cell fate determination circuitry (62). It seems likely that Th-POK, acting as a critical CD4-lineage regulator, needs the orchestration from upstream expression of GATA3 and antagonism of Runx expression.

Other molecule such as HMG (high-mobility group) factor TOX has been known to promote the development to the CD8 lineage (67). HMG proteins are DNA-binding proteins that regulate gene expression by modulating local chromatin structure and recruiting other nuclear factor (68). TOX is an HMG box protein that was first discovered because it was found to be up-regulated in the TCR-signaled DP thymocytes and so was thought to have a role in positive selection and/or lineage choice (16). Experiments in TOX deficient mice revealed that positively selected thymocytes do not become CD4$^+$CD8^{low} cells, but instead

become CD4^{low}CD8^{low} cells that failed to differentiate into CD4$^+$ T cells (69). However, reversal of TOX deficiency through the introduction of a TOX transgene restores this phenotype and rescues the CD4-lineage (69). Thus, TOX seems to be important for maintaining or up-regulating CD4 expression in positively selected DP thymocytes (16).

It has been noted that chromatin organization and chromatin-modifying factors also play an important role in the control of coreceptor expression. A proposed model for *Cd4* silencing is that BAF, the mammalian chromatin remodeling complex, opens the *Cd4* silencer to binding by Runx3, which in turns mediates recruitment of histone deacetylases and corepressors. Sequential deacetylation and CpG methylation accompanied by repositioning to heterochromatin, possibly mediated by transcription factor Ikaros, then leads to a permanently silenced state (46).

1.2.5   Central tolerance in the thymus

Central tolerance refers to those events in the early life of a lymphocyte that focus the adaptive immune system on pathogens and steer it away from healthy tissue. It is induced at the primary sites of lymphocyte development – the bone marrow for a developing B cell and the thymus for a developing T cell – and it encompasses all of the mechanism by which antigen-receptor recognition of self-antigen at these sites results in self-tolerance (70). However, central-tolerance mechanisms are not perfect, and self-reactive lymphocytes can escape from this scrutiny and migrate into peripheral tissues. Therefore, peripheral tolerance mechanisms must exist to regulate the activity of self-reactive T cells that have escape central tolerance (70).

The hallmark of T cell central tolerance is clonal deletion: that is, suicide of T-cell progenitors that have high affinity for self-antigen (71). Other processes have been described, including anergy and receptor editing, but these are thought to have a lesser role (70). These three processes impair or eliminate high-affinity self-reactive cells and are considered to be negative selection mechanisms. However, not all central tolerance mechanisms cripple self-

reactive T cells. Recently, there is growing evidence for a fourth alternative fate, rather than being positively selected for useful T cells, being negatively selected for harmful T cells or death by neglect, during the thymic development (72). It has been proposed that, instead of actively induced cell death, some developing thymocytes expressing high affinity TCR against self-peptide/MHC can be positively selected to become regulatory T cells; this process is also referred to as agonist selection (Fig. 1.5). $CD4^+CD25^+$ regulatory T cells, $CD8\alpha\alpha^+$ intestinal epithelial lymphocytes and natural killer T cells have been suggested as T regulatory cells (70). These additional mechanisms are required for reducing the threat of autoimmunity inherent in the adaptive immune responses.

Inherited autoimmune syndromes due to impaired transcriptional factors have emphasize the crucial role for these two distinct central-tolerance mechanisms (73). In one case, mutations in autoimmune regulator (AIRE) protein lead to defective clonal deletion of T cells. AIRE deficiency results a disease known as autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome (APECED), a syndrome that is characterized by the presence of autoantibodies that are specific for multiple self-antigens (70, 71); this leads to lymphocytic infiltration of endocrine glands and autoimmune disorders localized at these organs (73). Mice with mutation in the *Aire* gene have pathological autoimmune features that are similar to those of patients with APECED. Remarkably, mTECs from AIRE-deficient mice have a significant decrease in the expression of multiple transcripts encoding peripheral tissue-specific antigens (TSAs) (74), including the insulin 2 (*Ins2*) and salivary protein 1 (*Spt1*) genes, which seem to be under the direct control of AIRE (73). Experiments using TCR transgenic mice that are specific for neo-self antigens have provided compelling evidence that AIRE is essential for efficient deletion of self-reactive T cells (75, 76). However, in the same AIRE-deficient mice, aggressive diabetes at birth was observed due to the escape of autoreactive cells expressing transgenic TCR agonist to the antigen expressed by the pancreatic β-cells (75, 76). This suggests that T cells that escape negative selection are capable of inducing autoimmunity. Thus, the consequence of AIRE deficiency is thought to be due to deficient negative selection of T cells that are specific for self-antigens, which could be caused by defective expression of self-antigens by thymic epithelial cells (73).

Another inherited disease results from mutations in the forkhead box P3 (*Foxp3*) gene which leads to impaired development of Treg cells and cause the syndrome known as immuno-dysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) (77). The occurrence of IPEX reflects impaired dominant central-tolerance mechanisms which we will cover the details in following chapters. In summary, although there are many peripheral mechanisms that control lymphocyte reactivity, central mechanisms, which operate during development, seem to be essential for the maintenance of self-tolerance (70).

1.3   Role of RasGRP family in lymphocyte development and effector function

1.3.1   RasGRP1 signaling pathway in T cell selection and activation

T cells communicate with their environment through a variety of cell-surface receptors that recognize and bind molecules present in the extracellular environment. After engagement of the T cell receptor (TCR) by antigen-presenting cells, multiple T cell signaling systems come into play, which ultimately alter the cell's behavior. The simultaneous deployment of several signaling cascades is thought to result in the efficient transcription of genes encoding cytokines and cytokine receptors, which promote further activation and proliferation of mature T lymphocytes (78, 79).

One of these signaling pathways involves the small GTPase Ras, which was originally identified as an oncogene able to induce cell transformation (80, 81). Ras exists as a key binary switch between "off" and "on" by the receptor-transduced incoming stimuli, and propagate signals further downstream. This equilibrium is regulated by the rate of guanyl nucleotide exchange and the rate of GTP hydrolysis. Ras guanyl nucleotide exchange factors (Ras GEFs) catalyze the release of GDP and replacement of GTP. Conversely, Ras proteins can be inactivated by either their own intrinsic GTPase activity or by pairing with GTPase-activating proteins (Ras GAPs). In unstimulated cells, including T cells, Ras exists predominantly in its inactive GDP-bound form. However, in response to TCR stimulation,

Ras-GTP accumulates, leading to the activation of effectors such as mitogen-activated protein kinase (MAPK) pathway and, ultimately, to changes in gene expression (82).

In T cells, Ras activation is mediated by at least two Ras GEFs, the well-characterized Sos (83) and the recently identified RasGRP1 (82). Recognition of major histocompatibility complex (MHC)-peptide complexes by TCR results in activation of the protein tyrosine kinases Lck (84) and ZAP-70 (85). Phosphorylation of the adaptor LAT (linker for activated T cells) by ZAP-70 leads to the recruitment of numerous proteins. On one hand, LAT serves as a docking site for Grb2 that brings Sos to the membrane leading to an increase in GTP-bound Ras. On the other hand, phospho-LAT also recruits phospholipase Cγ1 (PLCγ1) (86). Activated PLCγ1 converts phosphotidylinositol 4,5 bisphosphate (PIP2) into inositol 3,4,5 triphosphate (IP3) and diacylglycerol (DAG), resulting in membrane localization for RasGRP1 through its DAG binding C1 domain (87, 88). DAG analogs, such as phorbol 12-myristate 13-acetate (PMA), can bypass the TCR mediated pathway to directly recruit RasGRP1 to the membrane which leads to the activation of Ras, as well as the downstream signaling (82). It has recently been demonstrated that PLCγ1 selectively activates RasGRP1 on the Golgi membrane and is therefore, distinct from the Grb2/Sos pathway that activates Ras at the Plasma membrane (PM) (89-92) (Fig. 1.6). This surprising finding adds further complexity to Ras signaling, potential cellular responses and the induction of developmental programs. In addition to the differential localization, Ras activation also displays delayed or sustained kinetics at the Golgi relative to the PM (91).

The RasGRP1 protein (Ras guanyl nucleotide-releasing protein, also termed CalDAG-GEFII) is the first characterized protein within the RasGRP family (88) (other RasGRP1 related molecules (93) include RasGRP2, RasGRP3 and RasGRP4). All RasGRP family members share similar domain structures such as the catalytic region consisting of the Ras exchange motif (REM), the CDC25 box, the regulatory domains that include a DAG-phorbol ester binding domain and a pair of calcium-binding EF hands (88). Since PMA-induced Ras activation is absent in RasGRP1$^{-/-}$ mice (82), this Ras GEF may be the principal molecule of its type acting in thymocytes .

Ras signaling can result in the activation of three families of MAPKs, extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38. These MAPKs have been implicated in providing different contributions to the thymic selection (94). The Ras-ERK pathway that activates Raf-1, MAPK kinase (MEK1) and ERK has been determined to play an important role in the positive selection of T cells whereas JNK and p38 activation have been correlated with negative selection (95-98). More recent studies using $Grb2^{+/-}$ mice (83) demonstrated that the activation threshold for ERK was lower than that for JNK and p38. These data suggest that relatively weak TCR signals only activate ERK activation and lead to positive selection whereas strong TCR signals result in activation of the full range of MAPKs and negative selection (93).

RasGRP1-deficient mice display a block at the DP stage of thymocyte development suggesting a defect in the positive selection of T cells (99). This observation led to the hypothesis that the principal mechanism for distinguishing between positive and negative selection signals is coupled to the utilization of the RasGRP1 and Grb2/Sos pathways and hence, the MAPKs that get activated (100). According to this model, thymocytes that express low affinity TCR for their selecting ligands will activate Ras and ERK via RasGRP1 leading to positive selection; while thymocytes expressing high affinity TCR for the selecting ligands will activate Ras, ERK, JNK and p38 via the Grb2/Sos pathway leading to negative selection. To test this hypothesis, we generated two lines of $RasGRP1^{-/-}$ MHC class I-restricted TCR Tg mice, expressing either a weakly or strongly selecting TCR (93). Our findings indicate that a weakly selecting TCR is critically dependent on RasGRP1 for its positive selection. However, ERK activation and positive selection of thymocytes expressing a strongly selecting TCR are much less dependent on RasGRP1. By contrast, RasGRP1 deficiency has no effect on JNK and p38 MAPK activation or negative selection (93). Therefore, the positive selection of thymocytes expressing a strongly selecting TCR lacking RasGRP1 also offered us the unique opportunity to investigate the role of RasGRP1 in peripheral T cell homeostasis and function. Our previous studies suggested that RasGRP1 is important for transducing low-grade TCR signals which are necessary for survival and differentiation of naïve peripheral T cells, while strong TCR signals, responsible for the induction of early phase of Ag-driven growth, are RasGRP1 independent (93).

As a complement of the loss of function studies, experiments using mice with transgenic overexpression of RasGRP1 have suggested its role in compensating deficient pre-TCR signaling in vivo (101). In this study, it has been found that RasGRP1 expression is rapidly up-regulated in DN thymocytes following pre-TCR ligation. DN thymocytes from RasGRP1 transgenic mice overcome the deficiency of RAG2 expression and are able to mature to the DP stage (101). In addition, RasGRP1 transgenic mice favors thymocyte development towards the CD8 lineage and lowers the threshold of TCR signaling needed to initiate the proliferation of SP thymocytes. Together with studies using RasGRP1-deficient mice, it emphasizes the critical role of RasGRP1 in orchestrating optimal TCR signaling during T cell selection and activation.

1.3.2   RasGRP1 and RasGRP3 link BCR signaling to Ras in B lymphocyte

Although RasGRP1 has been widely studied for its crucial role in T lymphocyte development and function, recent studies using DNA microarray highlight the expression of RasGRP1 in other cell types both in mice and humans, such as NK cells, dendritic cells and B lymphocytes (102). However, except for B cells, the function of RasGRP1 in those cell types is unknown. In addition, other members of the RasGRP family have been detected in various cell types other than T and B lymphocyte. RasGRP2 acts on the Ras-related protein Rap and functions in platelet adhesion, whereas RasGRP4 is expressed in mast cells and certain myeloid leukemia cells (103).

Recently, RasGRP1 and RasGRP3 have been found to be coexpressed in primary murine B cells. In B cells, both RasGRP1 and RasGRP3 play a similar role downstream of the B cell receptor (BCR) linking BCR to Ras-ERK signaling (104). By using RasGRP1 and RasGRP3 single and double null mutant mice, recent studies have suggested that RasGRP1 and RasGRP3 both contribute to the BCR-induced Ras activation, although RasGRP3 alone is responsible for maintaining basal Ras-GTP levels in unstimulated cells (104). Surprisingly, in a sharp contrast to T cells, RasGRP-mediated Ras activation is not essential for B cell development since this process occurs normally in double-mutant mice (104). However,

RasGRP deficiencies affect humoral responses to specific immunogens and their presence is required for BCR-induced proliferation in vitro (104). Collectively, these data suggest that depending on the strength and duration of signaling event, as well as the developmental stage and contingent signaling through other pathways, various immune cell processes are facilitated by the controlled activation of Ras effector systems (103).

1.4   Naïve T cell activation and homeostasis-induced proliferation

1.4.1   Activation of naïve T cells

After entering the periphery, T cells become the adaptive immune systems' decisive recipients of messages from the innate immune system. Thus, their effective and efficient mobilization is crucial to the host's ability to respond to antigenic challenge (105). As naïve T cells, they rest in peripheral lymphoid organs until they encounter activating signals, such as foreign antigen presented by antigen-presenting cells (APCs), and gain the ability to enter sites of inflammation. While a CD4$^+$ T cell response emanates from the interaction of a TCR with its cognate peptide bound to an MHC class II molecule on the surface of an antigen-presenting cell, a CD8$^+$ T cell response originates from the engagement between a TCR and peptide/MHC class I complex. Stimulation through the TCR alone is insufficient to induce full activation of T cells. To prevent inappropriate activation that might be deleterious to the host, T cells require a second costimulatory signal to link proximal and distal signaling cascades effectively, which results in the transcription of a wide variety of downstream genes (105). However, in the absence of the costimulatory signals derived from the interaction of CD28 on T cells and B7 (CD80/CD86) on APCs, aberrant activation of TCR can produce a long-lived state of functional unresponsiveness, known as anergy. Specifically, anergized T cell clones produce negligible amounts of IL-2, which is crucial for clonal expansion following the T cell activation (106). Activated T cells then up-regulate the expression of high affinity IL-2 receptor α chain (CD25), and respond promptly to IL-2 in autocrine or paracrine fashion. They undergo vigorous clonal expansion, and finally acquire effector functions. With respect to CD8$^+$ T cells, the effector T cells become cytolytic and armed with

killer function, whereas CD4$^+$ T cells produce cytokines that can mediate direct effects on target cells or assist in the activating other arms of the immune system.

After massive proliferation and lineage-specific differentiation into effector cells, most activated T cells undergo activation-induced cell death (AICD), a process in which activated TCR restimulated T cells die after the engagement of cell-death receptors, such as CD95 or tumor necrosis factor receptor (TNFR). Only a small fraction (~5-10%) of the clonally expanded T cells survive the contraction phase and become long-lived memory cells. They experience a return to homeostasis mediated by balancing a low level of T cell proliferation with survival and death. Upon secondary challenge, those memory T cells can respond in a faster rate and more strongly to eliminate the antigens. Cytokines, especially the common γ-chain-cytokine family, including IL-2, IL-7 and IL-15 are thought to play critical roles during the four major events of an immune response, that is initiation, clonal expansion, contraction and memory generation (107). After ligation by peptide/MHC, substantial T cell clonal expansion occurs and might be driven, in part by IL-2. IL-15 might also enhance the proliferation of antigen-specific T cells. During the late phase of clonal expansion, IL-2 is thought to induce T cell death. The massive cell death that occurs during the contraction phase results in the loss of most antigen-specific T cells. Both IL-15 and IL-7 might rescue T cells from cell death and allow memory T cell generation by promoting survival signaling, such as the induction of Bcl-2 expression (107).

1.4.2   Homeostatic proliferation of peripheral T cells

Via the process of selection events during the thymic education, the thymus generates a peripheral repertoire that is largely depleted of overtly autoreactive T cells but retains low but significant reactivity for self-MHC molecules (108). Retaining weak affinity for self-MHC/peptide ligands is not only required for T cells to optimally recognize foreign antigens in the context of self-MHC molecules, but also helps to maintain the survival and homeostatic proliferation of naïve T cells (109).

Although it has long been realized that mature T cells are regulated at a population level by homeostatic mechanisms that maintain the total size of the T cell pool at a near-constant level (110-112), the knowledge of homeostatic proliferation originally came from experiments in which a small number of naïve T cells were adoptively transferred into immunodeficient hosts (108, 113). However, using host mice deficient in MHC class I or II molecules, this slow proliferation was hampered, suggesting the homeostatic proliferation of CD4$^+$ and CD8$^+$ cells requires contact with self-MHC class II and I molecules, respectively (108). Recently, there is compelling evidence that homeostatic proliferation of naïve T cells is driven by the low-affinity interaction with self-MHC molecules loaded with self-peptides (114-116). In addition, IL-7 signaling is thought to be important by promoting cell survival during the homeostatic proliferation of naïve T cells (117). In contrast to responses to high-affinity foreign peptides, homeostatic proliferation of naïve T cells to self-peptide/MHC complex is relatively slow and is not associated with the up-regulation of acute activation markers such as CD25 and CD69 (114-116). Instead, the dividing cells acquire cell surface markers typically expressed on memory T cells, such as CD44 and Ly6C, although cells become CD44^{hi} only after multiple rounds of cell division (108). However, whether these memory phenotype cells equated with "real" memory cells arising after an immune response to foreign antigens is still uncertain. In homeostasis-induced proliferation, the dividing cells can acquire effector functions, such as cytotoxicity and the capacity to rapidly secrete inflammatory cytokines (108, 117). This may pose risk to lymphopenic hosts, as it may skew the TCR repertoire of resident pool of T cells toward autoreactivity and furthermore, restrict its diversity.

1.5   T regulatory cell development and function

The random generation of antigen receptors in developing thymocytes results in a considerable risk of autoimmunity. The immune system has evolved several mechanisms to establish and sustain unresponsiveness to self-antigens (immunological self-tolerance), including physical elimination or functional inactivation of self-reactive lymphocytes (clonal deletion and anergy, respectively) (77). There is also substantial evidence that T cell-

mediated active suppression of self-reactive T cells is another essential mechanism of self-tolerance (118, 119). Although the idea of T cells that negatively control immune responses is not new for immunologists, there has been great controversy as to whether they actually constitute a functionally distinct cellular entity in the immune system and, if they exist, whether they are important in controlling autoimmune diseases (77). Recent years, however, have witnessed resurgent interest in suppressor or regulatory T (Treg) cells in many fields of basic and clinical immunology (118). Foxp3-expressing $CD4^+CD25^+$ Treg cells (77, 120), $CD8\alpha\alpha$ intestinal epithelial lymphocytes (121) and natural killer T (NKT) cells (122) have been suggested to possess the regulatory function. All are thought to be induced by high affinity interactions between self-peptide/MHC with TCRs on developing thymocytes (70). Surprisingly, recent studies indicate a subset of CD8 T cell population expressing CD122 (IL-2 receptor β) and high levels of CD44 also possesses regulatory functions since its deficiency is associated with severe autoimmune diseases (123-126). These results suggest $CD8^+CD122^+$ T cells contain novel population that can function as Treg cells. However, these results are controversial since they cannot be reproduced by other labs.

1.5.1 Naturally arising Foxp3-expressing Treg cells

A crucial experiment for addressing the function of Treg cells in natural self-tolerance is to determine whether their removal from the normal immune system can break self-tolerance, resulting in autoimmune disease (77). Efforts were made in mid-1980s to identify the Treg population by expression of particular cell surface molecules, such as CD5, CD45RB and CD25 (119). It has been shown that the IL-2 receptor-α chain (CD25), which is constitutively expressed by 5-10% of $CD4^+$ T cells and 5% of CD4 SP mature thymocytes in normal naïve mice, is able to be used as a marker for autoimmune-preventive Treg cells (118). These "naturally arising" $CD4^+CD25^+$ Treg cells became the best candidates for the T cell population mediating dominant tolerance to self. To emphasize their origin and importance, Treg cell production has been called the third function of the thymus (127).

The observation that autoimmunity can be prevented by transfer of peripheral CD4$^+$ T cells in neonatally thymectomized mice provided evidence for the thymic origin of Treg cells (119). Subsequent studies suggested that the CD25$^+$ CD4 SP thymocytes are capable of suppression in adoptive transfer models and in vitro suppression assay (128). In addition to its suppressive capacity and CD25 expression, this thymocyte subset displays markers characteristics of peripheral Treg cells, including increased expression of CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), glucocorticoid-inducible tumor necrosis factor receptor (GITR) and OX40 (129). The essential function for TCR signals in the development of Treg cells was suggested by the finding that TCR Tg mice on a RAG-1/2 deficient background do not develop Treg cells, whereas most TCR Tg mice expressing functional RAG-1/2 contains varying numbers of Treg cells (130). In addition, it has been shown that the costimulatory signals mediated by CD28 engagement of CD80 or CD86 molecules are important in shaping the repertoire and size of the Treg cell compartment (131). Furthermore, signals mediated by IL-2-IL-2R have been linked to the development, maintenance, survival and expansion of Treg population (132). Deficiency in IL-2 (133, 134), IL-2Rα (135) or IL-2Rβ (126) produce similar fatal lymphoproliferative inflammatory disease with an autoimmune component, generally called IL-2 deficiency syndrome (77). Notably, the number of CD4 Tregs is selectively reduced in the thymus and periphery of IL-2-deficient mice (133). IL-2 neutralization also inhibits the physiological proliferation of CD4 Tregs that are presumably responding to normal self-antigens (136). Although CD28 and IL-2 have a considerable effect on the size of developing Treg cell subset, the nature of the putative non-redundant signal involved in Treg cell lineage commitment in the thymus together with the TCR remains to be identified.

A dramatic feature shared by CD4 Treg cells and T cells with autoimmune potential is the ability to recognize self-antigens (137). Recognition of self-antigens by CD4 Tregs was initially suggested by observations indicating that the presence of a particular organ was important for the maintenance of CD4 Tregs-mediated tolerance to that organ (138). Further support comes from studies of mice transgenic for the αβ TCR, in which the development of CD4 Treg cells expressing the transgenic TCRs, is enhanced by thymic expression of the cognate antigen to the Tg TCR (139, 140). Finally, non-regulatory T cells transduced with

Treg cell-derived TCRs rapidly expand their populations in vivo and induce autoimmune disease in lymphopenic hosts (137). These data collectively demonstrate that self-reactivity is a prominent feature of naturally arising Treg cells' TCR repertoire.

Although CD25 expression has been useful in defining the Treg cell population in mice and humans, the most distinguishing feature of CD4 Tregs is the expression of a member of the forkhead family of transcription factor, Foxp3 (141). The discovery of Foxp3 as a critical gene in immune regulation came from the studies of the X-linked immunodeficiency syndrome IPEX and analogous X-linked recessive inflammatory disease in scurfy mutant mouse (131). Foxp3-deficient mice fail to develop $CD4^{+}CD25^{+}$ Treg cells and succumb to scurfy-like inflammatory diseases, which can be prevented by transfer of normal $CD4^{+}$ $CD25^{+}$ Treg cells (131). It is suggested that in the absence of CD4 Treg cells, some CD4 T cells expressed self-specific TCRs that are usually expressed by CD4 Tregs and these cells contribute to the pathology of Foxp3-deficient mice (142). Analysis of Foxp3 expression in T cells at both the mRNA and protein level has shown high expression in $CD4^{+}CD25^{+}$ Treg cells and low expression of Foxp3 in naïve and activated $CD4^{+}$ T cells (131). Furthermore, analysis of the origin of $CD4^{+}CD25^{+}$ Treg cells in chimeric mice containing a 1:1 mixture of allelically marked bone marrow stem cells derived from Foxp3-deficient and wild type mice showed that Foxp3-deficient bone marrow cannot give rise to $CD4^{+}CD25^{+}$ Treg cells, thus demonstrating that $CD4^{+}CD25^{+}$ Treg cell development is critically dependent on Foxp3 expression (120). These and other studies together suggest that Foxp3 functions as the Treg cell lineage specific factor, which is both necessary and sufficient for their development and function (131, 143).

The precise molecular mechanism by which CD4 Tregs suppress the activation and proliferation of other T cells is controversial and under active investigation (118). Some findings support essential roles of cytokines, such as IL-10, TGF-β and IL-35 in the regulation; others suggest the crosslinking of B7 (CD80 and CD86) molecules on the surface of APC or expression of CTLA-4 on Treg cells is required for suppression (131). It is likely the predominant effector mechanism of Treg cell-mediated suppression may vary depending

on the specific tissue and inflammation type being studied. Treg cell-specific gene targeting may help to elucidate the suppressive mechanisms operating in vivo.

1.5.2 Inducible regulatory CD4 T cells

Evidence suggests that regulatory T cells expressing the transcription factor Foxp3 develop extrathymically and intrathymically (144). Although neonatal thymectomy on day 3 substantially reduces peripheral $CD4^+CD25^+$ T cells number, emphasizing the important function of thymus in the production of Treg cells, intrathymic generation does not seem to represent the only mode by which $CD4^+CD25^+Foxp3^+$ T cells can be generated. It has been found that Treg cells can also be produced de novo from naïve $CD4^+CD25^-$ T cell by continuous supply of subimmunogenic doses of agonist peptides (145). In those experiments, generation of suppressor T cells was not due to the proliferation and/or local accumulation of preexisting $Foxp3^+$ cells, as they used TCR Tg T cells on $RAG-2^{-/-}$ background, which cannot produce Foxp3-expressing T cells. A recent report has shown that the conversion of truly naïve $CD4^+$ T cells into suppressor cells expressing Foxp3 can be achieved by targeting of peptide-agonist ligands to dendritic cells (146). In addition, the fact that TGF-β could help the conversion of in vitro- stimulated peripheral $CD4^+CD25^-$ T cells into suppressor cells suggests that TGF-β signaling could be involved in the antigen-driven conversion of suppressor cells in vivo (147, 148). Consistent with that idea, mice deficient in components of the TGF-β-TGF-β receptor system have reduced numbers of peripheral $CD4^+CD25^+$ T cells, but it is not apparent whether the deficiency in suppressor cells in these mice is exclusively due to reduced survival and/or proliferation or whether a deficit in de novo induction in peripheral lymphoid tissues (149). Overall, these studies suggest that there is mechanism for converting peripheral $CD4^+CD25^-$ T cells possessing high-affinity TCRs into Foxp3-expressing suppressor T cells in the presence of TGF-β, although the details of this conversion still remain unknown.

1.5.3 Unconventional CD8 T cells with regulatory function

TCR Tg mice in which CD8 T cells of defined specificity are generated provides a powerful tool for studying T cell development and function (150). In collaboration with Harald von Boehmer, we used mice that express the Tg H-Y TCR to study the mechanisms of T cells development. The H-Y TCR is specific for the male (H-Y) peptide presented by H-2D^b (20, 21). In male H-2^b H-Y TCR Tg mice, immature CD4$^+$CD8$^+$ thymocytes that express the H-Y TCR are deleted (151). However, surprisingly, large numbers of peripheral T cells that express low levels of CD8 (CD8^{lo}) and exclusively the H-Y TCRs, are present in the spleen and lymph nodes of male H-2^b H-Y TCR Tg mice (152). These self-specific CD8^{lo} cells possess a memory phenotype characterized by high expression of CD44, CD122 and Ly6C (153) and a lower level of TCR expression than naïve CD8 T cells (154). Interaction with cognate self-antigen was required for the optimal expansion of these cells in peripheral lymphoid organs (153). Furthermore, the CD8^{lo} cells are hyporesponsive to antigen stimulation relative to naïve CD8 T cells, while they can proliferate vigorously in response to either IL-2 or IL-15 (153). Upon activation by these cytokines, CD8^{lo} cells up-regulate NKG2D and DAP12, thus, are capable to perform killing in a NKG2D dependent fashion.

Although those studies were done using Tg mice, it has been shown that in normal B6 mice, there is a certain population of peripheral CD8 T cells possessing memory phenotype that share many functional similarities with the CD8^{lo} cells in H-Y mice (154, 155). It is generally assumed that these memory phenotype CD8 T cells in B6 mice are the progenies of conventional CD8 T cells following exposure to foreign antigens. However, similar frequencies of these memory CD8 T cells are also found in germ-free and antigen-free colonies, arguing they are unlikely memory cells. Recent data have identified the Tec kinases, Itk and Rlk as crucial regulators of CD8$^+$ T cell development into the conventional lymphocyte lineage (156). Studies using Itk, Rlk and IL-15 knockout mice support the hypothesis that the CD122$^+$ unconventional CD8 cells represent a cell lineage that is distinct from conventional CD8 T cells (157-159). These studies show that whereas IL-15$^{-/-}$ mice lack CD44^{hi}CD122$^+$ CD8$^+$ T cells, Itk$^{-/-}$Rlk$^{-/-}$ lack CD44^{lo}CD122$^-$CD8$^+$ T cells. Furthermore, the same defects were observed during thymus development. They also confirmed that these

unconventional CD8$^+$ T cells express NK receptors and possess innate immune function whereas CD44^{lo}CD122$^-$CD8$^+$ T cells do not express NK receptors. These findings lead to the hypothesis that altered TCR signaling is the basis for the development of unconventional T cell lineage (156).

While there is a vast amount of literature on the developmental biology, function and TCR repertoires of CD4 Treg cells, little is known about the developmental biology, function and TCR repertoires of regulatory CD8 T cells, Studies in CD122-deficient mice have implicated the existence of CD8 Tregs that preferentially regulate the immune functions of CD8 T cells. CD122-deficient mice exhibit severe hyperimmunity (126), which is associated with the expansion of abnormally activated T cells (160). Suzuki el al (161) proposed that the lack of certain CD8 regulatory T cells might be responsible for the abnormally activated T cells, This notion was supported by the observation that the transfer of highly purified CD8$^+$CD122$^+$ T cells from normal mice into CD122-deficient neonates prevented the development of the abnormally activated T cells leading to a normal phenotype in the treated mice (124), although this result cannot be reproduced by other labs. This result suggests that CD8$^+$CD122$^+$ T cells contain novel Tregs that effectively regulate the activity of CD122-deficient CD8 T cells. Furthermore, RAG-2$^{-/-}$ mice that received wild type CD8$^+$CD122$^-$ cells die within 10 wk after transfer, suggesting that these normal CD8 T cells subpopulation can become dangerously activated in the absence of CD8$^+$CD122$^+$ Tregs (124). Follow-up studies indicate that the suppressor activity of CD8$^+$CD122$^+$ Tregs is mediated by IL-10 that is produced by these cells (123). Therefore, evidence supporting the existence of CD8 Tregs is beginning to emerge. However, much more work is needed to further characterize the molecular mechanisms how these CD8 Treg cells perform the suppressive function.

1.6   Thesis objectives

Our hypothesis of thesis is that RasGRP1 plays crucial roles in T cell development and peripheral function. Its deficiency will cause partial T-cell immunodeficiency as well as impaired central and peripheral tolerance.

The objectives of this thesis are:

1) Demonstrate the role of RasGRP1 in CD4 T cell development;

2) Characterize CD4 T cells with an activated phenotype in the periphery of B6 RasGRP1$^{-/-}$ mice;

3) Investigate potential link between the impaired CD4 T cell development and the activated CD4 phenotype in B6 RasGRP1$^{-/-}$ mice;

4) Determine the role of RasGRP1 in naïve CD8 T cell activation;

5) Determine the role of RasGRP1 in the development and function of CD4 and CD8 Treg cells.

Chapter 2 starts by examining the role of RasGRP1 in CD4 T cell development by using MHC class II-restricted AND TCR Tg mice. Chapter 3 characterizes the acutely activated and proliferating CD4 T cell population in the periphery of RasGRP1$^{-/-}$ mice, elucidates the consequences, such as homeostatic proliferation, generation of TCR repertoire and T cell response against infectious pathogen, due to the impaired T cell development in B6 RasGRP1$^{-/-}$ mice. Chapter 4 describes how RasGRP1 lowers the CD8 T cell activation threshold via controlling the IL-2 signaling, using MHC class I-restricted 2C TCR Tg mice. Chapter 5 describes the analysis of both CD4 and CD8 Treg cell development and function in B6 RasGRP1$^{-/-}$ mice.

1.7   Figures

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Figure 1.1 The thymus architecture. A. The human thymus section from a newborn child was stained with haematoxylin and eosin. B. The thymus section from an adult C57BL/6 mouse was stained for CD4 (green), CD8 (blue), and medullary thymic epithelial cells defined using Ulex europaeus 1 (red). Cells in cyan indicate the coexpression of CD4 and CD8, indicating $CD4^+CD8^+$ DP cells.

Figure 1.2 Stages in early T‑cell development. Cross-section through an adult thymic lobule showing the migration path of T‑cell precursors during development. Immigrant precursors initially enter the thymus through blood vessels near the cortico–medullary junction, the early T-cell precursors (ETP) subsequently migrate, and differentiate from double negative (DN) to double positive (DP) to single positive (SP) stages, through the distinct micro-environments of the thymus.. ETPs, DN2, DN3 and DN4 T-cell subsets: note that at any given stage of the cell cycle, the ETPs appear to have the options either to continue their expansion with minimal differentiation in the cortico–medullary junction region or to differentiate into DN2 cells (presumably DN2a cells as shown) that can begin their migration from the site of entry deep within the cortex to the outer rim of the cortex. β‑selection occurs during the accumulation of the DN3 T cells in the extreme outer portion of the thymus (that is, the subcapsular zone). A directional reversal of migration back across the cortex towards the medulla occurs at the later stages of thymocyte development. Broken arrows depict alternative developmental pathways that are still possible for ETPs and for different subsets of DN2 cells that likely correspond to DN2a and DN2b cells before they complete their commitment to the T-cell-lineage. CCR9, CC-chemokine receptor 9; ISP, immature single positive; LMPP, lymphoid primed multipotent progenitor; NK, natural killer; TCR, T‑cell receptor.

Figure 1.3 The classical models of CD4/CD8 lineage choice. A. The stochastic selection model postulates that positive-selecting T-cell receptor (TCR) signals randomly terminate the expression of one or the other co-receptor molecule, resulting in the generation of short-lived intermediate cells, which undergo programmed cell death unless they are rescued by a second TCR signal. Because the TCR-mediated rescue signal requires TCRs and co-receptors that are matched, 50% of positively selected thymocytes will fail to survive and mature. B. The strength-of-signal instructional model postulates that weak TCR signals terminate *Cd4* transcription, whereas strong TCR signals terminate *Cd8* transcription. Signaling by CD8 and MHC class I-restricted TCRs is weak and results in mature $CD8^+$ T cells, whereas signaling by CD4 and MHC class II-restricted TCRs is strong, which results in mature $CD4^+$ T cells. C. The duration-of-signal instructional model postulates that short and/or weak TCR signals terminate *Cd4* transcription, whereas long and/or strong TCR signals terminate *Cd8* transcription. MHC class I-restricted and MHC class II-restricted TCR signals are proposed to differ in both duration and intensity. DP, double positive.

Figure 1.4 The kinetic signaling model of CD4/CD8-lineage choice. Regardless of the specificity of their T-cell receptor (TCR), positively selecting TCR signals induce double positive (DP) thymocytes that are transcriptionally $Cd4^+Cd8^+$ to terminate *Cd8* gene expression and to convert into $Cd4^+Cd8^-$ intermediate thymocytes. Because of the absence of *Cd8* gene transcription, $Cd4^+Cd8^-$ intermediate thymocytes appear phenotypically as $CD4^+CD8^{low}$ cells, and these are the cells in which lineage choice is made. Persistence of TCR signalling in $Cd4^+Cd8^-$ intermediate thymocytes blocks interleukin-7 (IL-7)-mediated signalling and induces differentiation into mature $CD4^+$ T cells. Cessation or disruption of TCR signalling in $Cd4^+Cd8^-$ allows IL-7-mediated signalling, which induces $Cd4^+Cd8^-$ intermediate thymocytes to undergo coreceptor reversal, gain a $Cd4^-Cd8^+$ phenotype and differentiate into $CD8^+$ T cells.

Figure 1.5 Central tolerance mechanisms. The affinity of the T-cell receptor (TCR) for self-peptide–MHC ligands is the crucial parameter that drives developmental outcome in the thymus. Progenitors that have no affinity or very low affinity die by neglect. This is thought

to be the fate of most thymocytes. If the TCR has a low affinity for self-peptide–MHC, then the progenitor survives and differentiates, a process that is known as positive selection. If the progenitor has a high affinity for self-peptide–MHC, then several outcomes are possible. First, the progenitor can be selected against, a process that is known as negative selection. The main mechanism of negative selection is clonal deletion, but receptor editing and anergy have also been described. Second, there seem to be mechanisms that select for high-affinity self-reactive cells and result in differentiation into a 'regulatory'-cell phenotype. It is not known what determines whether a T cell is tolerized by negative selection or is selected to become a regulatory T cell. IEL, intestinal epithelial lymphocyte; NKT cell, natural killer T cell; Treg cell, $CD4^+CD25^+$ regulatory T cell.

Figure 1.6 Switching on Ras in different cellular locations. **a,** When receptors on the cell surface are activated, they recruit the enzyme Src. Simultaneously, another enzyme, phospholipase $C\gamma1$ (PLC-γ1), is recruited and phosphorylated by Src (represented by a circled 'P'), activating it. **b,** PLC-γ1 leads to the generation of diacylglycerol (DAG) and an increase in the level of Ca^{2+} ions. **c,** DAG and Ca^{2+} cause a cytoplasmic protein, RasGRP1, to move to the Golgi. This protein activates Golgi-associated Ras, by catalysing the exchange of GTP for GDP. This new pathway coexists in the cell with the 'textbook' pathway that links the cell surface receptor to Grb2 and Sos; the latter protein activates Ras at the plasma membrane (**d**) in a Ca^{2+}-independent way. **e,** The increase in Ca^{2+} levels also leads to the activation of CAPRI, and possibly to its movement to the plasma membrane. **f,** CAPRI inhibits Ras by stimulating its intrinsic GTPase activity, which hydrolyses GTP into GDP.

a Human

Capsule   Trabeculae   Hassall's corpuscles

Subcapsular zone   Cortex   Cortico–medullary junction   Medulla

b Mouse

Subcapsular zone   Cortex   Cortico–medullary junction   Trabeculae

Capsule   Medulla
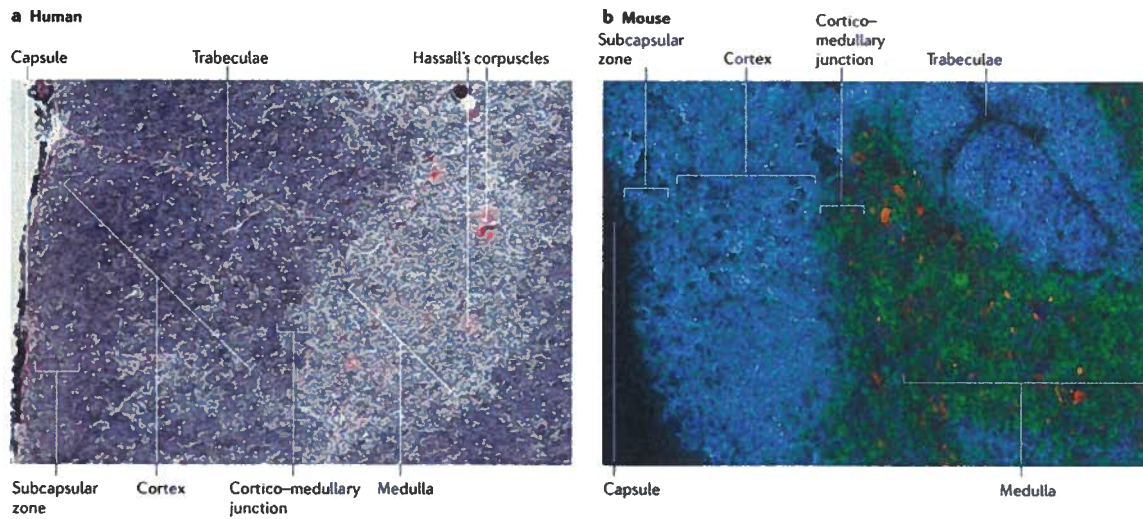
Figure 1.1 The thymus architecture. Figure adapted from Takahama, Y. 2006. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* 6: 127-135 (5).
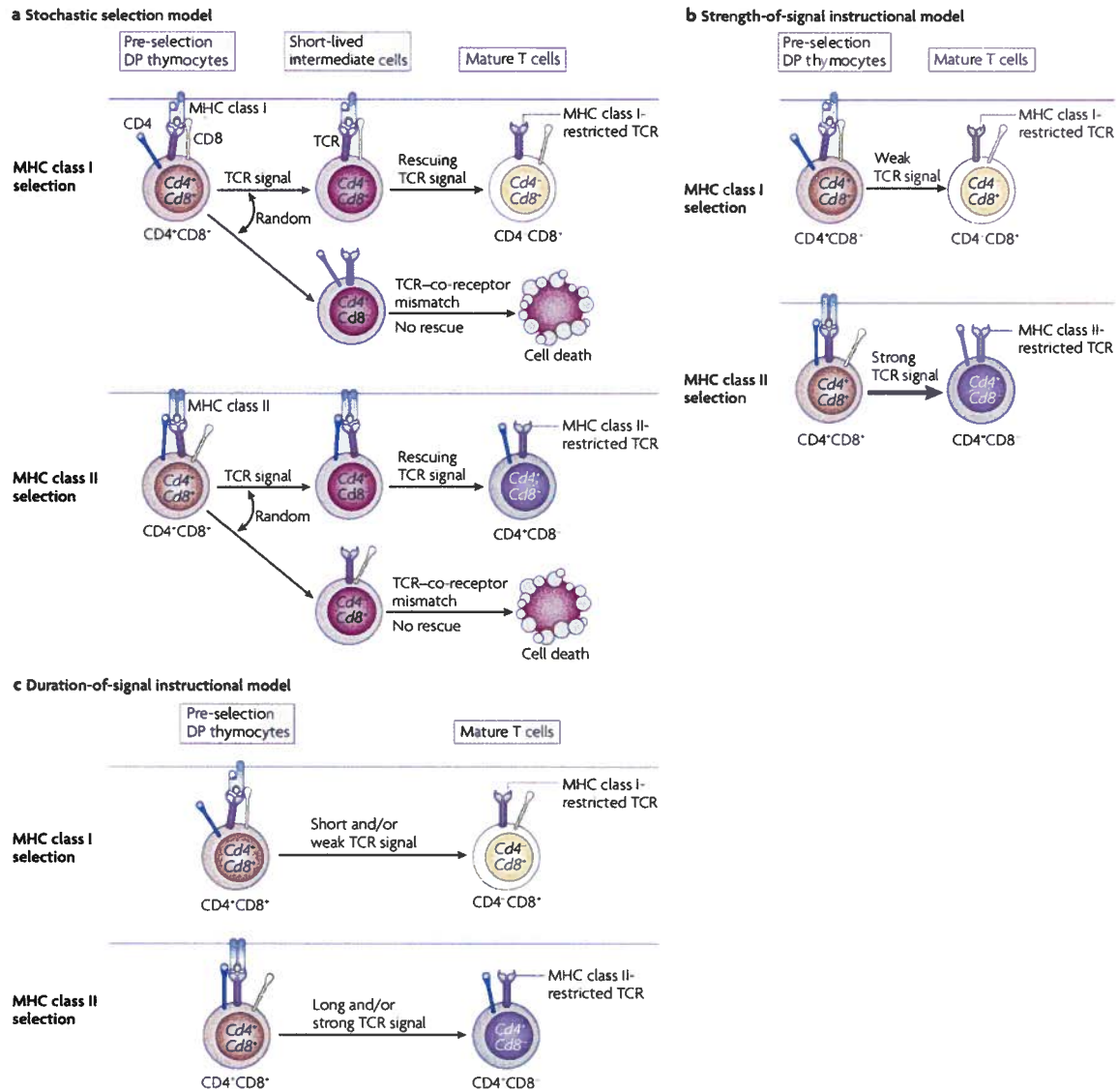
Figure 1.2 Stages in early T–cell development. Figure adapted from Rothenberg, E.V., J.E. Moore and M.A. Yui. 2008. Lauching the T-cell-lineage developmental programme. *Nat Rev Immunol* 8: 9-21 (1).

Figure 1.3 The classical models of CD4/CD8 lineage choice. Figure adapted from Singer, A., S. Adoro and J.-H. Park. 2008. Lineage fate and intense debate: myth, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol* 8: 788-801 (16).
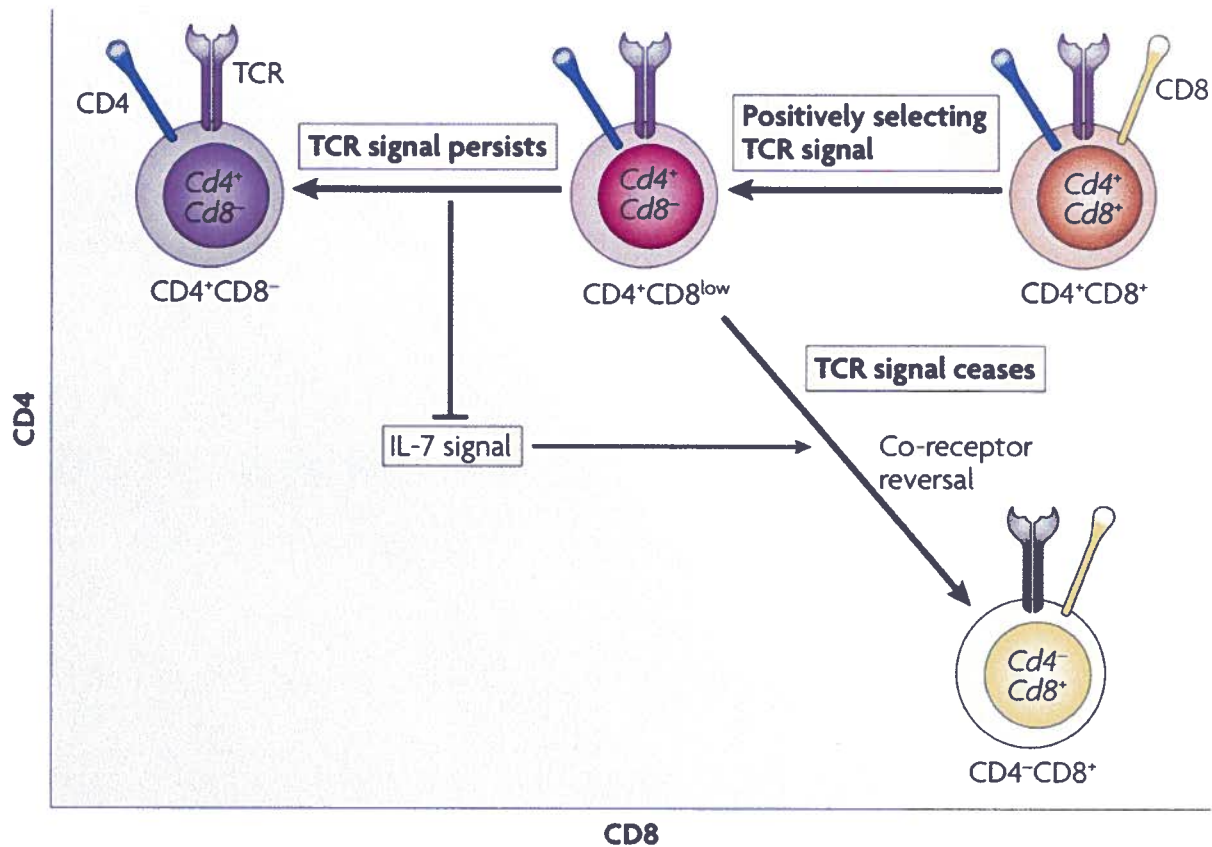
Figure 1.4 The kinetic signaling model of CD4/CD8-lineage choice. Figure adapted from Singer, A., S. Adoro and J.-H. Park. 2008. Lineage fate and intense debate: myth, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol* 8: 788-801 (16).
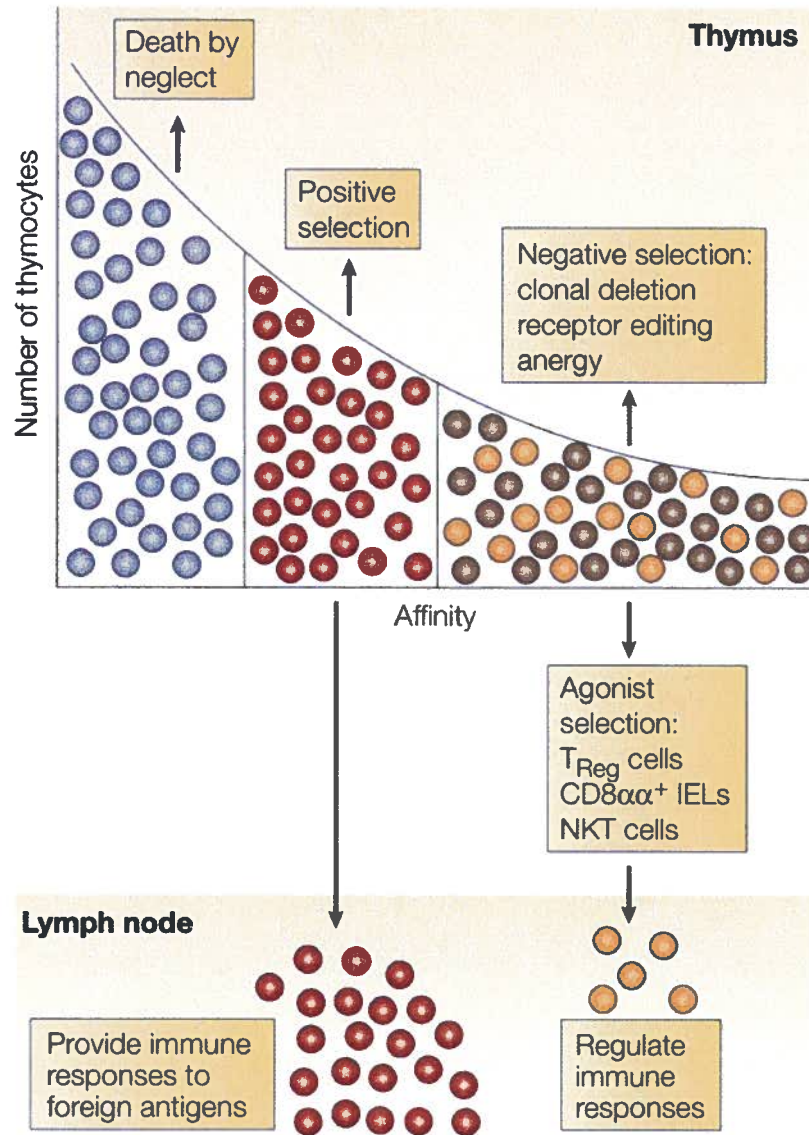
Figure 1.5 Central tolerance mechanisms. Figure adapted from Hogquist, K.A., T.A. Baldwin and S.C. Jameson. 2005. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 5: 772-782 (70).
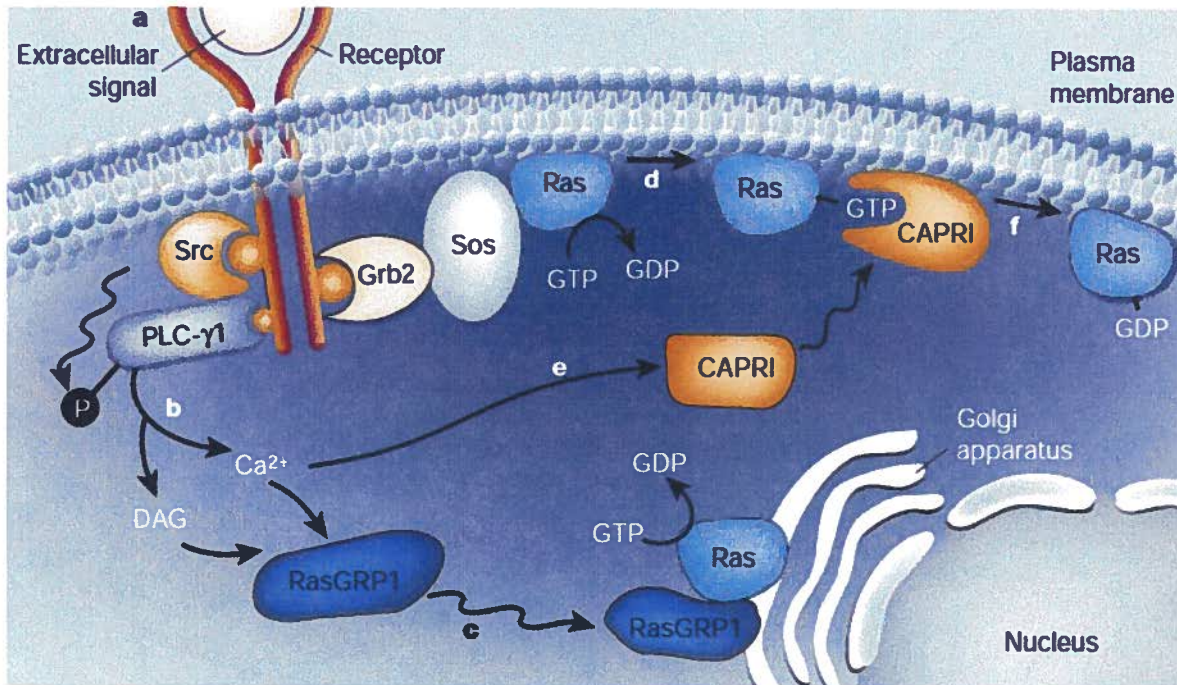
Figure 1.6 Switching on Ras in different cellular locations. Figure adapted from P. P. Di Fiore. 2003. Signal transduction: Life on Mars, cellularly speaking. *Nature* 424: 624-625 (91).

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CHAPTER 2   RASGRP1 TRANSMITS PRODIFFERENTIATION TCR SIGNALING THAT IS CRUCIAL FOR CD4 T CELL DEVELOPMENT[1]

2.1   Introduction

Developing double-positive (DP) thymocytes are subjected to extreme selection pressures since the thymus culls through millions of these cells each day; killing the masses and selecting a precious few for survival (1). The positive outcome of this process, a diverse TCR repertoire that is both self-restricted and self-tolerant, is vital to fight infection and prevent autoimmunity. Current evidence supports the "strength of signaling" hypothesis, proposing that quantitative attributes of TCR signaling instruct cell fate during thymocyte selection (2, 3). The intensity of interaction between the TCR and its ligands on thymic cortical epithelial cells and bone marrow-derived cells determines the magnitude of signaling. According to this model, weak TCR signaling instructs survival and differentiation, also known as positive selection. In contrast, a lack of TCR signaling and strong TCR signaling results in cell death programming, death by neglect, and negative selection, respectively.

Expression of CD4 or CD8 defines two distinct T cell lineages that differ both by their MHC specificity and function. Most CD4^{+} T cells are MHC class II-restricted and function as Th cells, whereas CD8^{+} T cells are MHC class I-restricted and differentiate into cytolytic effectors after TCR engagement. Because CD4^{+} and CD8^{+} T cells arise from a common precursor pool of DP thymocytes, the question arises as to how the concordance between MHC specificity and lineage is established. Experiments manipulating TCR signaling, such as mutations affecting the coreceptor (4-8) and the tyrosine kinase Lck (9, 10) function in developing thymocytes, have provided strong support for the notion that CD4/CD8-lineage choice is also dictated by quantitative attributes of TCR signaling. The strength of signaling

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model as applied to lineage selection states that stronger TCR signaling promotes CD4 T cell fate, whereas weaker TCR signaling directs CD8 T cell fate (2, 3).

The expression of either constitutively active or dominantly interfering forms of Ras (11), Raf-1 (12), and MEK1 (13) in transgenic (tg) mice have demonstrated the critical role of the Ras/MAPK pathway in positive but not negative selection. However, recent in vitro studies suggest that the strength and/or duration of Ras/ERK signaling may be critical in determining whether a thymocyte adopts a positive or negative selection program (14-16). Positively selecting ligands were found to induce a sustained, low-level ERK activity, whereas negatively selecting ones brought about stronger but transient signals. Furthermore, these findings have now been corroborated to also occur in fetal thymic organ cultures (17). One function for sustained ERK signaling in positive selection may be to provide survival signaling. Studies using MAPK inhibitors have demonstrated that ERK activity is critical for the up-regulation of the antiapoptotic protein Bcl-2 (18, 19). In addition, ERK has been shown to phosphorylate the BH3-only Bcl-2 family member Bim, preventing its association with Bax and disabling it from inducing cell death (20). Therefore, sustained ERK activation may also facilitate T cell maturation by phosphorylating Bim and subsequently blocking its proapoptotic activities.

The role of ERK signaling in lineage commitment has been the center of some controversy. Although studies using Tg mice expressing either gain-of- or loss-of-function forms of Ras, Raf, and MEK1 were not found to sway CD4 vs CD8 differentiation (11-13), mice bearing a hypersensitive mutant form of ERK2 gene favor the development of CD4 single-positive (SP) thymocytes (21). Further support for ERK signaling directing thymocyte fate has come from investigations using MEK1 inhibitors in combination with fetal thymic organ cultures (21-23). Therefore, the amplitude and/or kinetics of ERK activation may also be key in determining lineage decision (21-23). Moreover, ERK activity has recently been found to positively regulate TCR signaling by modifying the Lck tyrosine kinase and subsequently preventing its inactivation by the Src homology protein-1 phosphatase (24). Thus, it is possible that ERK may affect TCR signaling strength by lengthening the duration of Lck activity through a feedback-loop mechanism.

In thymocytes, at least two Ras-guanyl nucleotide exchange factors, the well-studied Sos (25) and the more recently identified RasGRP1 (26), mediate Ras activation. The action of these two Ras-guanyl nucleotide exchange factors is dependent on their relocation to membranes by two distinct mechanisms (27). TCR stimulation and subsequent LAT (linker for activated T cells) phosphorylation result in the Src homology 2-domain-mediated targeting of Grb2/Sos complex to the plasma membrane (PM). In contrast, the movement of RasGRP1 is dependent on phospho-LAT's recruitment and activation of phospholipase Cγ1 (PLCγ1). The action of PLCγ1, converting phosphotidylinositol 4,5 bisphosphate into inositol 3,4,5 triphosphate and diacylglycerol, results in membrane localization for RasGRP1 through its diacylglycerol-binding C1 domain (28, 29). The surprising finding that RasGRP1 is recruited to the Golgi membrane (30-32), activating Golgi-associated Ras, rather than at the PM adds further complexity to Ras signaling, potential cellular responses, and the induction of developmental programs. In addition to the differential localization, Ras activation also displays delayed or sustained kinetics at the Golgi relative to the PM.

RasGRP1$^{-/-}$ mice bear a defect in late thymic selection, where mutant DP thymocytes undergo rearrangement of their TCRα genes and express normal levels of TCR$\alpha\beta$ /CD3 but are poorly promoted to the mature SP lineages (26, 33). Previously, we have determined that RasGRP1-dependent and -independent mechanisms exist for ERK activation and positive selection of MHC class I-restricted TCRs (33). To further characterize the role of RasGRP1 in thymocyte maturation, examining its relative importance in CD4 T cell development and lineage commitment, we generated RasGRP1$^{-/-}$ mice that express a Tg MHC class II-specific TCR. Here, we show that in mutant mice, the development of CD4 T cells expressing a Tg MHC class II-restricted TCR is completely abrogated. Therefore, we find that RasGRP1 deficiency affects CD4 T cell development more profoundly than the development of CD8 T cells. From in vitro TCR-stimulation experiments, RasGRP1$^{-/-}$ DP thymocytes display a diminished capacity to up-regulate Bcl-2. Based on these findings, we hypothesized that an important role for RasGRP1 may be to maintain cell viability and thus enable the differentiation of DP into SP thymocytes. However, the failure of forced Bcl-2 expression to restore CD4 T cell development in RasGRP1-deficient mice suggests that the RasGRP1-

Ras/MAPK pathway may be particularly important for imparting differentiation signals that are essential for CD4 T cell development.

2.2   Materials and methods

Mice

RasGRP1-null breeder mice were provided by J. C. Stone (University of Alberta, Edmonton, Alberta, Canada) and bred onto C57BL/6J background at least seven generations before mating with TCR Tg mice. The TcrAND (34) and TcrH-Y (35) Tg mice were bred onto the C57BL/6 (H-2^b) background. Breeders bearing the TcrAND transgene were purchased from Jackson ImmunoResearch Laboratories. The Eμ-Bcl-2-36 Tg mouse line expresses strongly in thymocytes and has been bred at least six generations onto the C57BL/6J background as described previously (36). The Eμ-Bcl-2-36 Tg mouse line was provided by C. J. Ong (University of British Columbia, Vancouver, British Columbia, Canada). B6.PL-Thy1a/Cy (Thy 1.1$^+$) mice were acquired from The Jackson Laboratory. All studies followed guidelines set by the Animal Care Committee at the University of British Columbia in conjunction with the Canadian Council on Animal Care.

Flow cytometry

Abs against CD4 (GK1.5), CD8 (53-6.7), H-Y TCR (T3.70), TCRβ (H57-597), CD3ε (2C11), heat-stable Ag (HSA; M1/69), CD127 (A7R34), CD69 (H1.2F3), CD44 (IM7), Thy1.1 (HIS51), and Thy1.2 (53-2.1) were purchased from eBioscience. Abs against CD5 (53-7.3), TCR Vα11 (RR8-1), TCR Vβ3 (KJ25), and Bcl-2 (3F11) were purchased from BD Biosciences. For phospho-ERK (P-ERK)-specific staining, cells were fixed in methanol-free 2% formaldehyde (Polysciences), washed, and made permeable by incubation in 90% methanol for 30 min on ice. Intracellular staining was conducted in FACS buffer (2% FCS in PBS) at room temperature for 30 min. Rabbit anti-P-ERK1/2 Ab (Cell Signaling Technology; catalog no. 9101) was detected with donkey anti-rabbit Ig F(ab')2-PE (Jackson ImmunoResearch Laboratories; catalog no. 711-116-152). After blocking unbound binding

sites of the secondary Ab with rabbit IgG, cells were subsequently incubated with anti-CD4-FITC and CD8-PerCP (BD Biosciences). Data were acquired using either a FACScan or FACSCalibur and CellQuest software (BD Biosciences). Data were analyzed with CellQuest or FCSPress software (<www.fcspress.com>).

Proliferation assays

SP thymocytes were identified by staining with anti-CD8-PE and anti-CD4-PE-Cy5 Abs and purified by cell sorting using a BD FACSVantage (BD Biosciences). For Ab stimulation, 96-well flat-bottom plates were coated with 10 µg/ml of either ant-CD3ε (2C11) or anti- Vα11 (RR8-1) in PBS for 2 h at 37°C. After washing wells three times with PBS, 40,000 sorted cells were seeded per well in complete IMEM (Invitrogen Life Technologies) containing 20 U/ml IL-2. For peptide stimulation, 20,000 sorted cells were incubated with 5×10^5 irradiated C3H (H-2^k) splenocytes in round-bottom 96-well plates with the indicated pigeon cytochrome c (PCC) concentration and IL-2 (20 U/ml). After 72 h, cultures were pulsed with 1 µCi of [^{3}H] thymidine for 6 h to assess proliferation.

CTL assays

SP thymocytes were identified by staining with anti-CD8-PE and anti-CD4-PE-Cy5 Abs and purified by cell sorting using a BD FACSVantage. To generate effector T cells, purified AND CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ SP thymocytes were stimulated for 3–4 days on anti-AND TCR-Ab-coated dishes in the presence of exogenous IL-2 (20 U/ml). The H-2^k-expressing CH12 lymphoma cells were ^{51}Cr labeled and subsequently incubated with various numbers of effectors in the presence and absence of 10 µM PCC peptide. After 5 h, supernatants were assayed for released ^{51}Cr and specific killing determined as described previously (37).

DP thymocyte stimulations

Tissue culture wells (24-well plate; Falcon) were coated with 0.5 ml of either PBS alone, 10 µg/ml anti-CD3ε (2C11), or 10 µg/ml anti-CD3ε plus 10 µg/ml anti-CD4 (GK1.5) Abs for 2 h at 37°C. After washing the wells three times with PBS, total thymocytes (2×10^6) were

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placed in culture in complete IMEM for 24 h, harvested, and stained with anti-CD4 (RM4-4), anti-CD8 (53-6.7), and 5 µg/ml 7-aminoactinomycin D (7-AAD; Calbiochem) for dead cell exclusion and various cell surface markers. For Bcl-2 expression, cells were fixed in 2% paraformaldehyde/ PBS solution for 15 min, made permeable by treatment with 0.2% Tween 20/PBS for 15 min and stained with either anti-Bcl-2 or isotype-control Abs conjugated to PE (BD Biosciences) for 30 min on ice. Following two washes with PBS, sample data acquisition was performed on a FACSCalibur.

Cell signaling studies and immunoblotting

Cell sorting, using a FACSVantage, was used to purify DP thymocytes. For activation, sorted DP thymocytes (10^7/ml) were coated with 10 µg/ml hamster anti-CD3ε Ab for 20 min on ice, washed, and suspended in prewarmed (37°C) medium containing 80 µg/ml goat anti-hamster Ab (Jackson ImmunoResearch Laboratories) for the indicated periods. Cells were lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, protease, and phosphatase inhibitors. Abs used for immunoblotting that recognize activated and total ERK, LAT, and Zap70 were purchased from Cell Signaling Technology.

Intrathymic injection (IT) experiments

DP thymocytes were electronically sorted exactly as described for coreceptor re-expression assay. Approximately 1 million sorted DP thymocytes (10 µl volume) were injected per thymic lobe of (nonirradiated) B6.PL-Thy1a/Cy (Thy1.1$^+$) mice. Two days postinjection, injected thymi were recovered and mashed into single-cell suspensions, at a concentration of 10^7 cells/ml. To deplete host cells, thymocytes were labeled with 0.5 µg/ml anti-Thy1.1 Ab, washed, and incubated with (25 µl/ml) sheep anti-mouse-Ig-coupled Dynabeads (catalog no. 110.31; Dynal Biotech). Following magnetic separation, the remaining cells were split into two portions: one aliquot for ex vivo coreceptor expression analyses of donor cells, whereas the second was pronase-treated for a coreceptor re-expression assay. Because binding of anti-CD4 mAb (GK1.5) may block the ability of pronase to subsequently strip surface CD4 molecules (38), we selected to assess CD4 and CD8 coreceptor re-expression by staining with Abs conjugated to fluorochromes distinct from those used for cell sorting and thus avoid

potential residual fluorescence. All samples were stained with anti-CD4-allophycocyanin, anti-CD8-PE, and Thy1.2-FITC-conjugated Abs, and the data were acquired on a BD LSR II benchtop (BD Biosciences) cytometer using FACSDiVa software (BD Biosciences) for both acquisition and analysis.

Coreceptor re-expression assay

Thymic single-cell suspensions were stained with PE-Cy7-conjugated anti-CD4 (GK1.5) and APC-Cy7-conjugated anti-CD8 (53-6.7) Abs and electronically sorted using either a FACSVantage or FACSAria flow cytometer (BD Biosciences). To cleave off surface coreceptor expression, purified DP thymocytes were pronase-treated as described previously (38, 39). Briefly, thymocytes were extensively washed with PBS and incubated in 0.01% pronase (Calbiochem) and 100 µg/ml DNase I (Sigma-Aldrich) for 15 min at 37°C. The enzymatic reaction was quenched by washing with medium containing 10% FCS and placed in overnight cultures at 37°C in complete medium. After culture, thymocytes were stained with anti-CD4-APC, anti-CD8-PE, and anti-Thy1.2-FITC Abs, and sample data were acquired with a BD LSR II cytometer (BD Biosciences) and FACSDiVa software (BD Biosciences). FCS used in these experiments had been depleted of endogenous steroids by pretreatment with 0.5% Norit A Charcoal (Sigma-Aldrich) and 0.05% dextran (Sigma-Aldrich).

2.3   Results

CD4 SP thymocytes are critically dependent on RasGRP1 for their development

To address the role of RasGRP1 in the development of thymocytes expressing a MHC class II-restricted TCR, we crossed the AND TCR transgene (TcrAND) onto RasGRP1-null animals. In C57BL/6 (H-2^b) mice, the AND TCR (Vα11, Vβ3) selects DP thymocytes on the MHC class II molecule I-A^b and directs the development of large numbers of CD4 SP thymocytes (34). Strikingly, analyses of thymic subpopulations, as visualized by labeling with anti-CD4 and anti-CD8 Abs, revealed that RasGRP1$^{-/-}$TcrAND mice are virtually

devoid of CD4 SP thymocytes (Fig. 2.1*A*). Associated with impaired thymic selection, RasGRP1$^{-/-}$TcrAND DP thymocytes possess reduced TCR expression, as judged by staining with anti-Tg TCRα, anti-TCRβ or anti-CD3ε Abs, and appear slightly immature by HSA and CD5 levels (Fig. 2.1*B*). By contrast with the MHC class II-restricted AND TCR, our previous results with RasGRP1-deficient mice expressing MHC class I-restricted TCRs exhibited a more modest effect on CD8 T cell development (33). Moreover, mutant mice expressing the weakly selecting H-Y TCR resulted in about a 4-fold reduction in CD8 SP thymocyte number, whereas the recovery of CD8 SP thymocytes bearing the strongly selecting 2C TCR resembled wild-type animals. These results indicate that RasGRP1-independent mechanisms cannot compensate for the positive selection of CD4 SP thymocytes by the AND TCR despite being able to mediate the development of CD8 T cells expressing either the H-Y or 2C TCRs (33).

Decreased ERK activity in RasGRP1$^{-/-}$TcrAND DP thymocytes is correlated with reductions in TCR and Bcl-2 expression

We have previously observed that RasGRP1-deficient DP thymocytes bearing MHC class I-restricted TCRs exhibit reduced levels of active ERK as compared with wild type (33). To examine the contribution of RasGRP1 on ERK activation under the defined TCR signaling conditions of a MHC class II TCR, we used a flow cytometric assay based on a phospho-specific ERK1/2 Ab as described previously (33). Thymocyte single-cell suspensions were rapidly generated in serum-free medium, fixed, and assayed for ERK activity (Fig. 2.2*A*). Analysis of RasGRP1$^{-/-}$TcrAND DP thymocytes revealed a considerable reduction in the frequency of P-ERK1/2^{high} cells (6.8 vs 20%, respectively; Fig. 2.2*A*). Next, we examined whether the level of active ERK correlated strongly with TCR up-regulation, an index of positive selection (Fig. 2.2*B*). Indeed, ERK activity corresponded well with TCR expression regardless of genotype. Wild-type TcrAND DP thymocytes exhibit a large fraction (36%) of P-ERK1/2^{high} AND TCR^{high} cells. Despite diminished positive selection in RasGRP1$^{-/-}$ TcrAND thymi, mutant DP thymocytes possess a smaller but significant fraction of P-ERK1/2^{high} AND TCR^{high} cells (7%). This observation is consistent with previous studies demonstrating a requirement for sustained ERK activity in T cell development (14-17). However, because our flow cytometric-based assay measures ERK activity during a narrow

window in time, it is not possible to determine whether the reductions in ERK signaling are the result of decreased intensity, duration, or both.

Previous studies using MEK1 inhibitors have suggested that ERK signaling is important for inducing Bcl-2 expression during thymic selection (18, 19). To examine whether RasGRP1 facilitates T cell differentiation by influencing Bcl-2 levels, we assayed Bcl-2 expression in TcrAND DP thymocytes via intracellular flow cytometry (Fig. 2C). As noted earlier (40), wild-type TcrAND DP thymocytes have elevated Bcl-2 levels as compared with DP thymocytes from a normal (non-TCR Tg) mouse, a likely consequence of the fact that every thymocyte in a TCR Tg mouse expresses a selectable TCR. By contrast, RasGRP1$^{-/-}$TcrAND DP thymocytes have considerably lower Bcl-2 expression as compared with wild-type TcrAND DP thymocytes (33.4 vs 74.1 mean fluorescence intensity (MFI); Fig. 2.2C). These findings suggest that RasGRP1 may transduce survival signaling via a Bcl-2-dependent mechanism.

RasGRP1$^{-/-}$ DP thymocytes demonstrate defective P-ERK, CD69, and Bcl-2 up-regulation following TCR engagement

ERK activation is a critical event in T cell development, and its relative activity correlates with the level of positive selection (41). Our analyses of TCR Tg mice demonstrate that RasGRP1-deficient DP thymocytes possess reduced levels of active ERK in isolated cells ex vivo (Ref. 33; Fig. 2). However, these reductions may be an indirect effect, a consequence of impaired positive selection in mutant animals. Therefore, we sought to investigate the direct effect of RasGRP1 deficiency on TCR-mediated ERK activation by stimulating DP thymocytes in vitro. A previous study (26) has shown that RasGRP1$^{-/-}$ thymocytes do not appreciably activate ERK upon either PMA or TCR stimulation. However, since this result was obtained from total thymocytes, it raises a concern that the differential composition of wild-type and mutant thymocyte populations may be responsible for these observations. Therefore, for TCR signaling studies, we sorted DP thymocytes from normal (non-TCR Tg) wild-type and mutant thymi and found that they expressed similar amounts of TCR/CD3ε (Fig. 2.3, A and B). Purified RasGRP1$^{+/+}$ and RasGRP1$^{-/-}$ DP thymocytes were subjected to anti-TCR cross-linking for the indicated times (Fig. 2.3C). Wild-type cells induced strong

ERK1/2 phosphorylation after TCR stimulation, whereas mutant DP thymocytes exhibited only a weak signal indicating that optimal TCR-induced ERK1/2 phosphorylation in these cells is dependent on RasGRP1. However, Zap70 and LAT, molecules upstream of RasGRP1, exhibit near-normal phosphorylation following TCR stimulation (Fig. 2.3C). This finding indicates that TCR-induced ERK activation is diminished in RasGRP1$^{-/-}$ DP thymocytes, whereas more proximal TCR signaling appears unaffected.

To examine whether RasGRP1 has direct impact on the expression of various markers of positive selection, we took DP thymocytes from normal (non-TCR Tg) wild-type and mutant animals and cultured them in vitro for 1 day in the presence or absence of TCR stimulation (Fig. 2.3D). Previous studies have shown that TCR engagement of DP thymocytes can induce many aspects of positive selection in vitro (42, 43). For our studies, we used two types of stimulation, either anti-TCR alone or anti-TCR plus anti-CD4 Abs, to mimic conditions of weak or strong TCR signaling. Analysis of CD5 induction revealed that RasGRP1$^{-/-}$ DP thymocytes behave similarly to wild-type cells under both conditions tested (Fig. 2.3D). In contrast, mutant thymocytes do not efficiently up-regulate CD69 upon stimulation with either TCR agonist. To address the RasGRP1 function in supporting survival signaling, we assessed the ability of mutant thymocytes to elevate Bcl-2 expression following TCR engagement. Using anti-TCR Ab alone, RasGRP1$^{-/-}$ DP thymocytes failed to up-regulate Bcl-2 significantly. However, when anti-TCR and anti-CD4 Abs are used in tandem, Bcl-2 can be induced in mutant thymocytes, although these levels are still lower than wild type (16.3 vs 35.8 MFI). These findings suggest that RasGRP1$^{-/-}$ DP thymocytes must receive strong TCR signaling for their positive selection and support the conclusion that RasGRP1 augments Bcl-2 expression.

Tg Bcl-2 expression fails to restore CD4 T cell development in RasGRP1-deficient mice
Positive selection entails the rescue of DP thymocytes from programmed cell death and is dependent on the appropriate level of TCR/self-MHC signaling to provide antiapoptotic cues, such as the induction of Bcl-2 (40, 44-46). Because our in vitro findings suggest that RasGRP1 promotes Bcl-2 levels (Fig. 2.3D), we sought to determine whether enforced Bcl-2 expression could rescue CD4 T cell development in RasGRP1-deficient mice. To test our

hypothesis, we introduced the Eμ-Bcl-2 transgene into both wild-type and RasGRP1$^{-/-}$ TcrAND mice (Fig. 2.4).

In RasGRP1-null animals, transgene-driven Bcl-2 expression results in a sharp increase in CD4 SP thymocyte number (6.1 ± 2.4 x 10^6 vs 0.31 ± 0.05 x 10^6), although still considerably lower than wild type (46.0 ± 7.6 x 10^6). Next, we examined whether Eμ-Bcl-2 RasGRP1$^{-/-}$ CD4 SP thymocytes display signs of differentiation. To address this question, we compared the level of TCR expression in Eμ-Bcl-2 RasGRP1$^{-/-}$TcrAND DP and CD4 SP thymocytes relative to wild-type TcrAND CD4 SP thymocytes (Fig. 2.4*C*). These analyses revealed that Eμ-Bcl-2 RasGRP1$^{-/-}$TcrAND CD4 SP thymocytes showed signs of positive selection because they had slightly elevated TCR expression vs DP thymocytes. This increase in TCR expression was observed regardless of whether cytometry was performed with anti-Tg TCRα-, anti-Tg TCRβ-, or anti-TCRβ constant region-specific Abs (Fig. 2.4*C* and data not shown). However, TCR expression by Eμ-Bcl-2 RasGRP1$^{-/-}$TcrAND CD4 SP thymocytes is considerably lower than the level that is exhibited by wild-type TcrAND CD4 SP thymocytes. Corroborating these studies, the analysis of non-TCR Tg animals lacking RasGRP1 also revealed that Bcl-2 overexpression fails to restore CD4 SP thymocyte proportion or numbers (data not shown). Therefore, we conclude that the developmental arrest of RasGRP1$^{-/-}$ TcrAND DP thymocytes cannot be rescued by provision of Tg Bcl-2 expression.

In agreement with a previous study using lck^{pr}-Bcl-2 TcrAND mice (40), the Eμ-Bcl-2 transgene has a dramatic effect on wild-type TcrAND thymi. These changes include an increase in total thymocyte number, the formation of massive numbers of AND TCR$^+$ CD8 SP thymocytes, and a modest augmentation of the CD4 SP thymocyte number (Fig. 2.4, *A* and *B*). Although Linette et al. (40) have suggested that these CD8 SP thymocytes are developmentally arrested, we have found that this population possesses characteristics of mature T cells—expressing high levels of AND TCR, bearing intermediate to low levels of HSA and proliferating when contacted by cognate Ag (data not shown). These findings suggest that limited survival signaling restricts the formation of CD8 SP thymocytes in wild-type TcrAND animals. However, the fact that enforced Bcl-2 expression results in only a modest increase in RasGRP1$^{-/-}$TcrAND CD8 SP thymocyte number (8.0 ± 3.8 x 10^6 vs 3.9 ±

0.8 x 10^6) argues that their inefficient positive selection is not primarily due to deficient Bcl-2 expression. Therefore, we conclude that RasGRP1 is necessary for development of massive numbers of CD8 SP thymocytes seen in wild-type Eµ-Bcl-2 TcrAND mice and provides developmental cues that are independent of Bcl-2 expression.

RasGRP1-deficient TcrAND CD8 SP thymocytes resemble CD8 SP thymocytes that develop in RasGRP1$^{-/-}$ mice expressing the MHC class I-restricted H-Y TCR

In addition to the lack of CD4 SP thymocytes, another feature of RasGRP1$^{-/-}$TcrAND thymus is a small population of CD8 SP thymocytes that expresses the CD8αβ coreceptor (Figs. 2.1*A* and 2.5*A* and data not shown). Uncannily, the thymic CD4/CD8 developmental profile of RasGRP1$^{-/-}$TcrAND mice seems reminiscent of mutant (female) animals expressing the MHC class I-restricted H-Y TCR (Fig. 2.5*A*). However, in contradistinction to the comparison of wild-type and RasGRP1$^{-/-}$TcrH-Y mice, there is no change in CD8 SP thymocyte frequency or cell number recovered from RasGRP1$^{-/-}$TcrAND mice relative to wild type (Fig. 2.5*B*). To examine whether RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes might be a product of positive selection, we compared surface TCR expression and markers of differentiation between RasGRP1-deficient CD8 SP thymocytes expressing either the AND or H-Y TCR (Fig. 2.5*C*). Regardless of the TCR expressed, mutant CD8 SP thymocytes share an immature cell surface phenotype (HSA^{high}) and possess low levels of TCR, CD5, and CD69. These observations suggest that RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes may be the product of weak and/or inefficient positive selection.

RasGRP1$^{-/-}$TcrAND DP thymocytes give rise to a few CD8 but not CD4 SP thymocytes after intrathymic transfer

Some unusual features of RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes may raise doubt regarding their developmental origin. Their rarity and relative immaturity (HSA^{high}, CD69^{low}, CD8^{low}) suggests the possibility that these cells may be immature SP thymocytes (ISPs), a transitional, developmental intermediate that exists between the double-negative (DN) and DP cell stages (47), rather than being progenies of DP thymocytes. To gain insight into the derivation of RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes, we used electronic cell sorting of DP

thymocytes in combination with a previously described coreceptor re-expression assay (38). The ability of coreceptor re-expression assay to assess lineage commitment is based on the fact that developing DP thymocytes committing to either the CD4 or CD8 lineage terminate synthesis of the inappropriate coreceptor molecule. The coreceptor re-expression assay enables one to discern what coreceptor molecules are actively being synthesized by individual cells by first stripping thymocytes of pre-existing coreceptor molecules by treatment with a protease called "pronase," followed by culturing overnight at 37°C to enable cells to re-express coreceptor proteins and finally assessing their newly synthesized CD4/CD8 surface phenotype by flow cytometry.

To assess the developmental potential of RasGRP1$^{-/-}$TcrAND DP thymocytes in vivo, we purified TcrAND DP thymocytes (Thy1.2$^+$) by cell sorting (Cell purity: >98% for wild type, >99% RasGRP1$^{-/-}$) before adoptively transferring them into B6 (Thy1.1$^+$) host thymi (Fig. 2.6). Two days post-IT, recovered thymi were assessed for CD4/CD8 surface phenotype of donor cells either before (untreated) or after pronase treatment and overnight culture at 37°C (pronase/37°C). Under these in vivo conditions, RasGRP1$^{-/-}$TcrAND DP thymocytes gave rise to CD8 SP thymocytes (4.4%) but no CD4 SP thymocytes (Fig. 2.6). Importantly, the frequency of RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes (3.75 ± 1.73%; n = 7) and CD4 SP thymocytes (0.02 ± 02%; n = 7) observed in seven independent DP thymocyte IT experiments are similar to what is observed in normal RasGRP1$^{-/-}$TcrAND thymi (Fig. 2.1). By contrast, wild-type TcrAND DP thymocytes gave rise to larger frequencies of both CD4 (9.9 ± 1.7%; n = 5) and CD8 SP thymocytes (12.3 ± 3.2%; n = 5). The observed increase in CD8 lineage cells is in line with a recent report, which showed that weakly selecting (or nonselecting) neighboring thymocytes can shift some AND TCR-expressing thymocytes toward the CD8 lineage (48). True to form, the coreceptor re-expression assay revealed an increased proportion of lineage-committed cells and conversely a decreased proportion of DP thymocytes in both wild-type and mutant samples. More importantly, this assay indicated that RasGRP1$^{-/-}$TcrAND DP thymocytes could give rise to only CD8 but not CD4 thymocytes. Oddly, a sizeable fraction of RasGRP1$^{-/-}$TcrAND DP thymocytes failed to express either surface CD4 or CD8 molecules, particularly after pronase treatment, and it is unclear whether the lack of RasGRP1 signaling has either aborted their development or taken

them toward a cryptic differentiation program with a CD4⁻CD8⁻ destiny. Previously, the derivation of CD4⁻CD8⁻ progeny has been associated with DP thymocytes cultured in medium alone (no signal) before their intrathymic transfer as compared with those treated with either PMA/ionomycin or anti-TCR plus anti-CD2 Abs (39). In conclusion, our IT experiments indicate that RasGRP1⁻ᐟ⁻TcrAND DP thymocytes yield a similar percentage of CD8 SP thymocytes as observed in the thymi of unmanipulated RasGRP1⁻ᐟ⁻TcrAND mice. Although these findings do not exclude an alternate origin, our observations are consistent with the notion that CD8 SP thymocytes present in RasGRP1⁻ᐟ⁻TcrAND animals are derived from DP thymocytes.

Mutant CD8 SP thymocytes express markers of positive selection, respond to a MHC class II-specific Ag, and differentiate into cytotoxic effectors

We have previously observed that the formation of CD8 SP thymocytes is associated with an elevated level of active ERK relative to DP thymocytes (33). Therefore, we sought to determine whether TcrAND CD8 SP thymocytes possess a similarly heightened ERK activity directly ex vivo (Fig. 2.7*A*). Indeed, both wild-type and mutant TcrAND CD8 SP thymocytes exhibited an elevated level of ERK activity, typical of positive selection. However, the nonconformity of CD8 SP thymocytes expressing a MHC class II-restricted TCR combined with their immature surface phenotype raises the question of whether these cells have truly undergone the positive selection process. To address this issue, we next examined whether RasGRP1⁻ᐟ⁻TcrAND CD8 SP thymocytes exhibit two other events associated with the formation of SP thymocytes, IL-7R expression and elevated Bcl-2 levels. Somewhat surprisingly, RasGRP1-sufficient TcrAND CD8 SP thymocytes do not express detectable levels of IL-7R and have atypically low quantities of Bcl-2 (Fig. 2.7*B*). Apparently, the selective formation of CD4 SP thymocytes impairs CD8 SP thymocyte development in wild-type TcrAND mice (34, 48) and results in very few peripheral CD8⁺ T cells (34). In contrast, RasGRP1⁻ᐟ⁻TcrAND CD8 SP thymocytes express IL-7R and display Bcl-2 amounts characteristic of SP thymocytes, suggesting that these cells have undergone positive selection. Again, RasGRP1⁻ᐟ⁻ CD8 SP thymocytes expressing the AND TCR share quite similar attributes to those bearing H-Y TCRs. Therefore, in contradistinction to CD8 SP thymocytes

from wild-type TcrAND mice, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes bear hallmarks of mature SP thymocytes.

A more definitive measure of positive selection is acquisition of mature T cell function. Therefore, we sought to establish whether the CD8 SP thymocytes that develop in RasGRP1$^{-/-}$TcrAND mice could proliferate upon TCR engagement. Upon placement into tissue culture wells coated with anti-TCR Ab (either anti-CD3ε or anti-TCR Vα11 Ab), mutant TcrAND CD8 SP thymocytes proliferated at least as strongly as wild-type TcrAND CD4 and CD8 SP thymocytes (Fig. 2.7C). Next, we tested the capacity of these subpopulations to react with the cognate Ag for the AND TCR, the PCC peptide (88–104) presented by MHC class II H-2E^k molecule (34). Equivalent numbers of responder T cells were incubated with various doses of peptide plus irradiated, H-2E^k-expressing stimulator splenocytes. In contrast to the proliferation assays based on plate-bound anti-TCR Abs, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes proliferated less vigorously than wild-type TcrAND CD4 SP thymocytes, particularly at low peptide concentrations (Fig. 2.7D). A plausible reason for the poorer response to peptide stimulation is that TcrAND CD8 SP thymocytes lack the appropriate coreceptor for corecognition of the I-E^k MHC class II molecule.

The acquisition of effector function is another stringent evaluation of positive selection. To address this question, TcrAND SP thymocytes were activated with anti-TCR Vα11 Ab and examined for hallmarks of CD8 T cell effector function, namely the expression of the cytotoxic-associated molecule granzyme B and cytotoxicity. In contrast to TcrAND CD4 SP thymocytes, TcrAND CD8 SP thymocytes, regardless of genotype, rapidly expressed high levels of granzyme B upon activation (Fig. 2.7E). Subsequently, we investigated whether activated TcrAND SP thymocytes could mediate peptide-specific cytotoxicity (Fig. 2.7F). Indeed, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes could kill ^{51}Cr-labeled targets in a MHC class II-restricted (PCC)-dependent fashion. Wild-type TcrAND CD4 SP thymocytes were at least 10-fold less potent killers (mutant cells have a greater kill at 1:1 E:T ratio compared with 10:1 for wild type). Thus, despite expressing a relatively immature cell surface phenotype, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes can efficiently kill Ag-coated target cells and thus are functionally mature.

RasGRP1$^{-/-}$TcrAND animals possess significant numbers of peripheral AND TCR$^+$ CD8$^+$ T
cells but very small numbers of AND TCR$^+$ CD4$^+$ T cells

To investigate the consequences of altered thymic selection on the peripheral lymphoid compartments, we examined the splenic T cell composition in these animals (Fig. 2.8*A*). In RasGRP1$^{-/-}$TcrAND mice, there is a 35-fold reduction in AND TCR$^+$ CD4 T cell numbers, whereas the recovery of AND TCR$^+$ CD8 T cells is increased 1.8-fold relative to wild-type mice (splenic cell recovery: RasGRP1$^{+/+}$TcrAND, 98.7 ± 11.2 x 10^6, n = 3; RasGRP1$^{-/-}$ TcrAND, 78.1 ± 7.1 x 10^6, n = 3). Curiously, mutant CD8 T cells express slightly lower levels of CD8α- and CD8β-chain (Fig. 2.8*A* and data not shown). It is also noteworthy that only 23% of peripheral CD4 T cells in RasGRP1$^{-/-}$ mice express high levels of the AND TCR, suggesting that the majority of these cells exploit endogenous TCRα-chains for positive selection (Fig. 2.8*B*). By contrast, peripheral CD8 T cells from RasGRP1$^{-/-}$TcrAND mice exclusively express the AND TCR (Fig. 2.8*B*), consistent with the notion that these cells use the AND TCR for their development. Moreover, RasGRP1$^{-/-}$TcrAND CD8 T cells share a comparable cell surface phenotype to functionally mature female RasGRP1$^{+/+}$TcrH-Y CD8 T cells (Fig. 2.8*C*). These observations support the conclusion that the few CD8 T cells that develop in RasGRP1$^{-/-}$TcrAND mice are the product of the positive selection process mediated by the AND TCR.

2.4   Discussion

Experiments with TCR Tg mice have demonstrated that thymocyte cell fate is dependent on whether the TCR of a developing DP thymocyte recognizes self-MHC class I or -MHC class II molecules. The strength of signaling model of lineage commitment proposes that intensity and/or duration of a TCR signaling determines whether a developing DP thymocyte chooses a helper CD4$^+$ or cytotoxic CD8$^+$ T cell fate; stronger and/or sustained TCR signaling results in CD4 SP thymocytes, whereas weaker and/or transient signaling produces CD8 SP thymocytes. Defining the molecular mechanisms controlling this cell fate decision has been a central focus of many investigations. Roles of the Ras/MAPK signaling pathway in positive selection have been implied by studies using either pharmacologic inhibitors of MEK1/2 in

vitro or Tg mice expressing copious amounts of either constitutively active or DN forms of Ras and downstream MAPK family members (49, 50). In addition, some studies have suggested that ERK signaling strength may also be deterministic in lineage commitment (21-23). Moreover, studies based on either transgene overexpression or pharmacologic inhibitors may be especially prone to pleiotropic effects. Thus, many questions remain on how surface TCR signal transduction connects to Ras activation, links up to MAPK signaling pathways, and contributes to signaling cues necessary for the survival and differentiation of DP thymocytes. Using RasGRP1-null mice, we have previously shown that RasGRP1-dependent and -independent mechanisms exist for ERK activation and positive selection of CD8 SP thymocytes (33). Because the formation of CD4 SP thymocytes is thought to be dependent on prolonged TCR signaling (51) and RasGRP1 induces Ras activation with sustained and/or delayed kinetics (30-32), we sought to investigate the relative importance of RasGRP1 in the development of thymocytes expressing a MHC class II-restricted TCR. Herein, we report that RasGRP1 is a crucial TCR signaling component for CD4 T cell development.

The surprising finding of CD8 SP thymocytes, instead of CD4 SP thymocytes, in RasGRP1$^{-/-}$ TcrAND raised questions regarding their nature and origin. The CD8 dull-phenotype of these cells suggests the possibility that they may belong to the γδ T cell lineage, because premature expression of the αβ TCR in mice bearing the TcrH-Y and TcrAND transgenes causes aberrant γδ T cell development (52, 53). Moreover, male TcrH-Y (H-2^b) mice notably contain large numbers of mature H-Y TCR$^+$ CD8^{low} and H-Y TCR$^+$ DN T cells with γδ T cell-like properties (52, 53), whereas TcrAND transgene is normally associated with an abundance of mature AND TCR$^+$ DN T cells possessing such traits (53). However, TcrH-Y (H-2^b) mice only develop CD8^{low} H-Y TCR$^+$ T cells when nominal Ag (male, H-2^b) is present because they are absent in the positively selecting (female, H-2^b) and nonselecting (male or female, H-2^d) backgrounds (54). Because TcrAND mice on a H-2^b background lack nominal Ag, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes are unlikely to be γδ-lineage "wannabe" cells analogous to those present in H-Y H-2^b male mice. Another possibility regarding the origin of RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes is raised by their immature phenotype (HSA^{high}, CD69^{low}) that is often characteristic of ISPs rather than "true" (mature) CD8 SP thymocytes. In contrast to ISPs, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes exhibit

mature functional attributes and can be derived from RasGRP1$^{-/-}$TcrAND DP thymocytes. Therefore, together with our intrathymic transfer experiments (Fig. 2.6), indicating that RasGRP1$^{-/-}$TcrAND DP thymocytes can give rise to some CD8 but not CD4 SP thymocytes, it is reasonable to presume that CD8 SP thymocytes observed in RasGRP1$^{-/-}$TcrAND mice are the product of inefficient positive selection and of the αβ lineage.

The fact that RasGRP1$^{-/-}$ DP thymocytes expressing a MHC class II-restricted TCR fail to develop into CD4 SP thymocytes but can form functional CD8 SP thymocytes suggests that CD4 T cell development may be much more dependent on RasGRP1 than the development of CD8 T cells. This conclusion is corroborated with our MHC class I-restricted TCR Tg studies (33), defining significant RasGRP1-independent mechanisms for the positive selection of CD8 SP thymocytes, and reduced ratio of CD4 vs CD8 SP thymocytes in both normal and TCR Tg RasGRP1$^{-/-}$ mice. The findings are also consistent with previous studies using thymocyte cultures in combination with MEK1 inhibitors to analyze the significance of the Ras/ERK pathway (21-23). These studies revealed that the formation of CD4 SP thymocytes is more dependent on ERK activation than CD8 SP thymocytes. Reinforcing these observations, a recent report on ERK-deficient mice has found that the elimination of ERK1 and ERK2 protein preferentially affects the CD4 lineage (55). Therefore, we propose that RasGRP1-deficiency reduces the propensity of TCR signaled-DP thymocytes to take the CD4 SP developmental path rather than directly influencing lineage commitment per se.

To examine whether RasGRP1 promotes CD4 T cell development by inducing Bcl-2 expression, we introduced the Eμ-Bcl-2 transgene into RasGRP1$^{-/-}$TcrAND animals. In Eμ-Bcl-2 RasGRP1$^{-/-}$TcrAND mice, we observed a substantive (20-fold) recovery of CD4 SP thymocytes vs RasGRP1$^{-/-}$TcrAND animals lacking the Bcl-2 transgene. Because enforced Bcl-2 expression cannot transform DP thymocytes into SP thymocytes in the absence of MHC molecules (40), the prosurvival effect of Bcl-2 without TCR signaling is insufficient for the formation of SP thymocytes. Therefore, the increased presence of CD4 SP thymocytes in Eμ-Bcl-2 RasGRP1$^{-/-}$TcrAND mice argue that RasGRP1 assists CD4 T cell development by providing survival signaling to DP thymocytes. However, the facts that the CD4 SP thymocyte number is still much less than wild type (7.5-fold decrease) and that the

cells that develop have lower TCR expression indicates that RasGRP1 transmits differentiation signals independent of those necessary for Bcl-2 induction. Collectively, our data suggest that positive selection in RasGRP1$^{-/-}$TcrAND is inefficient, with only a small proportion of DP thymocytes receiving TCR signaling strength necessary for differentiation.

The impaired T cell development in RasGRP1-deficient animals results in a large number of discernable alterations including decreased ERK activation, altered surface marker expression, and reduced Bcl-2 levels. Such changes may result either directly from RasGRP1 -deficiency or indirectly, a consequence of delayed positive selection. To determine whether RasGRP1 function was unequivocally connected to some of these changes, we cultured DP thymocytes from non-TCR Tg mice in the presence or absence of anti-TCR Ab stimulation to mimic the positive selection process in vitro as described previously (42, 43). Although CD5 induction is not affected, RasGRP1$^{-/-}$ DP thymocytes fail to up-regulate the positive selection marker CD69. This observation is consistent with a previous study demonstrating that this TCR-mediated event is dependent on Ras activation (56). Our experiment also revealed that RasGRP1$^{-/-}$ DP thymocytes require conditions of stronger TCR signaling to elevate Bcl-2 levels. As a consequence, these data suggest that RasGRP1$^{-/-}$ DP thymocytes must be strongly self-reactive to overcome their signaling deficits and differentiate into SP thymocytes. Notably, a recent report has linked T cells that develop in the absence of RasGRP1 to autoimmune consequences in a novel mouse strain, bearing a spontaneous mutation in RasGRP1 (57).

The relative rarity of TcrAND CD8 SP thymocytes in the RasGRP1$^{-/-}$ mice may be attributable to a couple of factors. First, the lack of concordance between TCR/MHC specificity and coreceptor expression has been postulated to result in reduced avidity for MHC class II molecules, inefficient TCR signaling, and subsequent failure of such mismatched cells to develop. The facts that MHC class II-restricted CD8 T cells can be positively selected and function in CD4-deficient mice argues against this conclusion (6-8). However, although these experiments demonstrate that MHC class II-specific CD8 T cells can develop, their formation and maintenance may still be compromised. Second, RasGRP1 is necessary for the efficient development of CD8 SP thymocytes (33). It is possible that the

down-regulation of CD4 and lack of RasGRP1 expression may collaborate to limit the formation of RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes. The inability of Tg Bcl-2 expression to greatly improve CD8 SP thymocyte numbers in RasGRP1$^{-/-}$TcrAND mice suggests that their limited quantity is not the result of inadequate levels of Bcl-2 but rather inefficient TCR signaling required for CD8 T cell differentiation. Since T cells require optimal TCR and CD4/CD8 coreceptor stimulation for their differentiation and survival, it serves not only to facilitate self-restriction but also maintains concordance between MHC specificity, effector function, and the CD4/CD8 phenotype of T cells in normal mice.

It is noted that small numbers of MHC class II-restricted CD8 T cells have been observed in wild-type MHC class II-restricted TCR Tg animals (6, 58). One potential explanation for the origin of these cells is that they are a product of limiting niches for selection of CD4 SP thymocytes in wild-type TcrAND mice. Using the strength of signal model of lineage commitment, some DP thymocytes may receive weakened TCR signaling due to the intense competition for positively selecting ligands (self-peptides/self-MHC class II) and thus be diverted to the CD8 lineage. Previous studies have shown that the development of MHC class II-restricted CD8 T cells in these animals requires MHC class II molecules but is independent of endogenous TCRα-chains (6, 58). Although we cannot formally rule out the participation of endogenous TCRα-chains in the derivation of RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes, the fact that virtually all RasGRP1$^{-/-}$TcrAND peripheral CD8 T cells express the AND TCR (Fig. 2.8B) implies that endogenous TCRα-chains are not critical for their development.

The emerging role of subcellular compartmentalization in cell signaling has impacted the Ras/MAPK pathway (59). Recent work has demonstrated that PLCγ1 selectively activates RasGRP1 on the Golgi and is therefore distinct from the Grb2/Sos pathway that activates Ras at the PM (30-32). Differential localization may serve to alter the duration of signaling and/or pair-activated Ras with a different subset of effectors. Pointedly, Golgi-activated Ras has been found to have a delayed or sustained kinetics relative to PM-activated Ras (30-32). Such prolonged signaling by RasGRP1 may be critical to efficiently induce the expression of Th-POK (also known as cKrox), a Krüppel transcription factor family member that is both

necessary and sufficient to direct thymocytes undergoing positive selection into CD4 lineage (60, 61). Collectively, our results position RasGRP1 and its product, Golgi-activated Ras, to play a crucial role in initiating the CD4 T cell differentiation program.

2.5   Acknowledgements

2.6 Figures

Figure 2.1 RasGRP1$^{-/-}$ mice expressing the MHC class II-restricted AND TCR lack CD4 SP thymocytes. *A*, Thymocytes from wild-type and mutant mice expressing MHC class II-restricted AND TCR were stained with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the dot plots. Thymocyte recovery from wild-type and RasGRP1-deficient TCR Tg animals were as follows: RasGRP1$^{+/+}$TcrAND, 131.2 ± 20.1 x 10^6 (n = 6); and RasGRP1$^{-/-}$TcrAND, 95.4 ± 17.3 x 10^6 (n = 7). *B*, Flow cytometric analyses of TCR and developmental marker expression displayed by wild-type (shaded, thin line) and mutant (bold-line) DP thymocytes.

Figure 2.2 RasGRP1-deficient TcrAND DP thymocytes display reductions in ERK activity and Bcl-2 expression. *A*, P-ERK reactivity in wild-type (+/+) and RasGRP1$^{-/-}$ (-/-) TcrAND DP thymocytes was assayed by flow cytometry directly ex vivo. Numbers within the histogram represent the proportion of P-ERK1/2^{high} cells. Shaded histogram represents staining observed with secondary Ab alone. *B*, Up-regulation of the AND TCR on DP thymocytes correlates with elevations in ERK activity. DP thymocytes from wild-type (+/+) and RasGRP1$^{-/-}$ (-/-) TcrAND mice were stained with anti-P-ERK1/2 and anti-AND TCR Abs as described in *Materials and Methods*. Percentage of cells residing within each quadrant is shown on the dot plots. *C*, Bcl-2 histograms representing RasGRP1$^{+/+}$TcrAND, RasGRP1$^{-/-}$TcrAND, and isotype-Ab control DP thymocyte staining (shaded) are shown. MFIs are indicated within the histogram.

Figure 2.3 RasGRP1$^{-/-}$ DP thymocytes exhibit a decreased capacity to up-regulate P-ERK, CD69, and Bcl-2 levels following TCR engagement. *A*, Normal (non-TCR Tg) RasGRP1$^{+/+}$, and RasGRP1$^{-/-}$ thymocytes were stained with anti-CD4 and anti-CD8 Abs and subjected to cell sorting. Percentage of cells residing within each quadrant is shown on the density plots. Boxed area indicates the gate location used to sort DP thymocytes for signaling studies. *B*, Sorted RasGRP1$^{+/+}$ (thin line) and RasGRP1$^{-/-}$ DP thymocytes (bold line) express similar

levels of CD3ε. *C*, Sorted DP thymocytes were stimulated for indicated times (min) with anti-CD3ε. Data from one representative experiment of four are shown. *D*, Total thymocytes from wild-type and mutant mice were incubated for 24 h in the presence (bold line) or absence (thin, dashed line) of plate-bound anti-TCR (αTCR) or anti-TCR plus anti-CD4 Abs (αTCR + αCD4). Data presented were electronically gated on viable DP thymocytes (CD4$^+$CD8$^+$ 7-AAD$^-$). Numbers within Bcl-2 histograms indicate the MFI with (right) and without (left) TCR stimulation. Shaded histograms indicate intracellular staining observed with an isotype-control Ab.

Figure 2.4 Tg Bcl-2 expression fails to restore CD4 T cell development in RasGRP1$^{-/-}$ TcrAND mice. *A*, Thymic developmental profiles for wild-type TcrAND and RasGRP1-deficient TcrAND thymocytes bearing the Eµ-Bcl-2 transgene were ascertained by staining with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the density plot. *B*, Cell numbers present within thymic subpopulations among various mice shown in *A*. *C*, RasGRP1$^{-/-}$TcrAND CD4 SP thymocytes (thin line) express intermediate TCR levels as compared with wild-type TcrAND CD4 SP thymocytes (shaded, thin line) and RasGRP1$^{-/-}$TcrAND DP SP thymocytes (bold line). Dotted, gray line represents background autofluorescence staining.

Figure 2.5 RasGRP1$^{-/-}$ animals share a similar thymic developmental profile regardless of whether they express either the MHC class II-restricted or MHC class I-restricted TCR. *A*, Thymocyes from RasGRP1$^{-/-}$TcrH-Y or TcrAND were stained with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the density plot. *B*, Cell numbers present within thymic subpopulations from both wild-type and RasGRP1-deficient TcrAND and TcrH-Y were determined. To facilitate comparison between various thymi, cell numbers present within thymic subpopulations were plotted in bar graph format as the percentage of cells relative to wild type. *C*, RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes express comparable levels of various maturation markers as RasGRP1$^{-/-}$TcrH-Y CD8 SP thymocytes.

Figure 2.6 RasGRP1$^{-/-}$TcrAND DP thymocytes exhibit limited developmental potential. Wild-type and RasGRP1$^{-/-}$TcrAND thymocytes were stained with anti-CD4 and CD8 Abs and subjected to electronic cell-sorting to purify DP thymocytes. The sorting gate is shown as a box superimposed on original thymocyte populations (left). To assess the potential of TcrAND DP thymocytes to differentiate in vivo, purified DP thymocytes were injected directly into the thymus of congenic (Thy1.1$^+$) B6 mice. Two days after injection, host thymi were harvested, and the coreceptor expression of donor cells (Thy1.2$^+$) was determined either directly ex vivo (Untreated) or after coreceptor re-expression assay (Pronase/37°C). In this assay, cells are treated with pronase extracellularly to strip pre-existing CD4 and CD8 coreceptor molecules and cultured overnight. After culture at 37°C, surface coreceptor expression is a consequence of coreceptor molecules being actively synthesized by the cell. Frequency of cells present within each quadrant is indicated.

Figure 2.7 CD8 SP thymocytes that develop in RasGRP1$^{-/-}$TcrAND mice respond to MHC class II-specific peptide and differentiate in cytolytic effectors. *A*, TcrAND CD8 SP thymocytes possess elevated ERK activity. Numbers within histograms represent background subtracted MFIs. Shaded histograms represent staining observed with secondary alone. *B*, CD127 (IL-7Rα) and Bcl-2 histograms representing RasGRP1$^{+/+}$, RasGRP1$^{-/-}$, and isotype-control staining of CD8 SP thymocytes. *C*, Sorted RasGRP1$^{+/+}$TcrAND CD4 SP, RasGRP1$^{+/+}$TcrAND CD8 SP, and RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes were cultured on plates coated with either anti-CD3ε or anti-Vα11 (AND TCR; clone RR8-1) Ab plus exogenous IL-2 as described in *Materials and Methods*. *D*, Sorted SP thymocytes, either RasGRP1$^{+/+}$TcrAND CD4 SP, RasGRP1$^{+/+}$TcrAND CD8 SP, or RasGRP1$^{-/-}$TcrAND CD8 SP, were stimulated with various doses of PCC and exogenous of IL-2 as described in *Materials and Methods*. *E*, Using intracellular flow cytometry, RR8-1-activated RasGRP1$^{+/+}$TcrAND CD4, RasGRP1$^{+/+}$TcrAND CD8, and RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes were stained with anti-granzyme B Ab. *F*, RR8-1-activated RasGRP1$^{+/+}$TcrAND CD4 SP and RasGRP1$^{-/-}$TcrAND CD8 SP thymocytes were tested for their ability to kill the CH12 (I-E^k expressing) lymphoma either in the presence or absence of PCC. Assays were

done in triplicate, and the error bars represent the SD. Data from one representative experiment of three are shown.

Figure 2.8 RasGRP1$^{-/-}$TcrAND animals possess significant numbers of peripheral AND TCR$^+$ CD8$^+$ T cells but very small numbers of AND TCR$^+$ CD4 T cells. *A*, Lymph node cells were stained with anti-CD4 and anti-CD8 Abs. Percentage of cells residing within each quadrant is shown on the density plots. *B*, Clonotypic TCR (anti-Vα11 Ab; clone RR8-1) expression by peripheral CD4 and CD8 T cells in with wild-type and RasGRP1$^{-/-}$TcrAND animals. Numbers within histograms indicate the proportion of T cells expressing high levels of the AND TCR. *C*, Comparison of differentiation markers and TCR expression by CD8 T cells from the lymph nodes of female RasGRP1$^{+/+}$TcrH-Y (shaded), female RasGRP1$^{-/-}$TcrH-Y (thin-line), and RasGRP1$^{-/-}$TcrAND (bold-line) mice. Data from one representative experiment of three is shown.
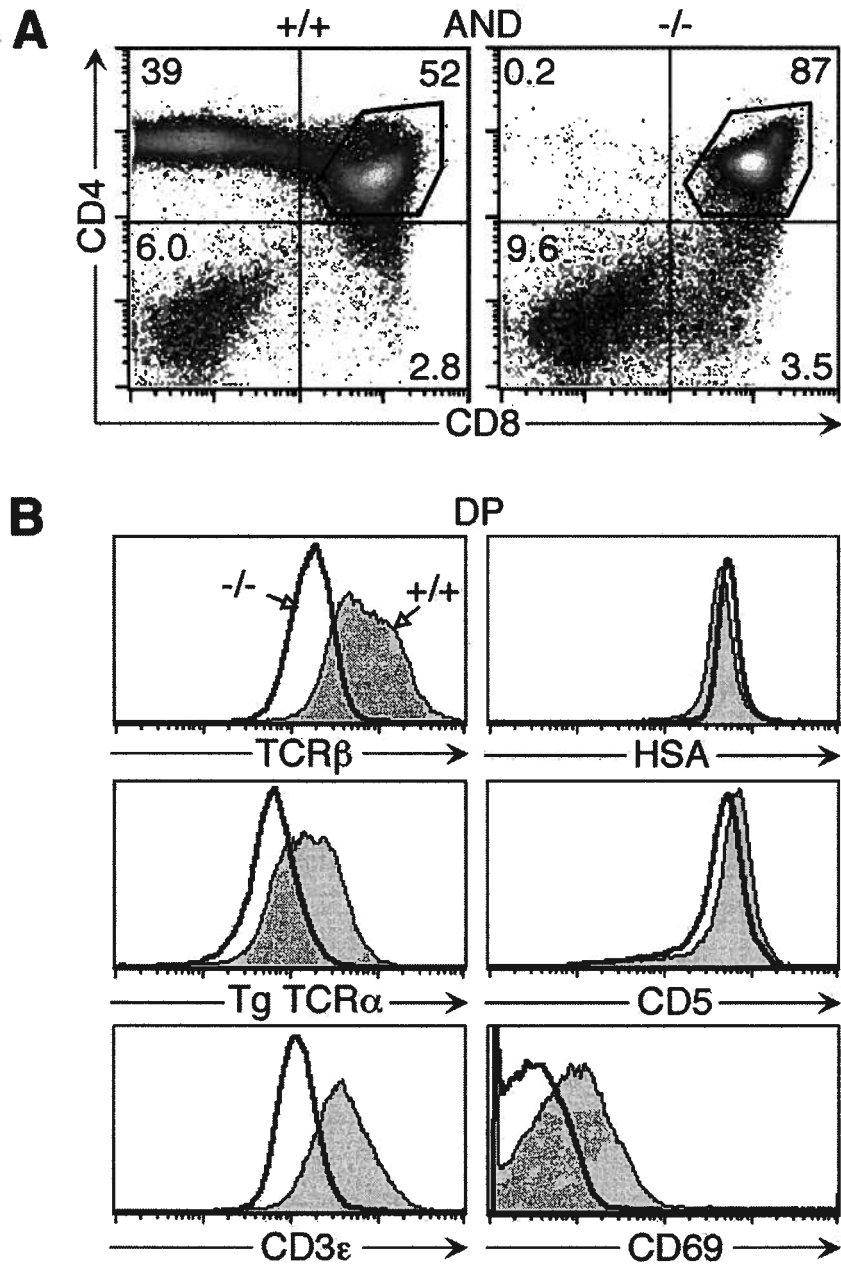
Figure 2.1 RasGRP1$^{-/-}$ mice expressing the MHC class II-restricted AND TCR lack CD4 SP thymocytes.
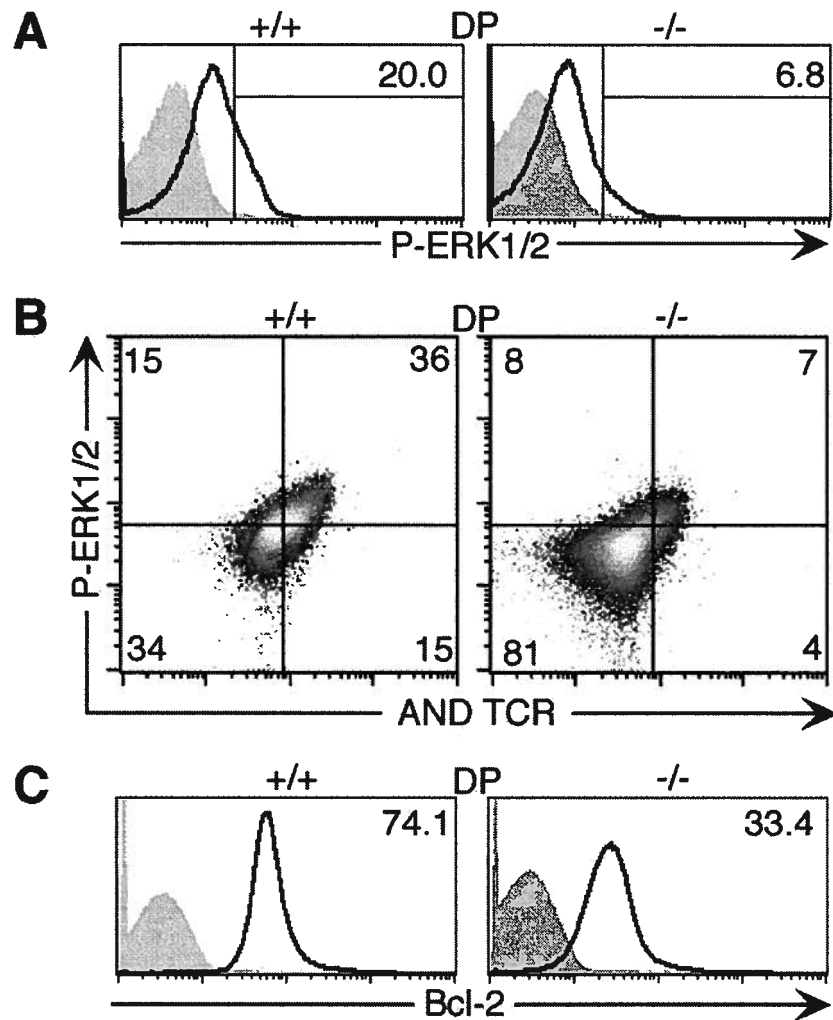
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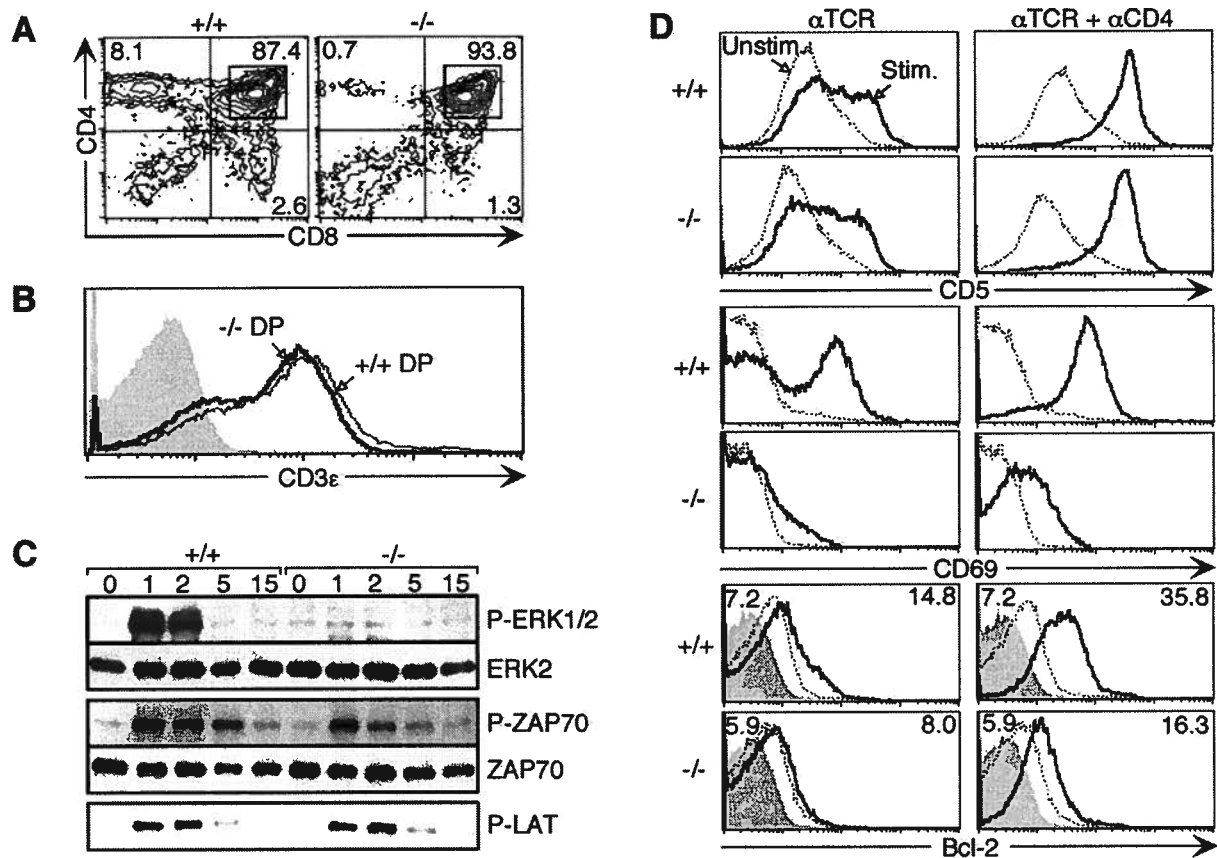
Figure 2.3 RasGRP1$^{-/-}$ DP thymocytes exhibit a decreased capacity to up-regulate P-ERK, CD69, and Bcl-2 levels following TCR engagement.
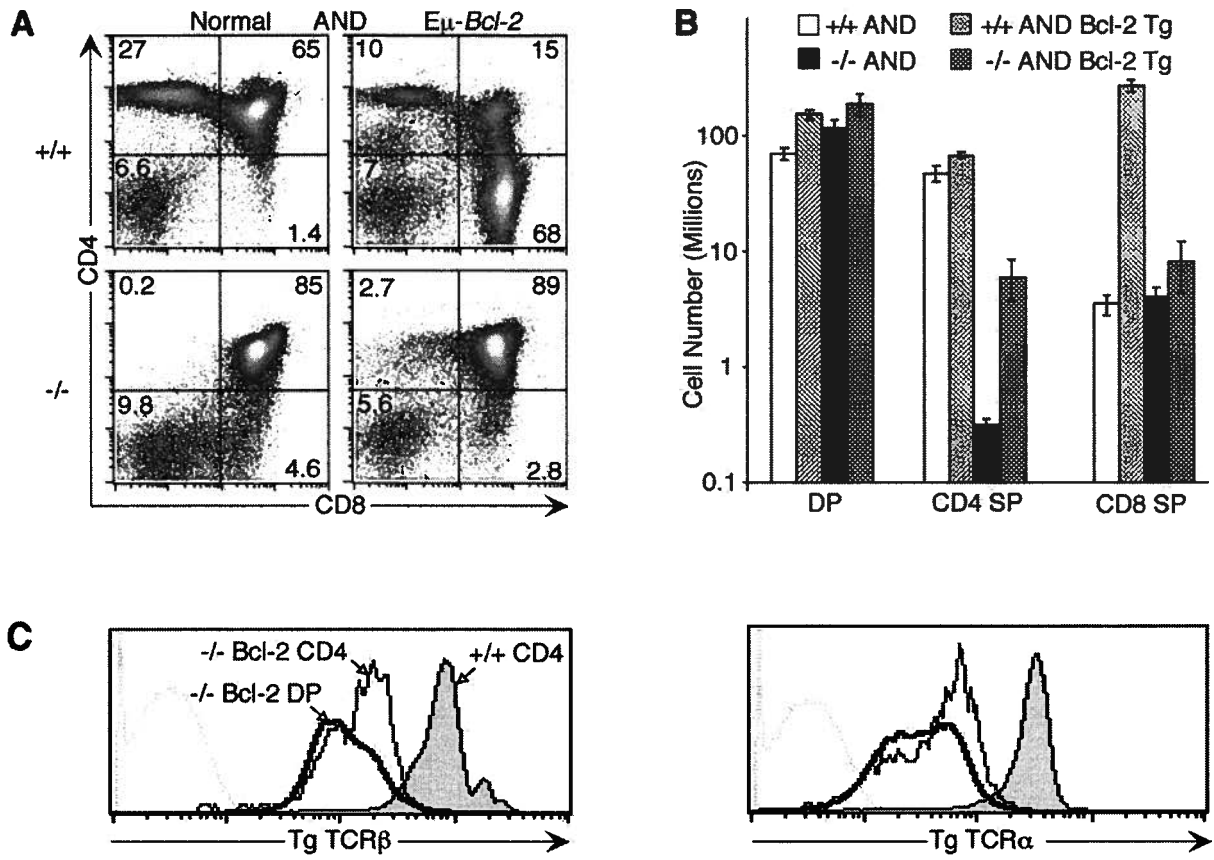
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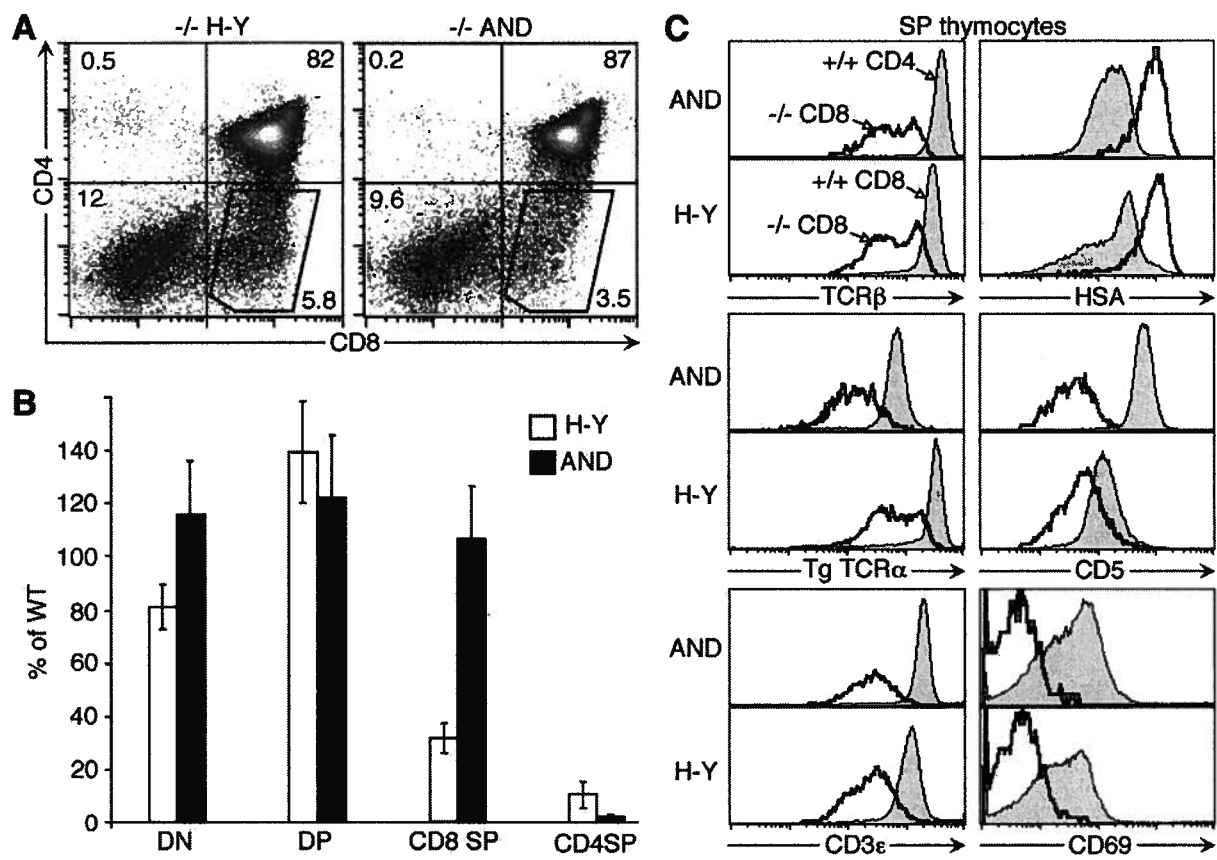
Figure 2.5 RasGRP1$^{-/-}$ animals share a similar thymic developmental profile regardless of whether they express either the MHC class II-restricted or MHC class I-restricted TCR.
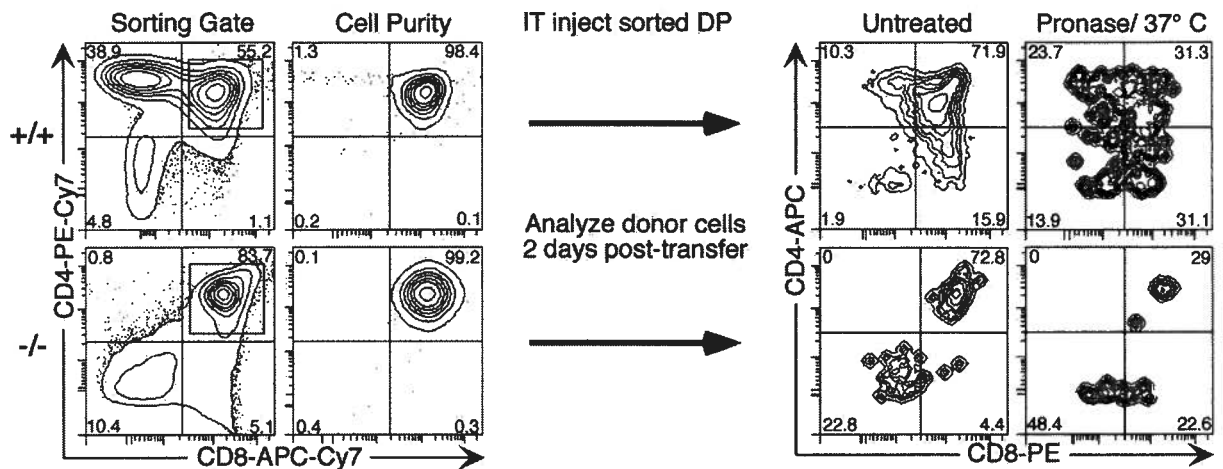
Figure 2.6 RasGRP1$^{-/-}$TcrAND DP thymocytes exhibit limited developmental potential.
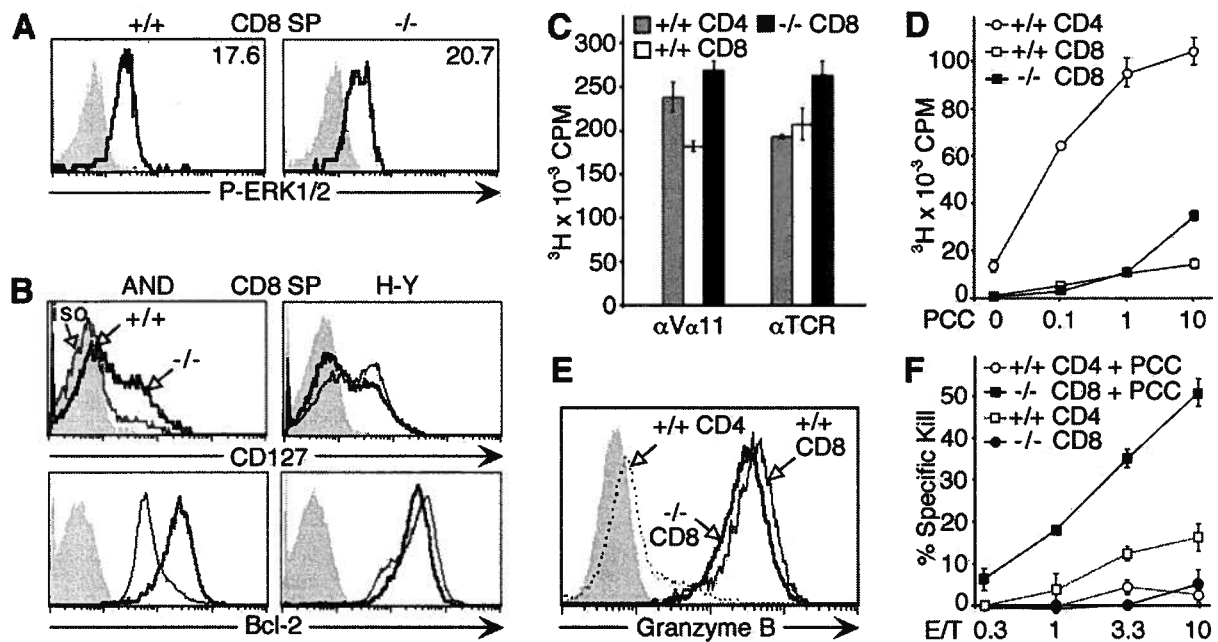
Figure 2.7 CD8 SP thymocytes that develop in RasGRP1$^{-/-}$TcrAND mice respond to MHC class II-specific peptide and differentiate in cytolytic effectors.
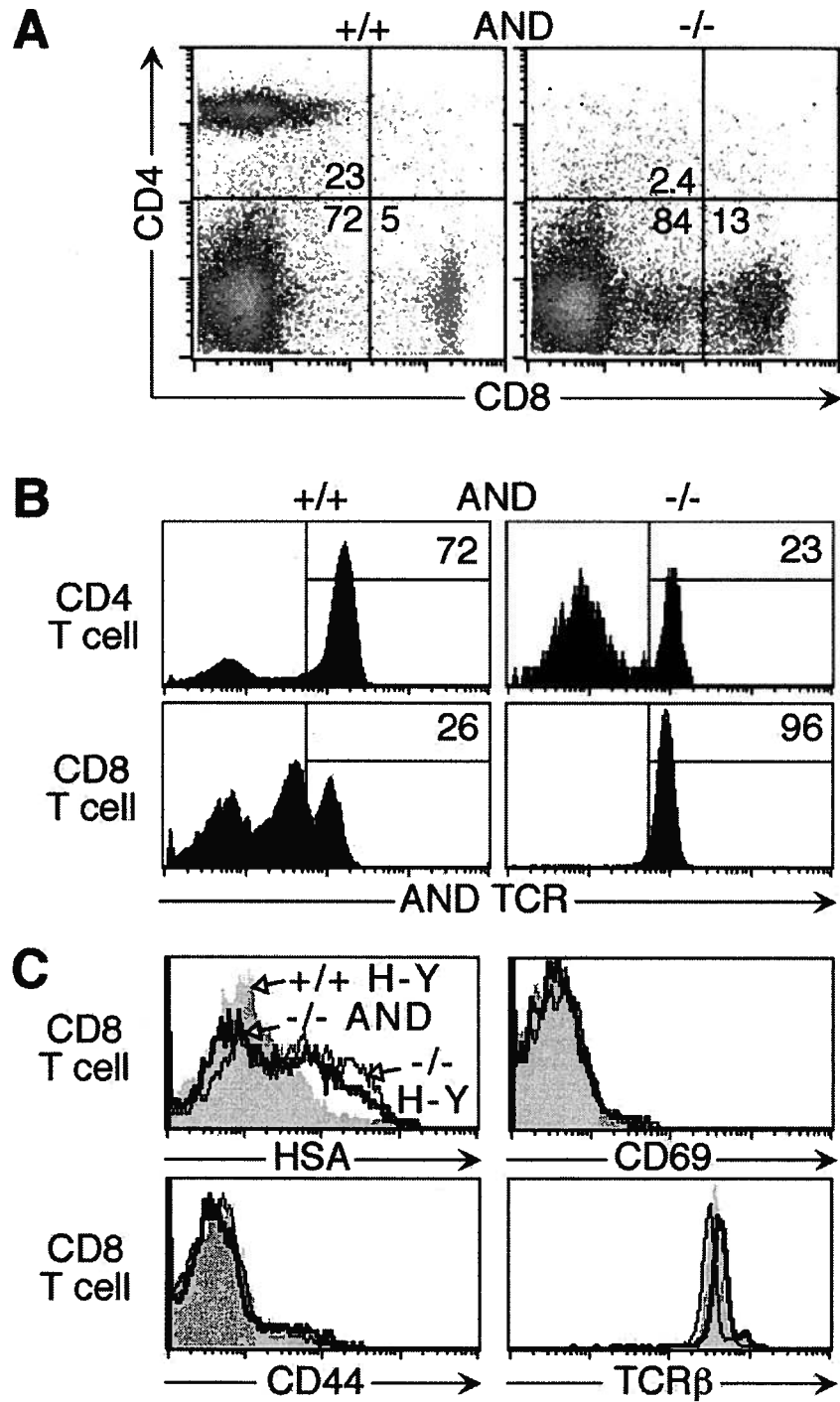
Figure 2.8 RasGRP1$^{-/-}$TcrAND animals possess significant numbers of peripheral AND TCR$^+$ CD8$^+$ T cells but very small numbers of AND TCR$^+$ CD4 T cells.

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CHAPTER 3   CHRONIC IMMUNODEFICIENCY IN MICE LACKING RASGRP1 RESULTS IN CD4 T CELL IMMUNE ACTIVATION AND EXHAUSTION[1]

3.1   Introduction

The two dominant forces maintaining homeostatic control on the size of naive T cell compartment are the availability of self-peptides/self-MHC ligands and the cytokine IL-7 (1, 2). Under normal T cell-sufficient conditions, low-avidity TCR interactions with self-peptides/self-MHC molecules along with IL-7R signaling promote T cell survival. During T cell lymphopenia, the increased abundance of these same elements is thought to induce slow T cell (homeostatic) proliferation in an attempt to increase peripheral T cell numbers. Therefore, homeostatic expansion is beneficial for preserving the size of the T cell population but it may pose risks because T cell proliferation is accompanied by acquisition of effector function, such as cytotoxicity and the capacity to rapidly secrete inflammatory cytokines (1, 2). Because this slow cellular division only applies to a subset of T cells, it is thought that the T cells recruited into cell cycle are the ones expressing TCRs with greater avidity for self-Ags (1, 2), Therefore, lymphopenia-induced T cell expansion could be dangerous as it may skew the TCR repertoire of the resident pool of T cells toward autoreactivity and, further, restrict its diversity.

The term "homeostatic proliferation" was originally coined to describe the slow T cell expansion observed when naive TCR-transgenic T cells were adoptively transferred into lymphopenic recipients, such as congenitally T cell-deficient $RAG^{-/-}$ mice or normal mice rendered lymphopenic by irradiation (2-4). Because donor TCR-transgenic T cells were RAG deficient, it ensured that these T cells were monoclonal, expressing a single $\alpha\beta$ TCR, and verified that this slow proliferation occurred in the absence of agonist peptides. Moreover,

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the product of this cell division possesses a memory T cell phenotype resembling the functional attributes and gene expression profiles of conventional memory T cells (2, 5). Despite these observations, recent work has suggested that foreign Ags may be responsible for some donor T cell proliferation observed following transplantation into congenitally T cell-deficient RAG$^{-/-}$, TCR$\alpha^{-/-}$, or SCID hosts (6, 7). Moreover, because mice lacking any $\alpha\beta$ T cells are severely immunocompromised, these hosts are highly susceptible to developing chronic infections and, as a result, may present a broad array of microbial Ags to donor T cells. In support of this hypothesis, the adoptive transfer of normal (polyclonal) T cells into a congenitally T cell-deficient host (chronically immunodeficient) results in a subset of T cells undergoing massive T cell expansion whereas only slow division is apparent when such T cells are transferred into wild-type hosts made lymphopenic by irradiation (acutely immunodeficient). The observation that donor TCR-transgenic (monoclonal) T cells undergo similar rates of division in these two disparate recipients demonstrates that rapid T cell proliferation only applies to a subset of T cells and suggests that it may be dependent on TCR specificity (6, 7). Because rapid T cell proliferation is lost when T cells are transferred into congenitally T cell-deficient, gnotobiotic (germfree) hosts (7), it argues that foreign Ags, likely derived from commensal microbes within the gut, are driving fast T cell proliferation in congenitally T cell-deficient animals.

The loss of T cell immunity is a common occurrence during chronic viral infections in both mice and humans (8). Recent evidence suggests that continual exposure to cognate Ag results in the overstimulation of viral-specific T cells and the development of an "exhausted" memory T cell phenotype. In contrast to acute infection, memory T cells derived from chronic infection exhibit Ag dependency, limited self-renewal capacity, diminished cytokine production, and reduced cytotoxicity (9). Therefore, these functional impairments displayed by chronically activated T cells may contribute to the failure to clear virus. Programmed death-1 (PD-1), a negative regulator of activated T cells (10), is strongly up-regulated on exhausted viral-specific CD8 T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection in mice and HIV infection in humans (11, 12). Because blockade of PD-1 interaction with its ligand PD-L1 can restore function in exhausted CD8 T cells (11), it

makes the case that PD-1 is not simply indicative of an exhausted state but also plays a key role in its maintenance.

In thymocytes, Sos (13) and RasGRP1 (14), two Ras-guanyl-nucleotide exchange factors, link Ras and MAPK activation to TCR signal transduction with their respective functions dependent on relocating to membranes by two distinct mechanisms (15). RasGRP1 mobilizes to membranes by binding the phospholipase Cγ1 product diacylglycerol through its C1 domain whereas Sos is recruited to the phosphorylated adaptor molecule linker for activated T cells by way of its association with the Src homology 2-domain-containing protein Grb2. RasGRP1^{-/-} thymocytes show signs of reduced TCR signaling (14, 16, 17) and a selective impairment of positive but not negative selection (16). Therefore, mice lacking RasGRP1 serve as a model lacking a positive regulator of TCR signaling. RasGRP1^{-/-} mice exhibit a marked T cell lymphopenia (14, 16), likely a consequence of decreased single-positive (SP) thymocyte maturation and T cell hyporesponsiveness (14, 16, 17). Paradoxically, a recently described novel mouse strain called RasGRP1[lag] (lymphoproliferation-autoimmunity glomerulonephritis), bearing a spontaneous mutation in RasGRP1, develops an autoimmune syndrome resembling systemic lupus erythematosus (SLE), exhibiting massive lymphoproliferation, high levels of serum autoantibodies and, eventually, advanced disease that required euthanasia (18). Therefore, RasGRP1 signaling may be critical for both thymocyte maturation and T cell tolerance.

In this study, we report that although RasGRP1^{-/-} mice remain T cell lymphopenic and free of overt disease until at least 1 year of age, they possess a population of proliferating CD4 T cells that display an exhausted phenotype, characteristic of chronic infection (8). Adoptive transfer experiments suggest that chronic immunodeficiency and foreign Ags might be responsible for inducing RasGRP1^{-/-} CD4 T cell proliferation rather than autoreactivity to self-Ags. Supporting the notion of a dysfunctional immune system, bacterial and viral challenge experiments revealed that RasGRP1^{-/-} mice exhibited impaired T cell responses and a delay in pathogen clearance. Lastly, we find that chronic T cell immunodeficiency in RasGRP1^{-/-} mice is likely a consequence of RasGRP1 protein loss in thymocytes and/or T cells rather than due to defects in innate immunity. In conclusion, these investigations

highlight the roles of RasGRP1 in determining a normal immune status and as an essential regulator of adaptive T cell immunity toward experimental infection.

3.2 Materials and methods

Mice

C57BL/6J (B6), B6.PL-Thy1a/Cy (Thy 1.1^+), B6.SJL-Ptprca Pep3b/BoyJ (Ly 5.1^+), B6.RAG-$1^{-/-}$, and B6.TCR$\alpha^{-/-}$ mice were acquired from The Jackson Laboratory. RasGRP1$^{-/-}$ breeder mice were provided by J. C. Stone (University of Alberta, Alberta, Canada) and bred onto a B6 background at least seven generations. To generate Thy1.1$^+$ 2C TCR-transgenic animals, the 2C TCR transgene was bred onto the B6.PL-Thy1a/Cy background (H-2^b, Thy1.1$^+$). All studies followed guidelines set by the Animal Care Committee at the University of British Columbia in conjunction with the Canadian Council on Animal Care.

Flow cytometry

Abs against CD4 (GK1.5), CD8 (53-6.7), CD5 (53-7.3), TCRβ (H57-597), CD62L (MEL-14), CD25 (PC61.5), CD69 (H1.2F3), CD45.1 (A20), Thy1.1 (HIS51), CD44 (IM7), CD62L (MEL-14), CD127 (AKR34), PD-1 (J43), PD-L1 (MIH5), PD-L2 (TY25), TNF-α (MP6-XT22), and IFN-γ (XMG1.2) were purchased from eBioscience. Annexin VPE, anti-Fas (DX2), anti-FasL (MFL3), anti-Ki-67 Abs (B56), and anti-TCR Vβ screening panel (no. 0143KK) reagent sets were purchased from BD Biosciences. For anti-Ki-67 staining, cells were fixed with 2% formaldehyde (Polysciences) for 10 min, permeabilized with 90% methanol, washed with 2% FCS/PBS and subsequently, incubated with anti-Ki-67 Ab for 30 min at room temperature. Isotype-control Ab (clone MOPC-21; BD Biosciences) staining was negligible (> 0.2%). Annexin V-PE staining was conducted as described previously (19). Data were acquired using either a FACScan or FACSCalibur and CellQuest software (BD Biosciences). Data were analyzed either with CellQuest, FCSPRESS, or FlowJo (Tree Star) software.

Adoptive transfer experiments

Wild-type splenic and lymph node T cells were purified from Thy1.1$^+$ animals and labeled with 1 μM CFSE (Molecular Probes) as previously described (20). Approximately 2 x 10^6 purified wild-type (polyclonal) Thy1.1$^+$ T cells or 1 x 10^6 Thy1.1$^+$ 2C TCR CD8 T cells were i.v. injected into Thy1.2$^+$ recipients, either irradiated (600 rad) wild-type B6 or nonirradiated wild-type B6, B6.RasGRP1$^{-/-}$, B6.RAG-1$^{-/-}$, and B6.TCRα$^{-/-}$ mice. Conversely, ~2 x 10^6 CFSE-labeled RasGRP1$^{-/-}$ T cells (Thy1.2$^+$) were transferred into either B6.Thy1.1$^+$, irradiated B6.Thy1.1$^+$, or B6.RAG-1$^{-/-}$ hosts. Spleens were recovered 1 wk posttransfer and proliferation of donor cells assessed by flow cytometry using a FACSCalibur (BD Biosciences).

Bacterial and viral infections

Mice were infected i.v. with ~10,000 CFU of a recombinant strain of *Listeria monocytogenes* engineered to express the 2C TCR agonist peptide SIYRYYGL (J. Priatel, L. Zenewicz, H. Shen, and H. Teh, manuscript in preparation). For viral infection, mice were injected i.p. with ~100,000 PFU of LCMV-Armstrong. Splenic viral titers were determined as described previously (21). For wild-type T cell infusion into RasGRP1$^{-/-}$ (Ly5.2$^+$) animals, purified (10 million) Ly5.1$^+$ T cells (~55:45% ratio of CD4 vs CD8 T cells) were i.v. injected into RasGRP1-deficient animals 1 day before infection with either rLM-SIY or LCMV.

Detection of IFN-γ production by intracellular flow cytometry

Spleens were harvested from mice at either day 7 (rLM-SIY) or day 8 (LCMV) postinfection, pressed through metal mesh to generate single-cell suspensions, and subjected to RBC lysis by ammonium chloride treatment. Splenocytes were cultured for 5 h in 96-well, flat-bottom plates, at a concentration of 2-4 x 10^6 cells/well, in 0.2 ml of complete medium supplemented with 1 μl/ml Golgi Plug (contains brefeldin A; BD Biosciences) to block cytokine secretion. Cells were stimulated with a concentration of 1 μM for the MHC class I peptides (SIY, SIYRYYGL; GP$_{33-41}$, KAVYNFATC; GP$_{34-43}$, AVYNFATCGI; NP$_{396-404}$, FQPQNGQFI; GP$_{276-286}$, SGVENPGGYCL; NP$_{205-212}$, YTVKYPNL; OVA$_{257-264}$, SIINFEKL) and 10 μM for the I-A^b MHC class II peptides (LLO$_{190-201}$, NEKYAQAYPNVS; GP$_{61-80}$,

GLKGPDIYKGVYQFKSVEFD). For anti-TCR stimulations, 2×10^6 splenocytes were incubated for 5 h in a 24-well plate that had been precoated with 10 μg/ml anti-CD3ε (145-2C11) Ab. After culture, cells were fixed for 15 min in 2% paraformaldehyde/PBS solution, permeabilized for 15 min with 0.2% Tween 20/PBS and stained with anti-CD4-allophycocyanin, anti-CD8-PE-Cy5, and anti-IFN-γ-FITC Abs (eBioscience). Data were acquired on a FACSCalibur using CellQuest software (BD Biosciences) and analyzed with FCSPress (www.fcspress.com). All peptides were synthesized at the University of British Columbia's Nucleic Acid Protein Service Unit.

Direct ex vivo CTL assays

After 7 days postinfection with rLM-SIY, splenic CD8 T cell effectors were isolated by staining total splenocytes with rat anti-mouse CD4 (GK1.5) Abs and subsequently depleted of $CD4^+$ and surface Ig^+ cells with anti-mouse (and rat-reactive) Ig-linked Dynabeads (catalog no. 110.02; Dynal Biotech). The target EL-4 cell line was labeled with ^{51}Cr, pulsed with SIY peptide, washed, and incubated with various numbers of effectors as previously described (20).

3.3   Results

RasGRP1$^{-/-}$ CD4 T cells exhibit markers of acute activation, exhaustion, proliferation, and spontaneous apoptosis

The impaired T cell development results in a T cell lymphopenia, exhibiting a 10-fold decreased abundance of peripheral T cells, in 1-mo-old RasGRP1$^{-/-}$ mice (14). To account for the limited positive selection in RasGRP1$^{-/-}$ animals, it had been proposed that thymocytes capable of being selected without RasGRP1 must express strongly self-reactive TCRs to overcome their signaling deficits (16-18). Notably, autoreactive CD4 T cells were suspected to be the root cause of massive lymphoproliferation and an underlying autoimmune disorder in RasGRP1^{lag} mice (18). However, although we observed some incidents of massive lymphoproliferation, splenomegaly, generalized lymphadenopathy, and a 20- to 30-fold

increase in total cellularity, in mice homozygous for the targeted mutation on a mixed C57BL/6J:129 background (our unpublished observations), this phenotype seems to have vanished after sequential backcrossing of the RasGRP1 gene-knockout allele to the C57BL/6J (B6) genetic background. In this report, we focus on RasGRP1$^{-/-}$ mice that have been bred at least seven generations onto the B6 mouse background.

B6-backcrossed RasGRP1$^{-/-}$ mice appear healthy until at least 1 year of age and do not develop massive lymphoproliferation. Sampling of 2- to 4-mo-old RasGRP1$^{-/-}$ mice revealed that they remain T cell lymphopenic, the recovery of both CD4 and CD8 T cells from spleens and pooled lymph nodes (LNs) were reduced vs age-matched wild-type mice (Fig. 3.1A). Comparison of secondary lymphoid organs revealed that RasGRP1$^{-/-}$ mice have similar-sized spleens with respect to wild type (Fig. 3.1B). Curiously, RasGRP1$^{-/-}$ mice possess small peripheral LN such as axillary, brachial and inguinal LNs whereas mesenteric LNs (MLN) from the mutant mice were enlarged as compared with age-matched wild-type animals (Fig. 3.1B). Next, flow cytometric analyses revealed that RasGRP1$^{-/-}$ CD4 and CD8 T cells express very high levels of CD44 as compared with wild type regardless of whether they were isolated from the spleen, peripheral LN, or MLN (Fig. 3.1C and our unpublished observations). In addition, a large proportion of RasGRP1$^{-/-}$ CD4 T cells also display signs of acute activation (CD69^{high}, CD127^{low}, CD62L^{low}, Fas^{high}, FasL^{high}). The activated and memory phenotype for RasGRP1$^{-/-}$ T cells is particularly conspicuous because our TCR-transgenic studies demonstrated that central tolerance was not affected by RasGRP1 deficiency and that RasGRP1$^{-/-}$ T cells displayed diminished capacities to undergo homeostatic expansion and respond to cognate Ag (16).

The activated state of RasGRP1$^{-/-}$ CD4 T cells is reminiscent of the exhausted phenotype described in mice and humans suffering from chronic viral infections (8). Therefore, we sought to determine whether RasGRP1$^{-/-}$ T cells expressed PD-1, a molecule that is strongly expressed by exhausted but not normal T cells (10). Strikingly, a large fraction of RasGRP1$^{-/-}$ CD4 T cells possess high levels of PD-1 on their surface (Fig. 3.1D). In addition, PD-L1, a PD-1 ligand that is constitutively expressed by most splenocytes (10), is markedly up-regulated by RasGRP1$^{-/-}$ CD4 T cells whereas it is more modestly elevated by mutant CD8 T

cells (Fig. 3.1*D*). By contrast, expression of PD-L2 does not appear to be altered in RasGRP1$^{-/-}$ mice (our unpublished observations). Next, we examined whether T cell activation in RasGRP1$^{-/-}$ mice was linked with cellular proliferation (Fig. 3.1*E*). Indeed, RasGRP1$^{-/-}$ CD4 T cells exhibit an elevated frequency of cells bearing the proliferation-associated nuclear Ag Ki-67 as compared with wild type (13.3 vs 5.0%). Because elevations in CD44 levels are intimately linked with peripheral T cell expansion (2), Ki-67 staining associates with increased CD44 expression in both wild-type and mutant mice (Fig. 3.1*E*). The difference in Ki-67 expression between wild-type and mutant CD4 T cells is significantly less pronounced when comparisons are based on the CD44^{high} subsets (wild type = 13.4%; mutant = 15.7%). Notably, the CD44 expression profiles shown for wild-type and mutant T cells (Fig. 3.1*E*) is altered as compared with Fig. 3.1*C* because it was performed after formaldehyde fixation and permeabilization with methanol to facilitate the detection of the intracellular Ag Ki-67 (see *Materials and Methods*). Studies have shown that formaldehyde/methanol treatment of cells results in a reduction in the intensity of CD44 staining, as detected by the clone IM7 mAb, and an increase in background cell staining levels (22). Subsequently, we sought to determine whether RasGRP1$^{-/-}$ CD4 T cells show signs of accelerated cell death because elevated Fas/FasL levels and exhausted T cell phenotypes are associated with increased rates of T cell apoptosis (Fig. 3.1*F*). Staining with the apoptotic marker annexin V revealed that RasGRP1$^{-/-}$ CD4 T cells are strongly reactive toward this reagent (RasGRP1$^{-/-}$ = 76.0% vs RasGRP1$^{+/+}$ = 24.1%). Collectively, these findings suggest that RasGRP1$^{-/-}$ CD4 T cells are highly activated, cycling, and apoptotic as compared with wild type.

RasGRP1$^{-/-}$ CD4 SP thymocytes display a naive cell surface phenotype

Mice deficient in RasGRP1 exhibit severely diminished numbers of mature SP thymocytes demonstrating that this molecule plays a critical role in thymopoiesis (14, 16, 18) (Fig. 3.2*A*). To examine whether RasGRP1$^{-/-}$ CD4 and CD8 T cells spontaneously acquire a memory phenotype from their development in the thymus, we stained thymocytes with Abs specific for CD4, CD8, and TCRβ to identify mature (TCRβ^{+}) SP (CD4^{+}CD8^{-} or CD4^{-}CD8^{+}) thymocyte subpopulations. In stark contrast to peripheral RasGRP1$^{-/-}$ T cells, RasGRP1$^{-/-}$ SP thymocytes express abnormally low amounts of CD44 and CD69 as compared with their

wild-type counterparts (Fig. 3.2B). However, in concordance with SP thymocyte maturation, RasGRP1$^{-/-}$ CD4 SP thymocytes bear equivalent expression of CD5, a marker of TCR signaling during positive selection (23), as well as similar levels of CD62L and Bcl-2 as compared with wild-type CD4 SP thymocytes (Fig. 3.2B and our unpublished observations). Strikingly, the CD44^{low} phenotype of most RasGRP1$^{-/-}$ CD4 SP thymocytes contrasts with the elevated CD44 expression levels previously reported for RasGRP1^{lag} CD4 SP thymocytes (18). To explain the contradiction between these findings, we hypothesize that the massive lymphoproliferation and lymphocytic tissue infiltration observed in RasGRP1^{lag} animals (18) results in activated peripheral CD4 T cells also infiltrating the thymus. Because B6-backcrossed RasGRP1$^{-/-}$ mice remain lymphopenic, fewer RasGRP1$^{-/-}$ CD4 T cells likely traffic to the thymus and contaminate the CD4 SP electronic gate. Therefore, we argue that RasGRP1 deficiency supports the development of naive CD4 SP thymocytes and that their conversion to a CD44^{high} phenotype in the periphery may result from homeostatic pressures.

RasGRP1$^{-/-}$ host animals support rapid donor T cell proliferation

A hypothesis for the origin of memory phenotype (CD44^{high}) RasGRP1-deficient T cells is that they are the product of slow homeostatic proliferation that result in the conversion of naive T cells into memory T cells. Because a substantially reduced number of SP thymocytes develop and are exported to the peripheral lymphoid organs in RasGRP1$^{-/-}$ mice (16), the few mature SP thymocytes that immigrate to the periphery are subjected to a T cell lymphopenic environment. Therefore, the availability of IL-7 and self-MHC interactions in RasGRP1$^{-/-}$ mice may be well-suited for inducing the peripheral T cell expansion. To test this hypothesis, we adoptively transferred equivalent numbers of wild-type T cells (Thy1.1^{+}), labeled with the mitotic tracker CFSE, into RasGRP1$^{-/-}$, wild-type B6, irradiated B6 (600 rad) and TCRα$^{-/-}$ host animals for a 1-wk period (Fig. 3.3A). As expected, the majority of CD4 and CD8 T cells recovered from normal (lymphoreplete) mice had failed to divide whereas those from irradiated recipients had undergone slow expansion that is characteristic of homeostatic proliferation, still retaining some fluorescence imparted by CFSE. In contrast, the outcome is substantially different when T cells are transferred into in congenitally T cell-deficient TCRα$^{-/-}$ or RAG-1$^{-/-}$ mice (6, 7). In these chronically immunodeficient animals, which are

completely devoid of any αβ TCR$^+$ cells, some donor T cells underwent "typical" homeostatic proliferation, cycling one to four times per week, while other donor T cells divide rapidly, greater than eight times within a week, and completely lost their CFSE fluorescence. The massive growth of these rapidly dividing cells results in their increased numbers and representation when looking at the distribution of donor cell CFSE fluorescence 1-wk posttransplantation (TCRα null; Fig. 3.3A). Interestingly, the donor cell division history in RasGRP1$^{-/-}$ recipients revealed a unique CFSE profile bearing similarities to both TCRα$^{-/-}$ and irradiated B6 hosts: a rapidly dividing population (52% of CD4$^+$ and 37% of CD8$^+$ T cells) and a slowly dividing population (21% of CD4$^+$ and 50% of CD8$^+$ T cells), respectively. These findings demonstrate that the lymphopenia present within RasGRP1$^{-/-}$ mice promotes spontaneous peripheral T cell expansion.

Foreign Ags, derived from commensal microbes, are thought to be responsible for the rapid naive T cell expansion observed after transfer of naive T cells into chronically immunodeficient mice RAG-1$^{-/-}$ and TCRα$^{-/-}$ mice (7). To support the idea that these rapidly dividing T cells in RasGRP1$^{-/-}$ recipients are undergoing an Ag-driven differentiation program, we charted CD62L (L-selectin), a marker that is down-modulated upon acute activation, vs CFSE fluorescence after 1-wk residence in vivo (Fig. 3.3B). Strikingly, a large proportion of the CFSE^{low} CD4 (69%) and CD8 (47%) T cells recovered from RasGRP1$^{-/-}$ mice have low CD62L expression. By comparison, T cells residing in irradiated B6 recipients maintain high levels of CD62L while the majority of T cells recovered from RAG-1$^{-/-}$ hosts have down-regulated this marker (Fig. 3.3B). Next, we examined whether RasGRP1$^{-/-}$ mice could also promote expansion of TCR-transgenic T cells by infusing wild-type 2C TCR-transgenic CD8 T cells (Thy1.1$^+$), labeled with CFSE, into normal B6, irradiated B6, RAG-1$^{-/-}$, and RasGRP1$^{-/-}$ host animals and measuring cellular proliferation 1 wk later (Fig. 3.3C). Notably, RasGRP1$^{-/-}$ recipients induce slow 2C T cell proliferation but this amount is considerably less than both RAG-1$^{-/-}$ and irradiated B6 mice (97 and 87%, respectively). Moreover, the observation that 2C T cells do not undergo rapid proliferation in either RasGRP1$^{-/-}$ or RAG-1$^{-/-}$ hosts suggests that this type of T cell expansion may apply to only a subset of T cells with unique TCR specificity. Together, these results suggest that

RasGRP1$^{-/-}$ mice are chronically immunodeficient and that forces driving wild-type T cell expansion in RasGRP1$^{-/-}$ hosts could be a combination of self- and foreign-Ags.

RasGRP1$^{-/-}$ CD4 T cells proliferate vigorously in chronically immunodeficient RAG-1$^{-/-}$ mice
The previous experiments examined how wild-type T cells respond following transplant into RasGRP1$^{-/-}$ hosts and therefore may not be reflective of how RasGRP1$^{-/-}$ T cells react to environmental cues. To define how mutant T cells respond in these different settings, CFSE-labeled RasGRP1$^{-/-}$ T cells (Thy1.2$^+$) were i.v. injected into normal B6 (Thy1.1$^+$), irradiated B6 (Thy1.1$^+$), and RAG-1$^{-/-}$ animals (Fig. 3.4A). Strikingly, the majority of RasGRP1$^{-/-}$ CD4 T cells recovered from either normal or irradiated B6 animals were not recruited into cell cycle (63 and 65%, respectively) while the large fraction of RasGRP1$^{-/-}$ CD8 T cells isolated from irradiated recipients were proliferating slowly (87%). By contrast, RasGRP1$^{-/-}$ CD4 and CD8 T cells transplanted into RAG-1$^{-/-}$ hosts proliferated vigorously. Furthermore, RasGRP1$^{-/-}$ T cells, particularly the CD4 T cells, recovered from RAG-1$^{-/-}$ recipients strongly down-regulated CD62L expression whereas those placed in irradiated B6 hosts resembled the surface phenotype before adoptive transfer (Figs. 3.4B and 3.1C). Next, we addressed whether the residence of RasGRP1$^{-/-}$ T cells in different hosts influenced effector function (Fig. 3.4C). Upon TCR stimulation, RasGRP1$^{-/-}$ T cells recovered from RAG-1$^{-/-}$ hosts possessed an increased frequency of cells capable of producing the proinflammatory cytokines TNF-α and IFN-γ vs those residing in irradiated wild-type recipients. In summation, these studies demonstrate that chronically immunodeficient hosts are capable of inducing RasGRP1$^{-/-}$ T cells to proliferate rapidly and boost effector function.

RasGRP1$^{-/-}$ mice generate poor pathogen-specific T cell responses
To test the hypothesis that RasGRP1$^{-/-}$ mice are immunodeficient, wild-type and mutant mice were infected with a novel recombinant strain of *L. monocytogenes* (rLM-SIY) expressing a MHC class I K^b-restricted peptide SIYRYYGL (SIY), an agonist for the 2C TCR (24). One week postinfection, splenocytes were stimulated with either the endogenous MHC class II-restricted peptide LLO$_{190-201}$ (listeriolysin O (LLO); Fig. 3.5A) or SIY (Fig. 3.5B) and Ag-specific T cell responses were monitored by IFN-γ production using intracellular flow

cytometry. As an additional control, splenocytes were also cultured on anti-TCR Ab-coated plates to test for the capacity to produce IFN-γ. Strikingly, RasGRP1$^{-/-}$ mice mounted a barely detectable immune response toward the LLO peptide (Fig. 3.5C). The fact that RasGRP1$^{-/-}$ CD4 T cells can respond to anti-TCR Abs suggests that the weak response by RasGRP1$^{-/-}$ mice is the result of a failure to generate LLO-reactive T cells rather than to secrete IFN-γ. By contrast, RasGRP1$^{-/-}$ CD8 T cells generate a strong anti-SIY response that is modestly reduced in Ag-specific T cell numbers as compared with wild type (Fig. 3.5C). In addition, we tested the function of RasGRP1$^{-/-}$ CD8 T cell effectors in a standard ^{51}Cr-release assay and found that these cells displayed cytotoxicity similar to their wild-type counterparts (Fig. 3.5D). These rLM-SIY infection studies suggest that RasGRP1 is particularly critical for generating MHC class II-restricted immune responses.

To address the possibility that the importance of RasGRP1 on mounting MHC class II-restricted immune responses may be restricted to the LLO peptide or bacterial infection, we used LCMV to infect wild-type and RasGRP1$^{-/-}$ mice. Eight days postinfection with a sublethal dose of LCMV, Ag-specific T cells were enumerated to viral immunodominant MHC class II-restricted GP$_{61-80}$ (Fig. 3.6A) and MHC class I-restricted peptides (GP$_{33-41}$, GP$_{34-43}$, GP$_{276-286}$, NP$_{205-212}$ and NP$_{396-404}$; Fig. 3.6B). Similar to the bacterial infection, RasGRP1$^{-/-}$ mice generated a weak MHC class II-restricted response although this time a reduced fraction of mutant CD4 T cells produced IFN-γ upon TCR stimulation (3.6 vs 13.0%). However, in contrast to rLM-SIY results, RasGRP1$^{-/-}$ mice mounted weak immune responses toward all five MHC class I-restricted viral peptides analyzed despite the fact that a similar proportion of wild-type vs mutant CD8 T cells can secrete IFN-γ upon TCR stimulation (48%). The fact that total T cell numbers were also sharply reduced in RasGRP1$^{-/-}$ animals makes the drop in Ag-specific T cell numbers even more dramatic (Fig. 3.6C). To evaluate whether the diminished T cell responses were physiologically relevant, splenic tissue, harvested 8 days postinfection, was screened for virus using plaque-forming assays (Fig. 3.6D). Indeed, 3 of 4 RasGRP1$^{-/-}$ spleens in this experiment (9 of 13 total) contained readily detectable virus at this late period postinfection, at a time point when virus is undetectable among wild-type mice. In conclusion, the weakened immune responses by RasGRP1$^{-/-}$ mice is associated with delayed viral clearance.

The ability of RasGRP1$^{-/-}$ mice to respond strongly to the SIY peptide but only weakly to immunodominant LCMV peptides led us to speculate whether the differential responses were intrinsic to the peptides or the pathogen. To resolve this issue, wild-type and RasGRP1$^{-/-}$ mice were infected with two other rLMs, either rLM-OVA or rLM-GP33, and the frequency of MHC class I-restricted anti-OVA$_{257-264}$- and anti-GP$_{33-41}$-specific T cells assessed 7 days postinfection. In stark contrast to the SIY peptide, RasGRP1$^{-/-}$ mice mounted weak, barely detectable responses toward both OVA$_{257-264}$ and GP$_{33-41}$ MHC class I-restricted peptides (our unpublished observations). These findings that RasGRP1$^{-/-}$ mice can respond to a limited set of immunodominant peptides suggest that its resident T cells may possess an altered TCR repertoire. To look for differences in the TCR repertoire between wild-type and RasGRP1$^{-/-}$ mice, Vβ TCR chain usages were determined by staining splenocytes with various anti-Vβ TCR chain-specific Abs in conjunction with Abs specific for CD4, CD8, and the constant region of the TCRβ chain (Fig. 3.7). Although we observed differences among the Vβ TCRs used by both RasGRP1$^{-/-}$ CD4 and CD8 T cells, the CD4 T cell population exhibited fewer statistically significant changes, due in part to a large deviation between RasGRP1$^{-/-}$ mice. It is not clear whether the TCR repertoire changes in RasGRP1$^{-/-}$ mice results from aberrant T cell development, homeostatic strain, or a mixture of both processes. Moreover, the possibility exists that CD4 T cell immune activation, resulting in persistent rounds of cellular proliferation and death, could contribute to great variability exhibited by RasGRP1$^{-/-}$ CD4 T cell TCR repertoire. Collectively, these studies raise the prospect that changes in the TCR repertoire in RasGRP1$^{-/-}$ mice could be responsible for the impaired generation of pathogen-specific T cells.

Failure of RasGRP1$^{-/-}$ mice to generate pathogen-specific T cells is the result of RasGRP1 loss in thymocytes and/or T cells

Because the engineered mutation in RasGRP1 results in a systemic loss of RasGRP1 function (14), it is possible that RasGRP1 deficiency in another cell type, besides T cells, may contribute to defective pathogen-specific responses. To investigate whether the innate immune system within RasGRP1$^{-/-}$ mice is capable of nurturing T cell responses, mutant mice (Ly5.2$^+$) were infused with purified wild-type T cells (Ly5.1$^+$) and infected the next day

with either rLM-SIY or LCMV (Fig. 3.8*A*). Seven days post-rLM-SIY infection, a sizable proportion (7.1%) of the wild-type CD4 T cells were LLO reactive whereas few RasGRP1$^{-/-}$ CD4 T cells produced IFN-γ upon peptide stimulation (Fig. 3.8*B*). Interestingly, RasGRP1$^{-/-}$ CD8 T cells (Ly5.1$^-$) mounted a weaker anti-SIY response in the presence of wild-type T cells (Figs. 3.8*B* and 3.5*B*), implying that they may not compete well for Ag. These findings suggest that the innate immune system in RasGRP1$^{-/-}$ mice is not to blame for the impaired generation of rLM-specific T cells.

To determine whether the wild-type T cells could also respond in mutant mice after viral infection, we performed an analogous wild-type T cell infusion experiment except this time we infected with LCMV. Consistent with our rLM-SIY observations, wild-type CD4 and CD8 T cells generate strong anti-LCMV responses toward immunodominant viral peptides (Fig. 3.8*C*). Interestingly, RasGRP1$^{-/-}$ mice seem to mount a better IFN-γ response to the NP396 peptide in the presence of wild-type donor T cells (12 vs 0.8%; Figs. 3.8*C* and 3.6*B*). A plausible explanation for this observation is that wild-type CD4 T cells help the mutant CD8 T cell response by producing cytokines or providing costimulation. Notably, we have previously shown that the addition of the cytokine IL-2 can restore Ag-driven proliferation by RasGRP1$^{-/-}$ 2C CD8 T cells in vitro (16). These experiments argue that poor T cell responses observed in RasGRP1$^{-/-}$ mice are the consequence of RasGRP1 deficiency in thymocytes and/or T cells.

The finding that the "empty" lymphoid compartment in RasGRP1$^{-/-}$ mice can induce spontaneous T cell expansion suggests the possibility that it could initiate the differentiation of donor T cells into pathogen-specific T cell effectors without infection. To explore the likelihood of this possibility, we compared cohorts of RasGRP1$^{-/-}$ mice receiving wild-type T cell infusions that were left untreated with those infected with LCMV. Nine days postdonor T cell infusion, mice that were left untreated did not exhibit significant numbers of viral-specific T cells (our unpublished observations) and the donor cell recovery was substantially lower than those infected with LCMV (3.4- ± 0.7-fold-decrease for CD4 T cells; 7.3- ± 1.2-fold-decrease for CD8 T cells). These experiments demonstrate that the adoptive transfer of

wild-type T cells into RasGRP1$^{-/-}$ mice does not induce their spontaneous differentiation into anti-LCMV T cell effectors.

3.4   Discussion

The prime directives of thymocyte development are to generate a TCR repertoire that is self-restricted, self-tolerant, and diverse, enabling responses toward a vast array of foreign peptides associated self-MHC molecules. Because the generation of the TCR repertoire is dependent on TCR signaling, mutations affecting signaling molecules downstream of the TCR may have deleterious effects on both T cell function and TCR repertoire. In this study, we report the consequences of RasGRP1 deficiency and reduced TCR-induced Ras signaling on peripheral T cell homeostasis and T cell immunity.

A recently described mouse strain called RasGRP1^{lag} suffers from massive lymphoproliferation and an autoimmune syndrome sharing similarities with SLE (18). Although young mice appeared normal, older RasGRP1^{lag} mice developed massive lymphoproliferation, displaying splenomegaly and lymphadenopathy, with an excess of 10-fold larger lymph node size and cell numbers as compared with age-matched controls (18). By 5–8 mo of age, RasGRP1^{lag} mice were found to be so anorexic and lethargic that it necessitated euthanasia (18). Although we observed RasGRP1$^{-/-}$ mice that developed substantial splenomegaly and lymphadenopathy (our unpublished observations), the penetrance of this phenotype disappeared after successive backcrossing of the targeted RasGRP1 mutation to the B6 background. Our B6 backcrossed RasGRP1$^{-/-}$ mice remain T cell lymphopenic and appear healthy until at least 1 year of age. However, despite the absence of massive lymphoproliferation, these RasGRP1$^{-/-}$ mice do possess elevated levels of serum autoantibodies (our unpublished observations). Because autoimmune disease often requires a complex mixture of genetics and environmental factors (25, 26), it is perhaps not surprising that a change in genetic background may be responsible for the contradictions between our findings and those previously reported (18). Moreover, a recent study has found that SLE can simply develop from a hybrid 129:B6 background rather than targeted gene

disruption (27). Therefore, we suspect that genetic modifiers from the 129/SvJ mouse strain may synergize with RasGRP1 deficiency to cause massive lymphoproliferation and exacerbate autoimmune disease.

It has been proposed that RasGRP1-deficient thymocytes capable of maturing into SP thymocytes need to express more strongly self-reactive TCRs to overcome their signaling deficits (16-18). Because central tolerance does not appear to be affected by RasGRP1 deficiency (16), it has led us to hypothesize that self-reactivity of TCRs mediating positive selection of RasGRP1$^{-/-}$ double-positive thymocytes (DP) must bridge the boundary between positive and negative selection. The question of why immune activation selectively affects the CD4 T cell lineage in RasGRP1$^{-/-}$ mice is unknown. Because CD4 T cell development has been proposed to be more highly dependent on RasGRP1/ERK signaling (17, 28), RasGRP1 deficiency may affect thymic ontogeny by selecting more strongly self-reactive CD4 than CD8 SP thymocytes. Alternatively, RasGRP1-dependent mechanisms preserving peripheral tolerance or a relentless homeostatic strain may preferentially induce the activation of RasGRP1$^{-/-}$ CD4 T cells. However, because RasGRP1$^{-/-}$ T cells are severely hyporesponsive as compared with wild-type cells (16), their autoimmune potential may be counterbalanced by their inefficient TCR signaling and reduced proliferation upon Ag encounter.

Homeostatic mechanisms that function to regulate peripheral T cell numbers may be basis for the association between autoimmunity and T cell lymphopenia observed in both animals and humans (4, 25). Our studies of the homeostatic mechanisms operating in RasGRP1$^{-/-}$ mice suggest that both self- and foreign-Ags could be driving T cell proliferation (Fig. 3.3). However, because the RasGRP1 mutation was made in 129/SvJ embryonic stem cells (14), it is plausible that 129/SvJ-derived alloantigens may be responsible for some donor wild-type B6 T cell proliferation observed after their transfer into RasGRP1$^{-/-}$ mice (Fig. 3.3). Although the RasGRP1$^{-/-}$ mice used in this study have been bred at least seven generations onto the B6 background, this mouse line may still contain a significant amount of 129/SvJ DNA that is likely closely linked to the targeted locus. To identify the forces driving the cell cycling of RasGRP1$^{-/-}$ CD4 T cells in vivo (Fig. 3.1E), we initiated a series of adoptive transfer

experiments using RasGRP1$^{-/-}$ donor T cells. Notably, it had been hypothesized that RasGRP1 deficiency allows autoreactive T cells to escape the thymus, proliferate upon encounter with peripheral self-Ags, and initiate autoimmunity (18). However, the failure of the majority of RasGRP1$^{-/-}$ CD4 T cells to proliferate after placement in wild-type hosts, either unmanipulated recipients or ones made lymphopenic through irradiation, suggests that self-Ags may not be responsible for their expansion (Fig. 3.4). By contrast, the observation that a subset of RasGRP1$^{-/-}$ CD4 T cells can undergo massive expansion in RAG-deficient hosts suggests that foreign Ags could be stimulating CD4 T cell proliferation in RasGRP1$^{-/-}$ animals (Fig. 3.4). Moreover, foreign Ags may be ideally suited to provoke weakly responsive RasGRP1-deficient CD4 T cells to proliferate vigorously because they can simultaneously act as a direct TCR stimulus, an activator of APCs and an inducer of inflammatory cytokine production. Therefore, these studies demonstrate that the environment within irradiated wild-type mice, possessing increased availability to both self-Ags and cytokines, is insufficient to recruit most RasGRP1$^{-/-}$ CD4 T cells into cell cycle.

The observation that RasGRP1$^{-/-}$ mice exhibit diminished T cell responses and delayed pathogen clearance suggests that they could be prone to developing chronic infections. Notably, RasGRP1$^{-/-}$ CD4 T cells share some attributes with functionally exhausted memory T cells found in both mice and humans during chronic infections (8, 9). First, RasGRP1$^{-/-}$ CD4 T cells possess markers of acute activation and T cell memory like exhausted T cells (Fig. 3.1C). Second, RasGRP1$^{-/-}$ CD4 T cells seem to have limited self-renewal capacity because they expand poorly in irradiated wild-type recipients, a cellular environment where the availability of the common γ-chain-linked cytokines IL-7 and IL-15 is increased (Fig. 3.4). RasGRP1$^{-/-}$ CD4 T cells also have reduced IL-7R expression (Fig. 3.1C). Third, RasGRP1$^{-/-}$ CD4 T cells strongly express PD-1, an inhibitory receptor that is coupled with exhausted viral-specific CD8 T cells in both mice and humans (11, 12), as well as its ligand PD-L1 (Fig. 3.1D). In contrast to the CD4 T cell phenotype, RasGRP1$^{-/-}$ CD8 T cells do not possess an exhausted phenotype because they do not exhibit signs of acute activation (CD69^{low}, CD62L^{high}, CD127^{high}; Fig. 3.1C), fail to express elevated levels of PD-1 (Fig. 3.1E) and can mount a significant anti-SIY T cell response (Fig. 3.5, B–D). A complication of housing chronically activated CD4 T cells is that it could promote autoimmunity in

RasGRP1$^{-/-}$ mice, perhaps through elevated FasL expression, inducing nonspecific cell death, or proinflammatory TNF-α production (Figs. 3.1C and 3.4C). Therefore, T cell immunodeficiency could predispose RasGRP1$^{-/-}$ mice to both chronic infections and autoimmunity.

The failure of RasGRP1$^{-/-}$ mice to generate normal numbers of Ag-specific CD4 and CD8 T cells after bacterial and viral infection likely results from a mixture of direct (T cell hyporesponsiveness) and indirect (altered thymic development and T cell homeostasis) influences of RasGRP1 deficiency. Decreased T cell responsiveness would be predicted to reduce the probability that a given T cell undergoes Ag-induced developmental programming whereas changes to T cell development and peripheral T cell homeostasis could alter the TCR repertoire and T cell differentiation. Notably, studies on RasGPR1$^{-/-}$ 2C CD8 T cells have found that RasGRP1 regulates homeostatic proliferation (16), TCR-signaling thresholds, and augments cytokine production (J. Priatel, X. Chen, L. Zenewicz, H. Shen, J. Coughlin, J. Stone, and H. Teh. manuscript in preparation). Because the precursor frequency of Ag-specific T cells is a critical parameter for the generation of effector and memory T cells (29, 30), we assessed Vβ TCR usage among splenic T cells from naive (uninfected) mice to look for alterations in the TCR repertoire between normal and mutant mice. Although our results demonstrate that the TCR repertoire of RasGRP1$^{-/-}$ mice is significantly different, they do not provide a measure of TCR diversity. Moreover, the CD44^{high} surface phenotype of RasGRP1$^{-/-}$ T cells (Fig. 3.1C) suggests the possibility that these cells may be derived from a considerable amount of peripheral expansion, a phenomena thought to restrict the TCR repertoire because it could result from the selective outgrowth of cells expressing a given TCR. In addition, the exhausted phenotype of RasGRP1$^{-/-}$ CD4 T cells suggests that their state of T cell differentiation could also contribute to their inaction. Therefore, reduced generation of Ag-specific T cells by RasGRP1$^{-/-}$ mice could result from changes in T cell differentiation, T cell function, and/or T cell TCR repertoire.

The fact that numerous T cell lymphopenic animals and humans exhibit T cell activation argues that an "empty" T cell compartment and changes to T cell homeostasis play a major role in the phenotype observed in RasGRP1$^{-/-}$ mice rather than being solely attributable to

RasGRP1 loss in peripheral T cells. Interestingly, T cell lymphopenia is also often associated with T cell hyporesponsiveness and autoimmunity (25) and as a consequence, it raises the question of what are common denominators between these phenomena. The knowledge that TCR signaling is critically important for both T cell development and T cell survival has provided insight into the pairing of these occurrences (1, 31). Moreover, mutations affecting TCR signaling may cause T cell activation by impacting central and/or peripheral tolerance (32). In addition, immunodeficiency may lead to disruptions in T cell homeostasis, development of chronic infections, persistent T cell activation, and T cell exhaustion (8, 10). Additionally, studies on two models of spontaneous autoimmune diabetes, the NOD mouse and BioBreeding diabetic rat, suggest that T cell lymphopenia may initiate disease by altering T cell homeostasis (33, 34).

In conclusion, our study highlights the dangers associated with RasGRP1 loss, including changes to T cell development, peripheral T cell homeostasis and T cell immunity. Because our findings suggest that chronic immunodeficiency promotes persistent CD4 T cell activation and constitutive proinflammatory cytokine production, they provide insight into why the conditions of T cell lymphopenia and T cell hyporesponsiveness are linked to autoimmunity in humans (25). An added complication of persistent T cell activation is that it may result in functional impairments by inducing a state of T cell exhaustion, a phenomenon commonly observed during chronic infection in mice and humans (8). Recently, PD-1, an inhibitory receptor that functions to put a brake on TCR signaling (10), has been shown to impair function of exhausted viral-specific CD8 T cells during chronic LCMV infection (11). Because CD4 T cells in HIV-infected individuals (12) and RasGRP1$^{-/-}$ mice express high levels of PD-1 (Fig. 3.1D), it leads us to speculate whether these exhausted states arise from similar forces despite the fact that CD4 T cell lymphopenia in these scenarios has disparate origins. In HIV-infected individuals, it has been hypothesized that CD4 T cell immune activation results from a combination of homeostatic strain and immunodeficiency at mucosal sites, facilitating infections by opportunistic pathogens (35, 36). Thus, it is possible that chronic immunodeficiency in RasGRP1$^{-/-}$ mice allows for translocation of commensal microflora across intestinal epithelium, causing local or systemic infections and CD4 T cell immune activation. Further study of RasGRP1$^{-/-}$ mice will provide additional mechanistic

insights into consequences of T cell immunodeficiency and contribute to understanding how such alterations predispose to autoimmune disease.

3.5   Acknowledgements

3.6 Figures

Figure 3.1 Peripheral CD4 T cells from RasGRP1$^{-/-}$ mice display signs of activation, exhaustion, proliferation, and spontaneous apoptosis. *A*, RasGRP1$^{-/-}$ animals possess a reduced frequency and numbers of peripheral CD4 and CD8 T cells. Decreased splenic CD4 and CD8 $\alpha\beta$ TCR$^+$ T cell numbers in mice lacking RasGRP1. The single (*) and double asterisk (**) represent p values, using an unpaired, two-tailed Student's t test, calculated at p < 0.02 and p < 0.005, respectively. *B*, Comparison of spleens, PLNs (brachial LN is shown), and MLN between wild-type and age-matched RasGRP1$^{-/-}$ mice. *C*, RasGRP1$^{-/-}$ CD4 and CD8 $\alpha\beta$ TCR$^+$ T cells (bold line) possess markers of memory and acute activation. Thin line, shaded histograms indicate staining pattern of wild-type T cell counterparts. *D*, RasGRP1$^{-/-}$ CD4 T cells (bold line) express elevated levels of PD-1 and its ligand PD-L1 as compared with wild type (thin line). Shaded histograms represent background autofluorescence of unstained cells. *E*, An increased frequency of RasGRP1$^{-/-}$ CD4 T cells exhibit expression of the proliferation-associated nuclear Ag Ki-67. *F*, RasGRP1$^{-/-}$ CD4 T cells bind high levels of apoptotic marker annexin V.

Figure 3.2 Aberrant-positive selection in RasGRP1-deficient mice results in a small population of SP thymocytes that bear a naive cell surface phenotype. *A*, Regions used for gating are indicated in the developmental profiles from wild-type and mutant mice. *B*, Expression of the CD5, CD44, CD62L, and CD69 differentiation markers are shown for wild-type (thin line) and RasGRP1$^{-/-}$ (bold line) DP, CD4 SP, and CD8 SP thymocyte subpopulations. Shaded histograms represent autofluorescence of unstained thymocyte subpopulations.

Figure 3.3 The cellular environment within RasGRP1-deficient animals promotes T cell expansion. *A*, Purified wild-type T cells (Thy 1.1$^+$) were labeled with CFSE and adoptively transferred into normal C57BL/6J (B6), irradiated B6, normal B6. RasGRP1$^{-/-}$, and B6.TCR$\alpha^{-/-}$ mice. Splenocytes were harvested 1-wk posttransfer and the proliferation of

donor CD4 and CD8 T cells measured by flow cytometry. *B*, Same type of experiment as in A except that contour plots are presented, displaying CD62L expression as a function of CFSE fluorescence. *C*, Same experiment as in *A* except that 2C CD8 T cells (Thy 1.1^+) were adoptively transferred into the indicated host animals and donor T cell proliferation tracked by gating on Thy1.1^+CD8$^+$ 2C TCR$^+$ cells.

Figure 3.4 RasGRP1$^{-/-}$ T cells expand vigorously after transfer into chronically immunodeficient RAG-1$^{-/-}$ hosts. *A*, CFSE-labeled RasGRP1$^{-/-}$ T cells (Thy 1.2^+) were adoptively transferred into normal B6 (Thy 1.1^+), irradiated B6 (Thy 1.1^+), or B6.RAG-1$^{-/-}$ host animals. After residing within recipient animals for 1 wk, donor T cell proliferation was measured by staining splenocytes with specific Abs to Thy1.2, CD4, CD8, and TCRβ and electronic gating. *B*, Same type of experiment as in A except that the relationship between cellular proliferation and CD62L expression is presented as contour plots. *C*, RasGRP1$^{-/-}$ T cells were recovered from either B6.RAG-1$^{-/-}$ or irradiated wild-type B6 recipients after 1-wk residence, stimulated on anti-TCR Ab-coated plates, and assessed for cytokine production.

Figure 3.5 RasGRP1$^{-/-}$ mice mount a poor MHC class II-restricted LLO response upon infection with *L. monocytogenes*. Wild-type and RasGRP1$^{-/-}$ animals were infected i.v. with rLM-SIY. Spleens of infected mice were harvested 7 days postinfection and assayed for immune responses toward the immunodominant MHC class II-restricted peptide LLO$_{190-201}$ (*A*) or the MHC class I-restricted peptide SIY (*B*) by measuring IFN-γ production using intracellular flow cytometry. Numbers within the plot reflects the frequency of CD8 or CD4 T cells responding to a particular condition. *C*, RasGRP1$^{-/-}$ mice possess reduced numbers of anti-LLO-reactive CD4 T cells. Error bars, SD. *D*, RasGRP1$^{-/-}$ CD8 T cell effectors display potent cytotoxic activity. Various numbers of splenic CD8 T cells from wild-type and mutant mice were incubated with a ^{51}Cr-labeled EL-4 target.

Figure 3.6 RasGRP1$^{-/-}$ animals generate a weak T cell response toward LCMV and exhibit delayed viral clearance. Wild-type and mutant mice were infected i.p. with the LCMV. At

day 8 postinfection, splenocytes were cultured in either medium alone (no peptide), stimulated with the indicated immunodominant viral peptide or placed in a well coated with anti-TCR Ab (αTCR). Anti-LCMV T cells were enumerated by IFN-γ production by gating on either CD4 (*A*) or CD8 (*B*) T cells. Numbers within the plot reflects the frequency of CD8 or CD4 T cells responding to a particular condition. *C*, RasGRP1$^{-/-}$ animals possess greatly reduced numbers of Ag-specific T cells. Error bars represent the SD. *D*, At day 8 postinfection, LCMV can still be detected in the spleens of some RasGRP1$^{-/-}$ animals. Dashed line indicates the approximate detection level of the assay.

Figure 3.7 Peripheral RasGRP1$^{-/-}$ T cells possess an altered TCR repertoire. The TCR Vβ usage of wild-type (n = 5) and mutant T cells (n = 7) was analyzed by using a panel of anti-TCR Vβ Abs, flow cytometry and electronic gating on either CD4$^+$ TCRβ^+ (*A*) or CD8$^+$ TCRβ^+ cells (*B*). To determine the significant differences observed between the two sets of animals, a two-tailed Student's t test (two-sample, unequal variance; heteroscedastic) was performed. The single asterisk (*) and double asterisk (**) represent values of p < 0.02 and p < 0.005, respectively.

Figure 3.8 Failure of RasGRP1$^{-/-}$ mice to generate pathogen-specific T cells is the result of RasGRP1 loss in thymocytes and/or T cells. *A*, RasGRP1$^{-/-}$ mice (Ly 5.2$^+$) were infused with ~10 million wild-type (Ly 5.1$^+$) T cells and infected 1 day later with either rLM-SIY or LCMV. Splenocytes were recovered from animals after infection with either rLM-SIY (*B*) or LCMV (*C*), stimulated with the indicated peptides in vitro, and IFN-γ production measured by flow cytometry. Data were electronically gated on either CD4 or CD8 and the frequency of responding wild-type (Ly5.1$^+$; right) or RasGRP1$^{-/-}$ T cells (Ly5.1$^-$; left) is indicated within the density plot.
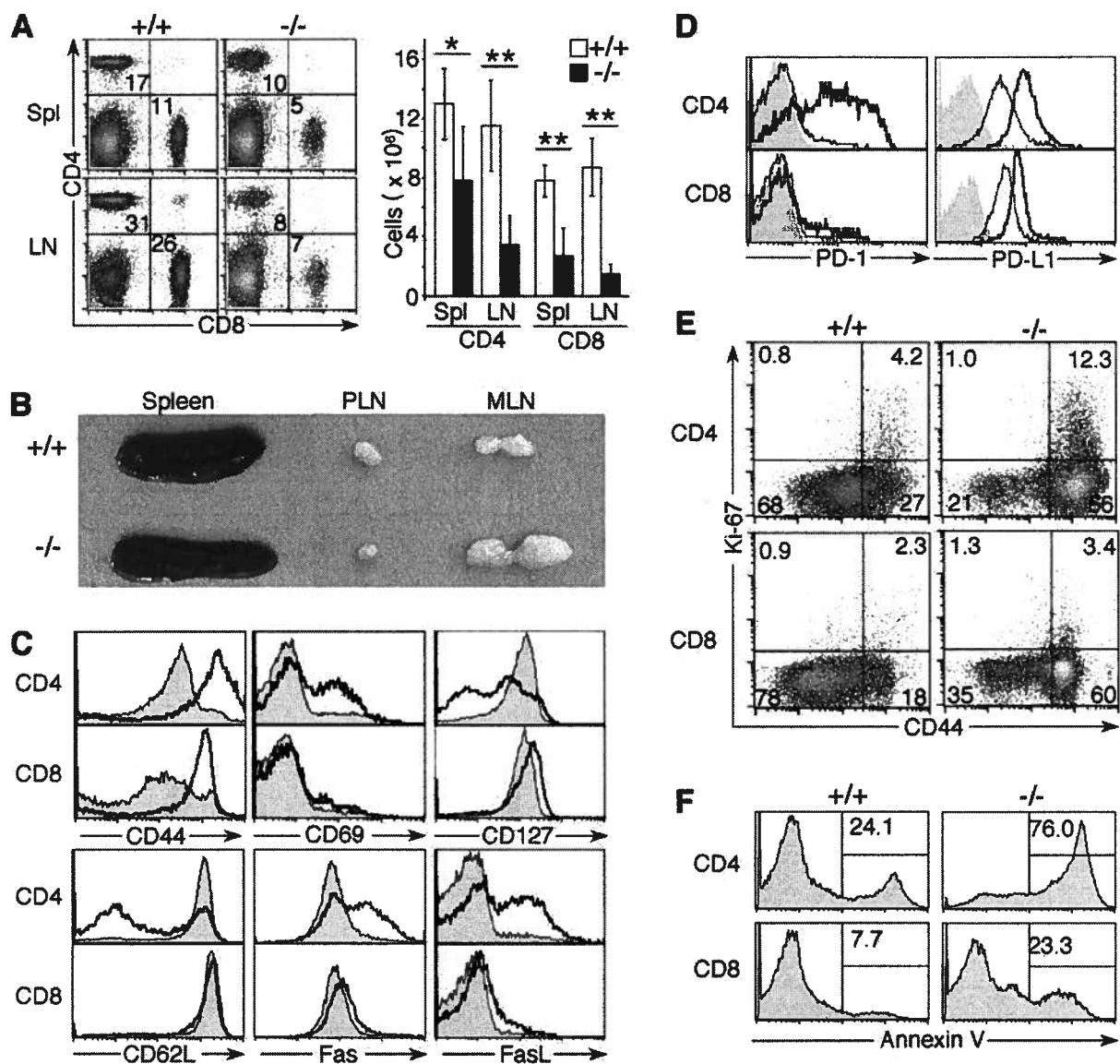
Figure 3.1 Peripheral CD4 T cells from RasGRP1$^{-/-}$ mice display signs of activation, exhaustion, proliferation, and spontaneous apoptosis.
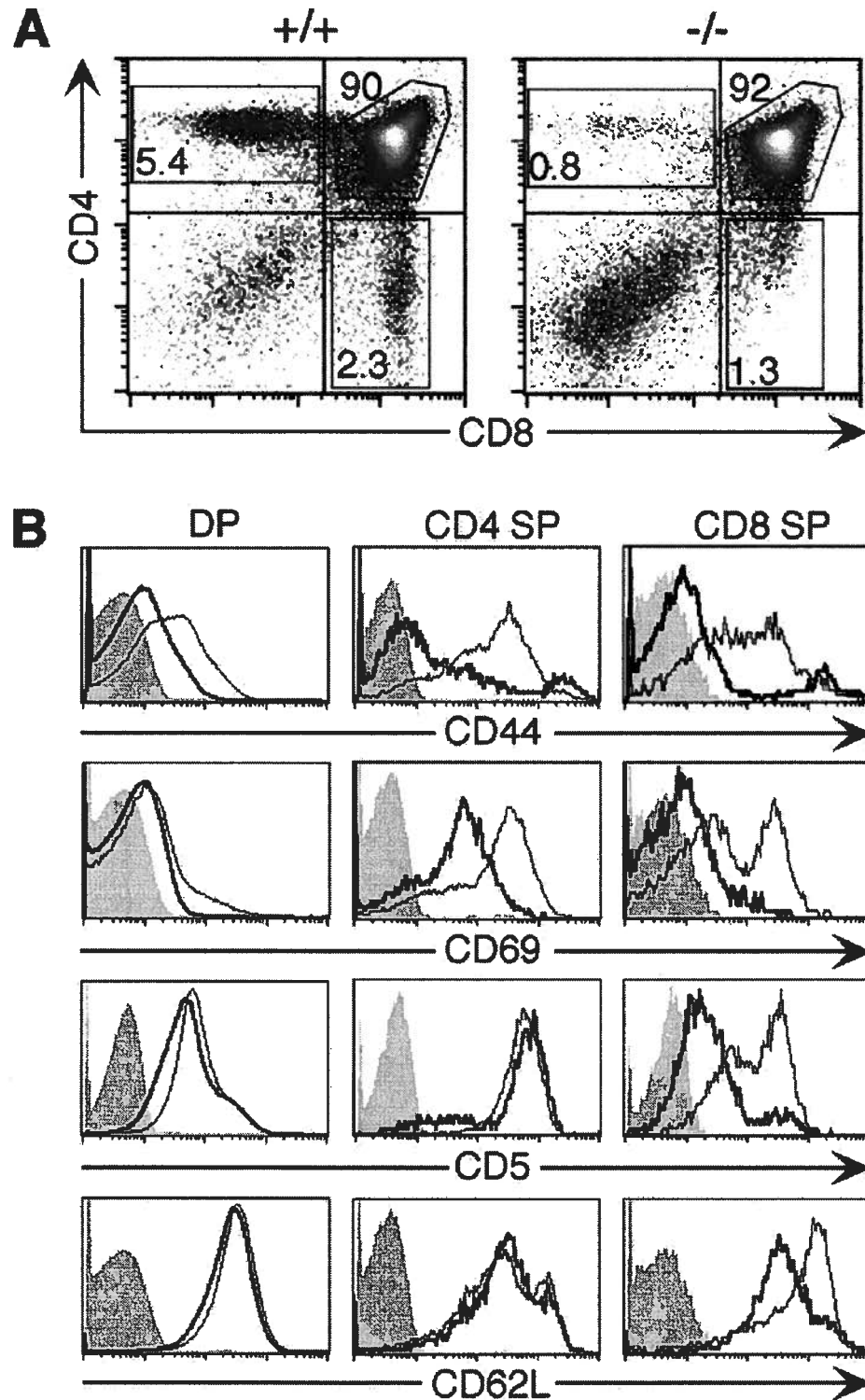
Figure 3.2 Aberrant-positive selection in RasGRP1-deficient mice results in a small population of SP thymocytes that bear a naive cell surface phenotype.

Figure 3.3 The cellular environment within RasGRP1-deficient animals promotes T cell expansion.

Figure 3.4 RasGRP1^{-/-} T cells expand vigorously after transfer into chronically immunodeficient RAG-1^{-/-} hosts.

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Figure 3.5 RasGRP1$^{-/-}$ mice mount a poor MHC class II-restricted LLO response upon infection with *L. monocytogenes*.

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Figure 3.6 RasGRP1$^{-/-}$ animals generate a weak T cell response toward LCMV and exhibit delayed viral clearance.
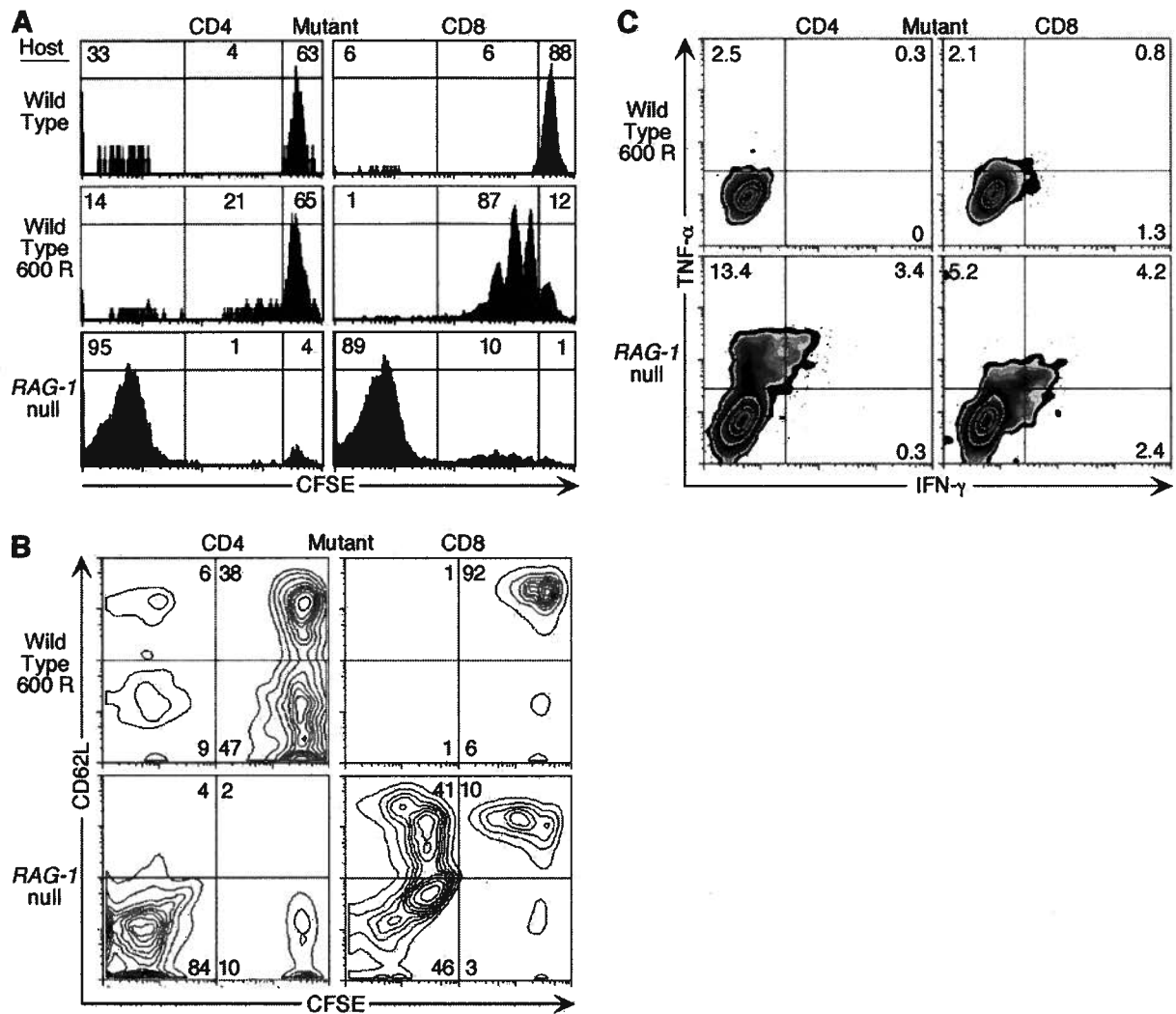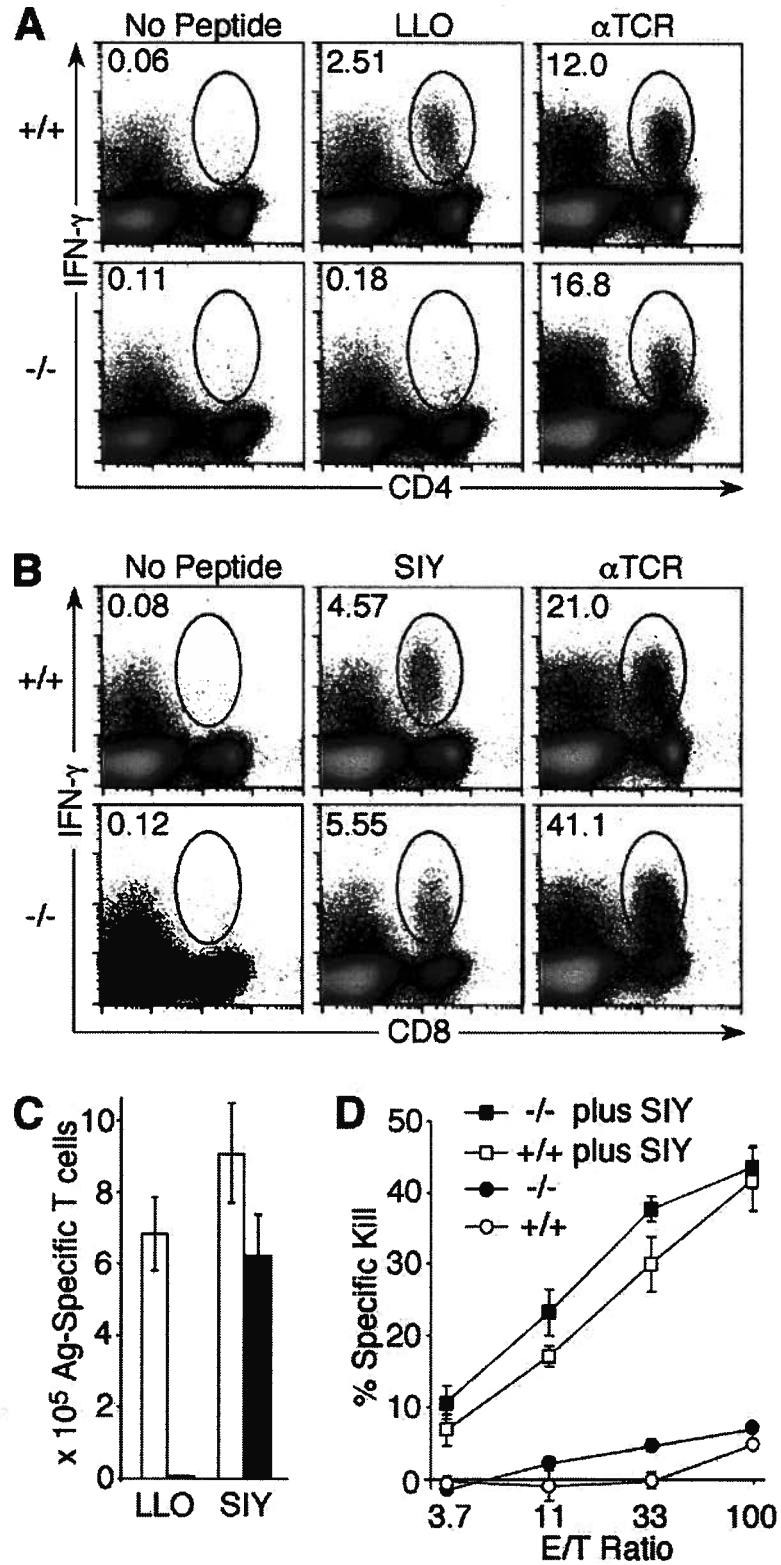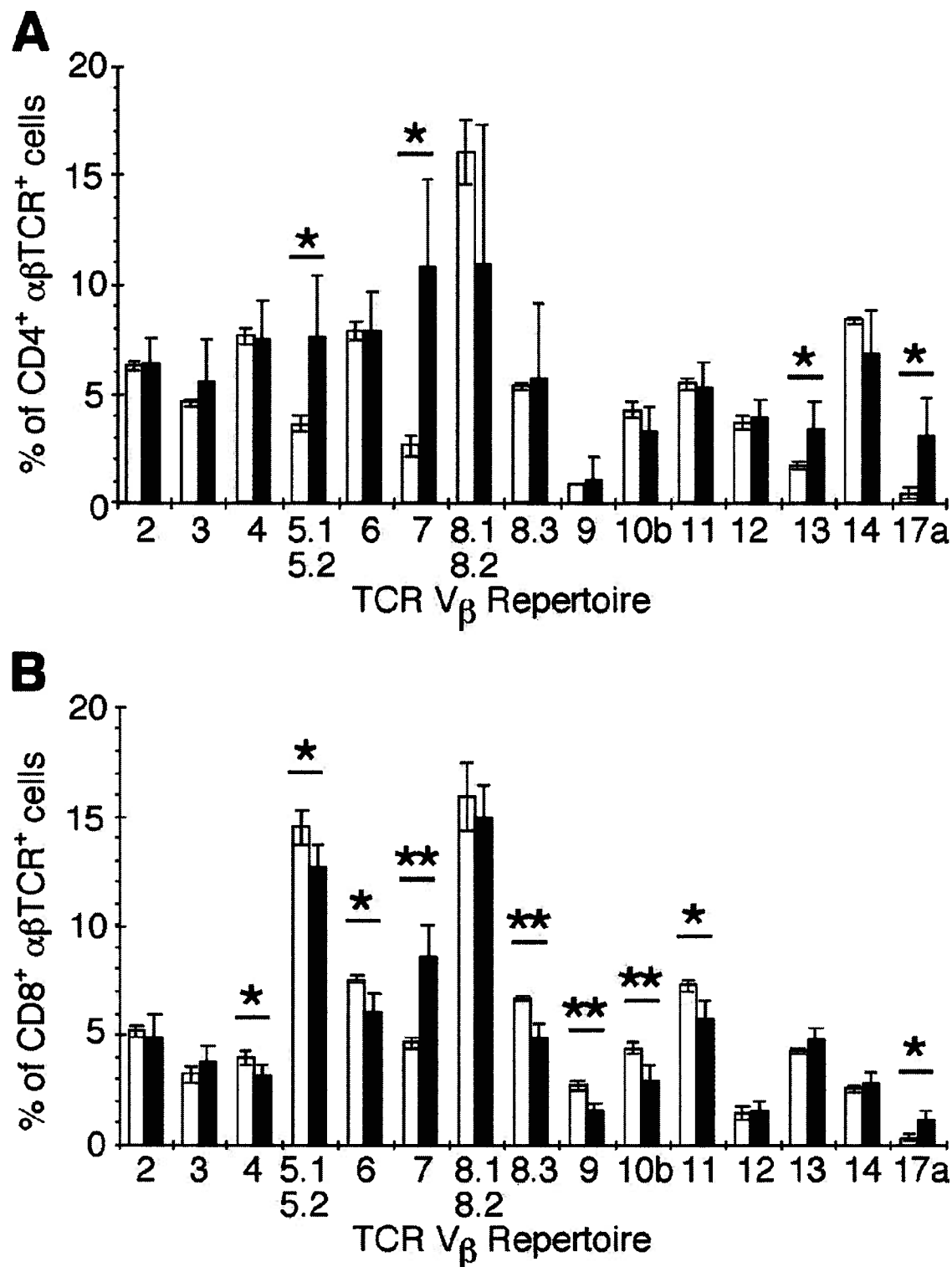
Figure 3.7 Peripheral RasGRP1$^{-/-}$ T cells possess an altered TCR repertoire.
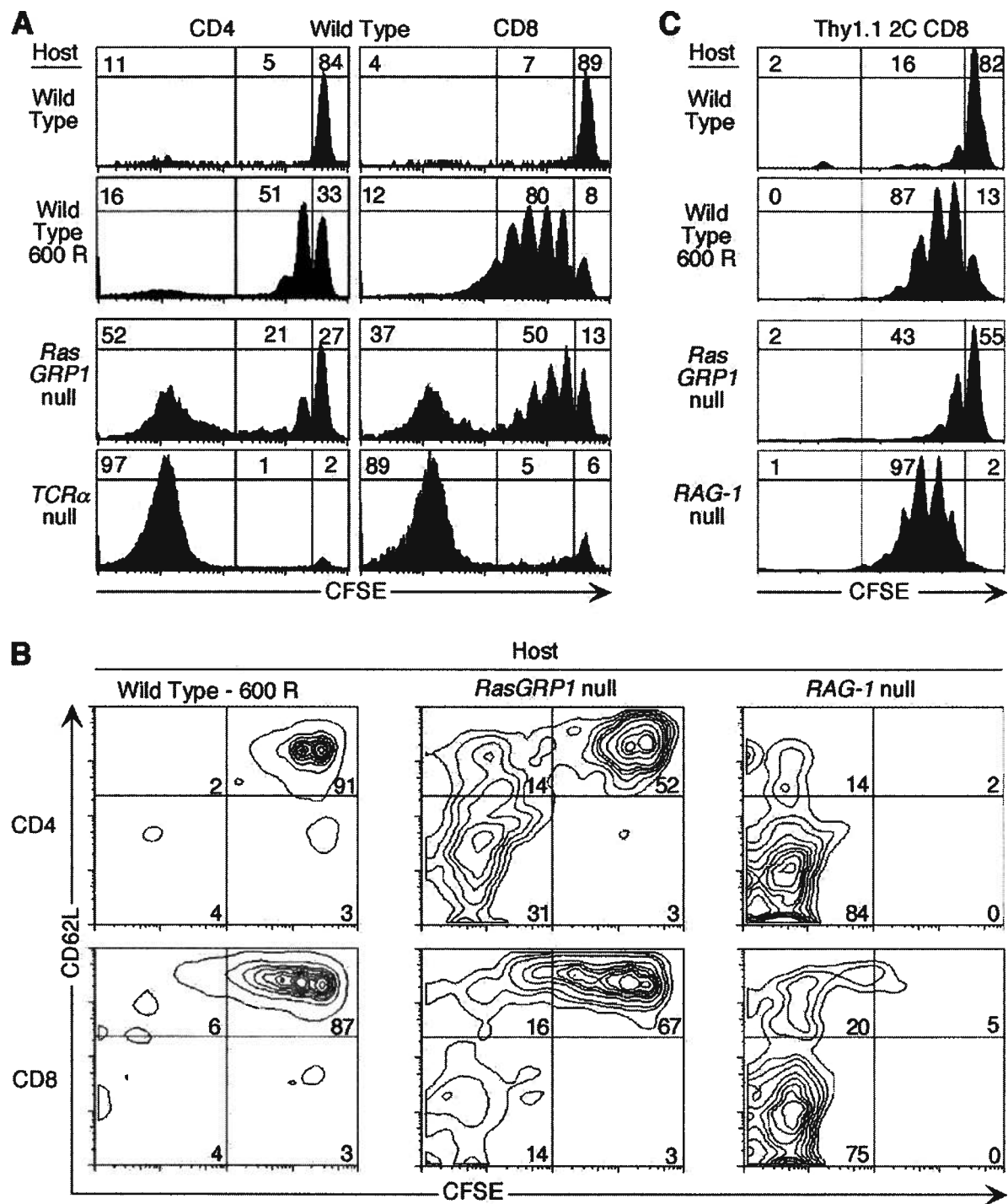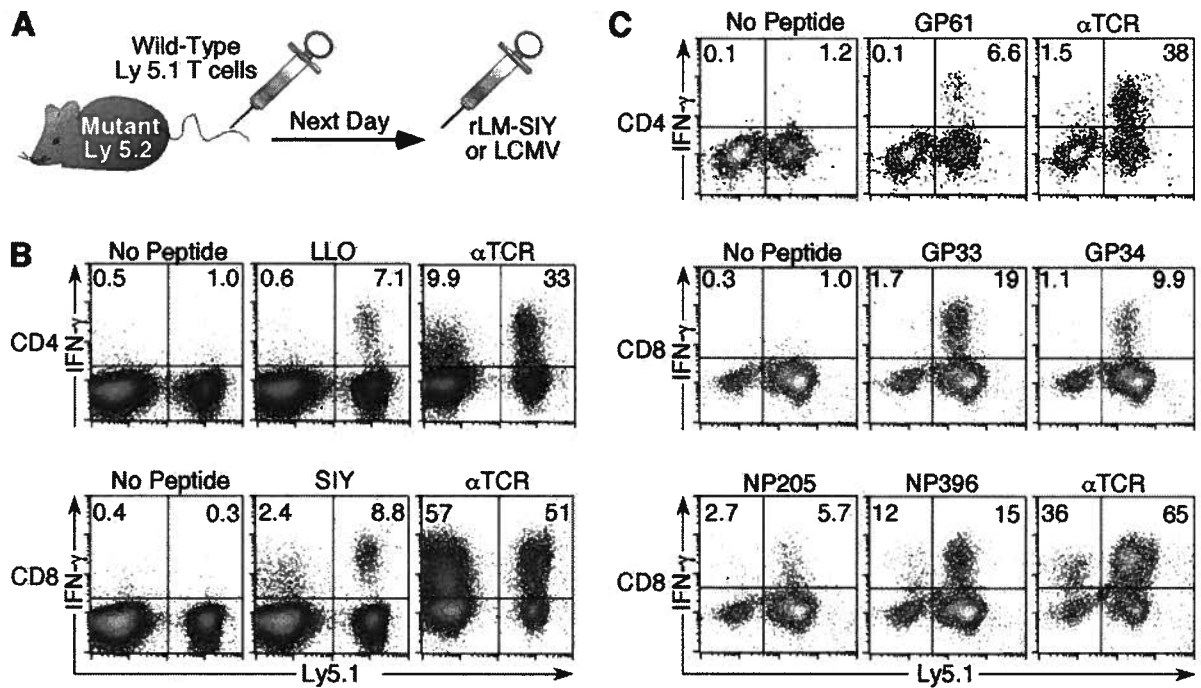
Figure 3.8 Failure of RasGRP1$^{-/-}$ mice to generate pathogen-specific T cells is the result of RasGRP1 loss in thymocytes and/or T cells.

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CHAPTER 4   RASGRP1 REGULATES THE RECRUITMENT OF NAÏVE CD8 T CELLS TO INITIATE ANTIGEN-INDUCED DEVELOPMENTAL PROGRAMMING[1]

4.1   Introduction

The TCR plays a fundamental role in both the development of mature T cells in the thymus and their function in the periphery (1, 2). During their ontogeny, the recognition of self-peptides/self-MHC molecules (self-Ags) by the TCR of a developing thymocyte is translated into a directive for either apoptosis or survival and differentiation. After maturation and export to the periphery, the interaction of a naïve CD8 T cell's TCR with self-Ags promotes their survival and peripheral maintenance whereas encounter with a specific foreign Ag (foreign peptide associated with self-MHC molecules) instructs a developmental program consisting of massive clonal expansion, effector cell formation, contraction through apoptosis and differentiation into long-lived memory cells. The basis by which the TCR instructs different outcomes onto a cell is mediated through graded signaling strengths, the affinity/avidity for its ligands determining the magnitude of signaling. Tight regulation of T cell homeostasis is essential for productive immune responses necessary for the clearance of pathogen and to prevent autoimmune-mediated self-destruction (3). The molecular mechanisms that regulate the recruitment of naïve T cells into cognate Ag-induced developmental program and navigate their transition from naïve to effector to memory cells are poorly understood.

TCR stimulation induces the rapid activation of small GTPase Ras whose signals are critical for orchestrating the positive selection of developing thymocytes (4, 5). Ras functions to link membrane receptor signaling to the internal MAPK cascades, cycling between an "off" (GDP-bound) and an "on" (GTP-bound) form. Ras guanine nucleotide exchange factors (Ras

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GEFs) greatly accelerate the release of GDP, facilitating Ras to bind GTP and the assumption of the active state. Conversely, Ras activity can be shut off by either its slow intrinsic GTPase activity or by pairing with GTPase-activating proteins (Ras GAPs). In thymocytes, the regulation of Ras activity by TCR signal transduction invokes at least two Ras GEFs, Sos and RasGRP1 (6-8). The respective functions of both Ras GEFs are dependent on relocating to membranes by two distinct mechanisms. RasGRP1 is recruited to membranes by binding the phospholipase Cγ1-product diacylglycerol (DAG) through its C1 domain whereas Sos is recruited to the phosphorylated adaptor molecule linker for activated T cells by way of its constitutive association with the SH2-domain-containing protein Grb2. RasGRP1 is likely the primary Ras GEF responsible for well-documented Ras activation observed following treatment of thymocytes with DAG analogs such as PMA (6, 9, 10).

The role of Ras activation in mature T cell function is less clear since transgenic mouse approaches of knocking down either Ras or its downstream MAPK cascade results in blocked thymocyte development accompanied with very few peripheral T cells and technical limitations of introducing genes into primary T cells (4). At least two distinct mechanisms have been postulated to regulate Ras activation upon TCR ligation in both human and mouse peripheral T cells (11, 12). Similar to the case of regulation of Ras in thymocytes, mature T cells are also thought to utilize the RasGRP1 and Sos for TCR-induced Ras/ERK activation (13, 14). However, the relative contribution of these two Ras GEFs to TCR-induced Ras signaling and their resulting impact on the activation and differentiation of mature CD8 T cells is unclear. Notably, defects in both Ras and ERK activation following TCR stimulation have been associated with anergized CD4 T cells and the inability to produce the T cell growth factor IL-2 (15, 16). A causal relationship between defective Ras signaling and T cell anergy has recently been demonstrated using novel techniques to transfect a constitutively active Ras into anergic T cells (17). Moreover, gain-of-function studies using the human Jurkat T cell line suggest that RasGRP1 promotes IL-2 transcription and thus, may stave off an anergic state (13).

We have previously generated two lines of TCR transgenic mice to study the role of RasGRP1 in CD8 T cell development under conditions of defined TCR signaling (9). Our

results found that RasGRP1 is critical for thymocytes expressing the weakly selecting H-Y TCR whereas those that express the strongly selecting 2C TCR are much less dependent on RasGRP1 for ERK activation and positive selection (9). The relatively efficient thymic development of 2C TCR CD8 T cells in mice lacking RasGRP1 along with their naïve peripheral phenotype and near-normal cell numbers afforded us with the unique opportunity to study the role of RasGRP1 in Ag-specific T cell responses. Here, we report that RasGRP1 plays a key role in the early activation events, such as CD25 up-regulation and IL-2 secretion, following exposure to cognate Ag. RasGRP1$^{-/-}$ 2C CD8 T cells exhibit greatly reduced sensitivity to TCR stimulation, failing to proliferate to either immobilized anti-TCR Abs or limiting doses of cognate Ag, and reduced burst size upon encounter with cognate Ag both in vitro or in vivo. These investigations highlight RasGRP1's function in regulating the growth of mature T cells and suggest that RasGRP1 may prove valuable as a potential drug target, suppressing T cell responses in transplantation and autoimmune disease settings.

4.2   Materials and methods

Mice

RasGRP1-deficient mice have been created and described previously by Dower *et al.* (6). C57BL/6J (B6) and B6.PL-*Thy1^a/Cy* (Thy 1.1$^+$) were acquired from The Jackson Laboratory (Bar Harbor, ME). RasGRP1-deficient 2C TCR transgenic mice were created by crossing with gene-targeted RasGRP1$^{-/-}$ mice that had been bred onto B6 background at least 10 generations. To generate Thy1.1$^+$/Thy1.2$^+$ 2C TCR transgenic animals, B6 mice bearing the 2C TCR transgene was mated with B6.PL-*Thy1^a/Cy* mice (H-2^b, Thy1.1$^+$). All studies followed guidelines set by the Animal Care Committee at the University of British Columbia in conjunction with the Canadian Council on Animal Care.

Flow cytometry

Antibodies against CD3 (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD8β (53-5.8), TCRβ (H57-597), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD122 (TM-

b1), CD127 (AKR34), Thy1.1 (HIS51), Thy1.2 (53-2.1), HSA (M1/69), PD-1 (J43), PD-L1 (MIH5), IL-2 (JES6-5H4), IL-4 (11B11), IL-10 (JES5-16E3), TNF-α (MP6-XT22) and IFN-γ (XMG1.2) were purchased from eBioscience. The clonotypic anti-2C TCR Ab was purified from the 1B2 hybridoma (from Dr. Herman Eisen, M.I.T.) and conjugated to either FITC or biotin using standard methods. For detection of intracellular cytokine, cells were stimulated in the presence of GolgiPlug (BD Biosciences; contains Brefeldin A) to block cytokine secretion as previously described (18). Anti-Ki-67 (B56) and isotype control (MOPC-21) Abs were purchased from BD Biosciences and the staining protocol employed was as published (18). Data was acquired with either a BD LSRII benchtop cytometer using FACSDiVa software (BD Biosciences) or FACSCalibur and CELLQUEST software (BD Biosciences). Data was analyzed with either CELLQUEST or FlowJo (Tree Star, Inc) software.

Purification and activation of 2C CD8 T cells

CD8 T cells were purified from spleens and lymph nodes using indirect magnetic bead-based separation according to manufacturer's instructions (MACS, Miltenyi Biotec). Briefly, CD8 T cells were labeled with anti-CD8β (53-5.8) Ab coupled to biotin, followed by incubation with Streptavidin-microbeads (Miltenyi Biotec) and column purification. For plate-bound anti-TCR Ab stimulations, tissue culture wells were coated with either 10 μg/ml of anti-CD3ε (145-2C11) Ab alone or 10 μg/ml of anti-CD3ε plus 5 μg/ml of anti-CD28 (37.51) mAbs. For cognate Ag stimulations, 2C CD8 T cells were incubated with irradiated B6 splenocytes that had been pulsed with 0.1 μg/ml of SIY peptide.

2C CD8 T cell stimulation using limiting doses of cognate Ag

CFSE-labeled wild type and RasGRP1$^{-/-}$ 2C CD8 T cells (50,000; Thy1.2$^+$) were cultured in vitro for 4 days with one million congenic CD8-depleted splenocytes (feeder; Thy1.1$^+$) and limiting numbers of SIY-coated APCs in flat-bottom 96-well plates. For preparation of SIY-coated APCs, CD8-depleted splenocytes from congenic (H-2^b; Thy1.1$^+$) mice were incubated with 0.1 μg/ml SIY peptide for 1 h at RT and subsequently, washed 3 times with PBS. For assessment of IFN-γ production, one million SIY-loaded splenocytes (Thy1.1$^+$) and the

golgi-transport inhibitor Golgi-Plug (BD Biosciences) were added to cultures 4 days post-stimulation for a 5 h period. Samples were stained and electronically gated on 2C CD8 (Thy1.2$^+$ CD8$^+$) cells to assess proliferation and markers of differentiation. All wild type and RasGRP1$^{-/-}$ Thy1.2$^+$ CD8$^+$ T cells expressed high levels of the 2C TCR as measured by anti-clonotypic 2C TCR mAb (1B2; data not shown).

Cell signaling studies and immunoblotting

2C CD8 T cells were purified by labeling pooled splenic/lymph node single cell suspensions with biotinylated anti-CD8β (53-5.8) Ab, incubated with streptavidin microbeads (Miltenyi Biotec) and subjected to magnetic separation. For TCR stimulations, CD8 T cells (10^7/ml) were prewarmed at 37 °C for at least 15 min in serum-free media and subsequently, treated with 10 μg/ml of anti-CD3ε Ab (145-2C11). Antibodies used to detect phosphorylated forms of ERK, LAT and ZAP-70 and total amounts of ERK2 have been described previously (19). For IL-2 signaling, T cell blasts were generated by activating purified 2C CD8 T cells for 2 d with 0.1 μg/ml SIY peptide plus irradiated splenocytes for APCs and subsequently, expanding them for 3-4 d with 20 U/ml IL-2. T cell blasts were serum-starved for 4 h prior to stimulation with either IL-2 (100 U/ml) or PMA (100 μM). At the indicated time, cells were rapidly precipitated by centrifugation, washed with ice-cold PBS and lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS, protease inhibitors and phosphatase inhibitors. Ras pulldown assays using Raf-GST fusion protein bound to glutathione-coupled beads were performed as described elsewhere (6, 13). A mixture of a pan-Ras (#R02120, Transduction Labs) and anti-K-ras (#30, Santa Cruz Biotec) Abs were used to measure Ras in both GST-Raf precipitates and total lysates. Blots were developed using ECL system (Amersham).

Generation of a recombinant strain of Listeria monocytogenes expressing the 2C agonist peptide SIY

A recombinant *Listeria monocytogenes* (rLM-SIY) was engineered to express the SIY peptide within a secreted DHFR fusion protein. The Ag cassette containing the SIY-peptide with flanking OVA and α-KG amino acid residues has been previously shown to induce

strong anti-SIY responses in B6 mice (20). After PCR amplification and cloning of the Ag cassette into the suicide vector pJJD180, the construct was introduced into the bacterial genome by homologous recombination as described previously (21, 22, 23).

Adoptive T cell transfers and bacterial infection experiments

To assess the capacity of rLM-SIY to induce 2C TCR CD8 T cell proliferation, one million purified wild type (Thy1.2$^+$/1.2$^+$) 2C TCR CD8 T cells were labeled with CFSE and adoptively transferred into congenic (Thy1.1$^+$/1.1$^+$) B6 mice via intravenous injection. Twenty-four h later, mice were either left untreated, infected with 10,000 CFU wild type LM (strain 10403S) or infected with 10,000 CFU rLM-SIY. One-week post-infection, donor 2C CD8 T cells were identified by staining with anti-Thy1.2 and anti-CD8 Abs and their proliferation measured by flow cytometry using a BD FACSCalibur or BD LSRII benchtop cytometer using CellQuest and FACSDiVa software respectively. For co-adoptive transfers experiments, a 50:50 mixture (10,000 of each genotype) of wild type (Thy1.1$^+$/1.2$^+$) and RasGRP1$^{-/-}$ (Thy1.2$^+$/1.2$^+$) 2C CD8 T cells were infused into syngenic (Thy1.1$^+$/1.1$^+$) B6 mice and infected the next day by intravenous injection using a dose of either 1,000 or 10,000 CFU of rLM-SIY in PBS. Bacterial doses were verified by plating the injectant on brain-heart infusion agar (BD Biosciences).

4.3   Results

RasGRP1$^{-/-}$ 2C CD8 T cells exhibit defects in early activation events and IL-2 production

To investigate the role of RasGRP1 in peripheral CD8 T cell function, we analyzed CD8 T cells from RasGRP1-deficient mice expressing the MHC class I-restricted 2C TCR transgene (24). For the study of cognate Ag-driven CD8 T cell responses, the 2C TCR recognizes the agonist peptide SIYRYYGL (SIY) bound to syngeneic H-2K^b MHC class I molecules with high affinity (25). Previously, we have observed that CD8 SP thymocytes were present at similar numbers in wild type and RasGRP1$^{-/-}$ 2C TCR thymi demonstrating that RasGRP1 is not critical for the development of mature cells by this TCR (9). Although peripheral

RasGRP1$^{-/-}$ 2C CD8 T cells are modestly reduced (about 50 %) relative to wild type, they possess a mature and naïve cell surface phenotype as determined by expression levels of 2C TCR, PD-1, PD-L1, CD3, CD24 (HSA), CD8α, CD8β, CD25, CD44, CD62L, CD69, CD122 and CD127 [(9), and data not shown]. To investigate for impairments in TCR signaling, we tested the ability of wild type and RasGRP1$^{-/-}$ 2C CD8 T cells to proliferate when cultured in wells coated with anti-TCR Ab. Proliferation was tracked 3 d post-stimulation by either analyzing dilution of CFSE-endowed fluorescence by flow cytometry (Fig. 4.1*A*) or measuring incorporated [^{3}H]-thymidine during a 6 h pulse period 3-days post-stimulation (Fig. 4.1*B*). By contrast to the SIY/APC response, RasGRP1$^{-/-}$ 2C CD8 T cells failed to even enter cell cycle when cultured immobilized anti-TCR Ab alone. Furthermore, complementing TCR signals with anti-CD28 Ab-mediated costimulation also failed to induce significant proliferation by RasGRP1-deficient 2C CD8 T cells (Fig. 4.1*A*, 4.1*B*). However, the proliferative responses by RasGRP1$^{-/-}$ 2C CD8 T cells resembled wild type T cells when either plate-bound anti-TCR Ab or anti-TCR plus anti-CD28 Abs stimulations were supplemented with exogenous IL-2. These data demonstrate RasGRP1-deficient 2C CD8 T cells display an anergic phenotype that is reversible upon the addition of exogenous IL-2.

The findings made with RasGRP1$^{-/-}$ 2C CD8 T cells using immobilized anti-TCR Ab as an agonist argue that RasGRP1 is important for early T cell activation and recruitment into cell cycle (Fig. 4.1*A*, 4.1*B*). However, our previous experiments, using soluble SIY peptide presented by syngeneic splenocytes to stimulate RasGRP1$^{-/-}$ 2C CD8 T cells, had suggested early activation and proliferation were RasGRP1-independent events (9). It is possible that the apparent discrepancy between these observations may be a consequence of the fact that APCs deliver additional signals, such as coreceptor engagement, costimulatory molecule interactions and cytokines, that anti-TCR plus anti-CD28 Abs alone do not provide. To investigate the respective role of RasGRP1 in mediating early T cell activation events and whether this role differs depending on the type of stimulation, wild type and RasGRP1$^{-/-}$ 2C CD8 T cells were stimulated with either SIY-pulsed splenocytes or anti-TCR Ab for 16 hours and subsequently, assessed for the induction of activation markers (Fig. 4.1*C*). A comparison of histograms and mean fluorescence intensities (MFIs) revealed that RasGRP1$^{-/-}$ 2C CD8 T cells exhibited impaired up-regulation of the activation markers as compared to wild type

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(CD25: 201 *vs.* 1280, 6.4x lower MFI; CD69: 295 *vs.* 776, 2.6x lower MFI; and PD-1: 295 *vs.* 630, 2.2 x lower MFI). By contrast, RasGRP1$^{-/-}$ 2C CD8 T cells exhibited a more normal activation marker expression profile when cells were treated with peptide/APC (CD25: 1182 *vs.* 1748, 1.5x lower MFI; CD69: 642 *vs.* 893, 1.4x lower MFI; PD-1: 370 *vs.* 464, 1.3x lower MFI). These findings argue that Ag receptor-induced expression of activation markers is heavily dependent on RasGRP1 when costimulatory or supplementary signals are limiting, such as the case with plate-bound anti-TCR Ab stimulation alone. Next, we sought to determine whether RasGRP1$^{-/-}$ 2C T cells show defects in the induction of IL-2 since its production by T cells is an early event upon encounter with cognate Ag and exogenously added cytokine rescues mutant T cell proliferation (Fig. 4.1*D*). Wild type and RasGRP1$^{-/-}$ 2C T cells were stimulated with SIY-pulsed splenocytes and monitored hourly for IL-2 expression by intracellular flow cytometry. At each time point post-stimulation, RasGRP1$^{-/-}$ 2C T cells displayed a significant reduction in the proportion of IL-2-positive cells. This finding implies that RasGRP1 affects T cell growth through the regulation of IL-2 transcription.

Addition of exogenous IL-2 can support the differentiation of naïve RasGRP1-deficient CD8 T cells into effector CTLs

Previously, we had observed that RasGRP1-deficient 2C CD8 T cells undergo early proliferation following exposure to cognate Ag, soluble SIY peptide and irradiated syngeneic splenocytes, but failed to sustain their growth (9). To investigate whether the impaired Ag-driven proliferation and developmental programming of RasGRP1$^{-/-}$ 2C CD8 T cells may be related to their decreased capacity to produce IL-2, we compared the differentiation status of wild type and mutant naïve 2C CD8 T cells stimulated with cognate antigen in vitro either in the absence or presence of exogenous IL-2 (Fig. 4.2*A*, 4.2*B*). After four days of culture with SIY-pulsed irradiated syngeneic splenocytes alone, wild type 2C CD8 T cells underwent considerable remodeling of their cells surface, elevating CD25, CD44 and the cytotoxic effector molecule Granzyme B while simultaneously down-regulating CD62L (Fig. 4.2*A*). Although RasGRP1-deficient 2C CD8 T cells lost CD62L expression, they exhibited markedly reduced expression of activation and differentiation markers relative to wild type (CD25: 91 *vs.* 301, 3.3x lower MFI; CD44: 450 *vs.* 1646, 3.6x lower MFI; Granzyme B: 138

vs. 294, 2.1x lower MFI). Next, we tested whether culturing in the presence of both SIY-pulsed irradiated syngeneic splenocytes and exogenous IL-2 could rescue the differentiation of RasGRP1$^{-/-}$ CD8 T cells (Fig. 4.2*B*). Wild type and RasGRP1$^{-/-}$ CD8 T cell effector profiles exhibited much less pronounced differences when exogenous IL-2 is added to cultures (CD25: 354 *vs.* 777, 2.2x lower MFI; CD44: 1431 *vs.* 1715, 1.2x lower MFI; Granzyme B: 384 *vs.* 392, similar MFI). Therefore, the differentiation of RasGRP1$^{-/-}$ CD8 T cells, as measured by surface markers and Granzyme B expression, can largely be restored by supplementation of medium with IL-2. Collectively, these experiments demonstrate that RasGRP1 plays a critical role in the efficient generation of CTL effectors and that its function can be compensated for by the addition of exogenous IL-2.

The stimulation of naïve T cells with cognate Ag results in their commitment to a developmental program linked with the production of effector cytokines such as IFN-γ (26). To determine whether signaling by RasGRP1 contributes to cytokine production elicited upon TCR engagement, CD8 T cell blasts, generated by culture with cognate Ag and exogenous IL-2 (Fig. 4.2B), were stimulated with SIY-pulsed splenocytes in vitro for 5 h. Regardless of genotype, the majority (~99 %) of CD8 T cell effectors produce IFN-γ after contact with cognate Ag (Fig. 4.2C). CD8 T cell effectors can be subdivided into IFN-γ-producing (Tc1) and IL-4-producing (Tc2) populations (27). As shown for naive RasGRP1$^{-/-}$ 2C T cells, RasGRP1$^{-/-}$ T cell blasts secrete less IL-2 than their wild-type counterparts. To investigate if RasGRP1-deficiency affects cytokine polarization of CD8 T cells, we also assessed the production of the Tc2 cytokines IL-4 and IL-10 (Fig. 4.2C). These results demonstrate that RasGRP1$^{-/-}$ CD8 T cell effectors expanded by cognate Ag and exogenous IL-2 produce IFN-γ and suggest that RasGRP1 function does not appear to influence Tc1 versus Tc2 polarization.

RasGRP1 regulates the antigen dose necessary to recruit naïve T cells to initiate proliferation and effector cell formation. The poor responsiveness to immobilized anti-TCR antibodies and decreased capacity to synthesize IL-2 suggest that RasGRP1$^{-/-}$ 2C T cells may be less sensitive to cognate Ag stimulation. To test this hypothesis, a constant number of CFSE-labeled wild type and RasGRP1$^{-/-}$ 2C T cells were cultured with limiting numbers of SIY-

pulsed APCs (Fig. 4.3*A*). After four days of culture, 150,000 total events were collected and CFSE fluorescence of 2C T cells plotted on histograms with cell numbers' scale indicated on the y-axis. As has been observed previously in an analogous experiment with P14 TCR CD8 T cells (28), increasing the number of cognate Ag-loaded APCs resulted in an increased frequency of TCR transgenic CD8 T cells recruited into cell cycle and correlated with an increased burst size (larger scale on y-axis). RasGRP1$^{-/-}$ 2C T cells were almost 10-fold less sensitive to cognate Ag relative to wild type since 2,000 SIY-loaded APCs were required to recruit greater than 40 % of cells into cycle while only 200 SIY-APCs were necessary for wild type T cells to reach this level of recruitment. In addition, recruited RasGRP1$^{-/-}$ 2C T cells retained more CFSE-endowed fluorescence suggesting these cells underwent fewer divisions than wild type (Fig. 4.3*A*). These findings imply that RasGRP1 strongly influences activation threshold of naïve CD8 T cells.

We next examined the relationship between the T cell differentiation marker CD44 and cell division in wild type and mutant 2C T cells stimulated with the indicated doses of SIY-coated APCs (Fig. 4.3*B*). Regardless of cell genotype, Ag-driven proliferation was accompanied with elevated levels of CD44. However, RasGRP1$^{-/-}$ 2C T cells had fewer CD44hi cells particularly at lower doses of cognate antigen. To determine whether CD44 up-regulation correlated with effector function in mutant T cells, cultures were prepared as above (Fig. 4.3*A*, 4.3*B*), stimulated with one million SIY-coated splenocytes for additional 5 h and assessed for IFN-γ production (Fig. 4.3*C*). Again, a reduced fraction of RasGRP1$^{-/-}$ 2C T cells were capable of secreting IFN-γ since a meager number of cells were mobilized to proliferate and differentiate when cognate antigen was limiting. Collectively, these findings argue that RasGRP1 regulates the recruitment of naïve CD8 T cells to initiate Ag-induced developmental programming.

RasGRP1$^{-/-}$ 2C CD8 T cells display transient ERK activation upon TCR ligation but normal ERK phosphorylation following IL-2 stimulation

Prior studies have shown that thymocytes lacking a functional RasGRP1 protein exhibit a greatly diminished capacity to activate ERK upon treatment with either DAG analogs (6, 9,

10) or anti-TCR antibodies (6, 10, 19). To examine the consequences of RasGRP1-deficiency on the ability of mature peripheral T cells to activate ERK, 2C CD8 T cells were purified using magnetic separation from wild type and mutant mice and stimulated with anti-TCR antibody for the indicated times (Fig. 4.4A). By contrast to the hypo-responsiveness of RasGRP1$^{-/-}$ immature thymocytes (6, 19), RasGRP1-deficient 2C CD8 T cells strongly phosphorylate ERK at an early time (3 min) post-stimulation at levels resembling wild type (Fig. 4.4A). However, ERK activation in mutant 2C CD8 T cells is shorter in duration, exhibiting significant deficits at 30 and 60 min post TCR stimulation. Analysis of the upstream signaling molecules argues that LAT and ZAP-70 phosphorylation are not substantially affected by RasGRP1-deficiency. These findings suggest that RasGRP1 may be critical for sustaining the kinetics of ERK activation following cognate antigen stimulation and that such prolonged signaling may be necessary for Ag-stimulated T cells to enter cell cycle.

Activation of the Ras/MAPK cascade in T cells not only accompanies Ag receptor ligation but is also invoked by IL-2 receptor signaling (29, 30). To examine whether RasGRP1 plays a role in IL-2-mediated Ras signaling, activated wild type and RasGRP1$^{-/-}$ 2C CD8 T cell blasts were serum-starved for 4 h prior to incubation with IL-2 for 10 min (Fig. 4.4B). Assessment of the activation status of Ras and ERK post-IL-2 stimulation revealed that the Ras/ERK cascade seemed largely intact in RasGRP1$^{-/-}$ cells despite the observation that they exhibited a 2-fold reduction in levels of the IL-2 receptor α chain CD25 (Fig. 4.4B; Fig. 4.2B). By contrast, RasGRP1$^{-/-}$ 2C T cell blasts displayed very weak Ras activation upon PMA treatment relative to wild type (Fig. 4.4B). These findings suggest that activation of Ras initiated by IL-2 receptor signaling does not directly depend on RasGRP1.

Recombinant Listeria monocytogenes expressing the SIY epitope induces wild type 2C T cells to proliferate in vivo

To study the consequences of RasGRP1-deficiency on Ag-driven proliferation, differentiation and memory formation of 2C CD8 T cells in vivo, we engineered *Listeria monocytogenes*, a well-characterized pathogen model for eliciting T cell responses (31, 32), to express the 2C agonist SIY peptide (Fig. 4.5A). The recombinant strain (called rLM-SIY),

derived by integration of an Ag cassette into the bacterial chromosome between the lecithinase and lactate dehydrogenase operons using techniques described previously (21-23), expresses the SIY peptide as a secreted DHFR fusion under the control of a virulence promoter (P_{hly}). To test the capacity of rLM-SIY to induce 2C T cell proliferation, one million CFSE-labeled wild type 2C T cells were infused to B6 mice (non-irradiated; Th1.1$^+$) and left untreated (naïve) or infected with 10,000 CFU of either rLM-SIY or wild type LM 10403S (Fig. 4.5*B*). One-week post-infection, the vast majority of splenic 2C CD8 T cells residing in either naïve mice or those infected with wild type LM failed to proliferate (95 % and 90 % respectively). By contrast, analysis of donor 2C CD8 T cells in rLM-SIY-infected mice revealed that these cells had strongly proliferated, virtually completely losing their CFSE-endowed fluorescence. In addition, donor 2C T cell proliferation induced by infection with rLM-SIY was accompanied by a greater than 100-fold increased recovery of donor 2C T cells relative to either naïve B6 hosts or those infected with wild type LM (data not shown). These findings demonstrate that rLM-SIY can effectively prime and induce the expansion of naïve wild type 2C T cells in vivo.

RasGRP1-deficient T cells exhibit a reduced rate of proliferation and diminished cytokine production upon cognate antigen stimulation in vivo

Naïve T cells encountering cognate Ag and the appropriate costimulatory signals initiate a development program, undergoing massive clonal expansion (26, 28). To test the proliferative capacity and function of RasGRP1$^{-/-}$ 2C CD8 T cells in vivo, equivalent numbers of CFSE-labeled wild type (Thy1.1$^+$/1.2$^+$) and mutant (Thy1.2$^+$/1.2$^+$) 2C CD8 T cells were infused into the same mouse, ensuring cells of each genotype were exposed to a similar environment and an equivalent amount of cognate Ag, and recipients infected the next day with 1,000 CFU of rLM-SIY (Fig. 4.5*C*). Since recent experiments have shown that naïve T cell precursor frequency profoundly affects the differentiation program taken by CD4 and CD8 T cells (33, 34), we injected greatly reduced numbers of 2C T cells (10,000/genotype) per mouse in comparison to the experiments presented in Figure 5*B* to more accurately resemble physiological levels of naïve T cells specific for a given Ag (35, 36). Using anti-Thy1.2 Ab to identify donor cells and anti-Thy1.1 Ab to discriminate genotype, RasGRP1$^{-/-}$ 2C CD8 T cells were found to be represented at a 4-fold decreased

proportion and total numbers as compared to wild type counterparts one-week post-infection (Fig. 4.5D, 4.5E). However, the majority of 2C T cells had undergone at least 7-8 rounds of proliferation regardless of genotype and therefore, lacked fluorescence bestowed by CFSE (Fig. 4.5F). Since tracking of cell division using CFSE fails to resolve additional information once the fluorescence of CFSE-labeled cells approaches the autofluorescence of unstained cells (37), expression of the proliferation-associated nuclear antigen Ki-67 was used to investigate whether decreased levels of cellular proliferation was associated with the reduced burst size by mutant CD8 T cells (Fig. 4.5G). A lower proportion of RasGRP1$^{-/-}$ 2C CD8 T cells expressed the Ki-67 marker as compared to wild type (10.7 ± 2.4 % vs. 26.9 ± 5.9 %), implying that their decreased representation is a consequence of slower cell cycling rates by this population of cells. These in vivo findings suggest that RasGRP1 is important in regulating the burst size of CD8 T cells responding towards cognate Ag.

The differentiation of naïve T cells resulting from Ag-driven clonal expansion is tightly linked with phenotypic and functional changes. This transformation includes increased expression of effector/memory markers, like CD44, and the acquisition of effector functions such as the capacity to produce the pro-inflammatory cytokines, like IFN-γ and TNF-α, and cytotoxicity (28, 26, 38). To investigate the cell surface phenotype of donor 2C T cells after cognate Ag exposure in vivo, splenocytes of host mice 7 days post-infection with rLM-SIY were stained with antibodies against various markers in addition to Thy1.1, Thy1.2 and CD8 (Fig. 4.5G). Analysis of donor 2C T cells (filled, black histograms) revealed that cells of either genotype had also strongly up-regulated CD44 expression as compared to naïve 2C cells (grey histograms), albeit the mutant cells expressed modestly reduced CD44 levels relative to wild type (2720 vs. 3790 MFI). To test the function of donor 2C T cell effectors, splenocytes isolated from host mice 7 days post-infection were placed in culture, stimulated for 5 h with the SIY peptide and subsequently, assessed for the production of the proinflammatory cytokines IFN-γ and TNF-α (Fig. 4.5I). By contrast to wild type, a significant fraction of RasGRP1$^{-/-}$ 2C CD8 T cell effectors failed to produce cytokine (21 %) and a diminished percentage co-expressed IFN-γ and TNF-α relative to wild type (38 % vs. 83 %). However, RasGRP1$^{-/-}$ 2C CD8 T cell effectors derived by expressed wild type levels of Granzyme B and were efficient killers of EL-4 targets pulsed with the SIY peptide (data

not shown). Next, we wanted to test whether the expression of RasGRP1 influenced the capacity of 2C T cell effectors to differentiate into memory. However, to our dismay, donor 2C T cells, either wild type or mutant, completely disappeared within 2 weeks post-rLM-SIY infection (data not shown). In addition, we were unable to visualize long-lived memory 2C T cells regardless of number of 2C T cells ($1 \times 10^3 - 2 \times 10^6$) adoptively transferred, the infectious dose of rLM-SIY ($2 \times 10^3 - 1 \times 10^5$ CFU) and the number of events acquired ($\geq 2 \times 10^7$). Since analogous experiments with OT-I TCR CD8 T cells and rLM-OVA infections yielded abundant numbers of long-lived memory OT-I CD8 T cells (data not shown), we believe that the inability to detect memory 2C CD8 T cells is not a technical issue. Moreover, the fact that rLM-SIY infection gives rise to long-lived anti-SIY CD8 memory T cells in normal (non-TCR transgenic) B6 mice [data not shown and (39)] leads us to suspect that the inability to find long-lived memory 2C CD8 T cells following rLM-SIY infection is somehow linked to the 2C TCR.

4.4   Discussion

Understanding the molecular controls of the immune system will yield insight into the basis of immunological diseases, improve the efficacy of vaccination and lead to the identification of molecular targets suitable for therapeutic intervention. Ras signaling pathways are known to play key roles in both the development of thymocytes and the regulation of mature T cell function. At least two important Ras GEFs, RasGRP1 and Sos, are known to play critical roles in TCR signal transduction in developing thymocytes and mature T cells (5). The findings that thymocyte positive selection is relatively inefficient in the absence of RasGRP1 demonstrate that these Ras GEFs are not functionally redundant (6, 9, 10). Moreover, aberrant positive selection in RasGRP1-deficient mice is associated with an abundance of activated- and memory-phenotype peripheral CD4 and CD8 T cells (10, 18, 40), a likely result of chronic immunodeficiency (18) and/or a breakdown in T cell tolerance (10). Therefore, studies of mature T cells in non-TCR transgenic RasGRP1$^{-/-}$ mice are tenuous since their differentiation status, a factor that influences TCR signal transduction (41), does not match wild type. Here, we report the use of RasGRP1$^{-/-}$ 2C TCR mice to investigate how

this Ras GEF participates in peripheral T cell function and differentiation since these animals produce sufficient quantities of 2C CD8 T cells, exhibiting a mature, naïve cell surface phenotype resembling those recovered from wild type 2C TCR animals (9).

Sufficient exposure of naïve T cells to cognate Ag commits them to an Ag-independent phase of proliferation that is intimately coupled to differentiation, composing of chromatin remodeling, gene expression changes and the acquisition of effector functions (26). We have found that RasGRP1-deficient 2C CD8 T cells require a much greater dose of cognate Ag to divide and when recruited into cell cycle undergo fewer rounds of proliferation (Fig. 4.3A). A net consequence of decreased sensitivity to TCR-stimulation is that a very low frequency of RasGRP1$^{-/-}$ 2C CD8 T cells differentiates into effectors at limiting doses of cognate Ag. A possible mechanism by which RasGRP1 may lower activation threshold and promote the growth of naïve T cells is through its regulation of IL-2 receptor α chain (CD25) expression and IL-2 production (Fig. 4.1C, 4.1D) since it has been shown that blocking of IL-2 signaling in Ag-stimulated TCR transgenic CD8 T cells can reduce the proportion of naïve T cells recruited to proliferate and the extent to which they divide (28). In addition, we found that the addition of exogenous IL-2 can promote both the proliferation and effector cell formation of TCR-stimulated RasGRP1$^{-/-}$ 2C CD8 T cells (Fig. 4.1, Fig. 4.2). These findings support a role of RasGRP1 in which it impacts T cell activation and growth, at least in part, through regulation of IL-2 production.

Anergy is a peripheral tolerance mechanism in which a T cell is rendered functionally inactive following encounter with cognate Ag. The hyporesponsive state of anergic T cells is acquired and maintained through intrinsic wholesale changes in TCR signaling. Moreover, a number of characteristics displayed by RasGRP1$^{-/-}$ 2C CD8 T cells are reminiscent of T cell anergy including hypo-responsiveness to cognate Ag stimulation, decreased IL-2 production and the apparent reversibility of this phenotype by the addition of exogenous IL-2. In addition, analyses of TCR signaling pathways in anergic T cells have often revealed defects in Ras/ERK activation and AP-1-induced gene transcription (42). Gene expression profiling experiments pinpointed the overexpression of diacylglycerol kinases (DGKs), negative regulators of Ras signaling that convert DAG to phosphatidic acid, as a possible cause of

aberrant TCR signaling in anergic T cells (17). Supporting this notion, enforced DGKζ in Jurkat cells has been found to inhibit TCR-induced activation of Ras, ERK and AP-1 (43). A cause-and-effect relationship between the overexpression of DGKs and anergy was demonstrated by findings that the introduction of DGKα into mouse T cells inhibited MAPK signaling, IL-2 production and the recruitment of RasGRP1 to membranes (17). Our data is consistent with the hypothesis that DGKs suppress T cell responsiveness by blocking RasGRP1's ability to activate Ras.

Cellular proliferation may be a critical factor in the differentiation of T cells since it may provide the opportunity for chromatin remodeling and thus, facilitate changes in gene expression (44, 45). To investigate whether the decreased proliferative potential of naïve RasGRP1$^{-/-}$ 2C CD8 T cells in vitro affected their ability to differentiate into effector and memory CD8 T cells in vivo, we engineered the model pathogen *Listeria monocytogenes* to express the 2C TCR agonist SIY peptide (Fig. 4.5A). Although RasGRP1$^{-/-}$ 2C CD8 T cells were found to exhibit a 4-fold reduction in the generation of Day 7 effectors relative to wild type (Fig. 4.5D), it is possible that the experimental conditions seen signaling defects in these T cells may have been partially masked under the experimental conditions since the presence of wild type host CD4 T cells and their accompanying helper-associated function of IL-2 secretion may compensate for RasGRP1 loss. Moreover, decreased numbers of RasGRP1$^{-/-}$ 2C T cell effectors was associated with reduced rates of proliferation (Fig. 4.5G) and inflammatory cytokine effector function (Fig. 4.5I), suggesting the possibility that signaling by RasGRP1 may be important for the formation of T cell memory. However, we were unable to test this hypothesis since we could not detect long-lived memory 2C T cells after rLM-SIY infection despite the observations that these cells had been induced to undergo numerous rounds of proliferation (Fig. 4.5F), elevate CD44 expression (Fig. 4.5H) and acquire the capacity to produce proinflammatory cytokines (Fig. 4.5I). Critical parameters mediating the differentiation of naïve CD8 T cells into effector and eventually, memory T cells are thought to be the Ag dose, Ag duration and the affinity of the TCR for a given cognate Ag (26). The findings that rLM-SIY infection in normal B6 mice results in the formation of long-lived memory anti-SIY CD8 T cells suggest that the 2C TCR may be somehow problematic for the development of CD8 T cell memory (39). However, a recent

study has demonstrated that 2C T cells can differentiate into long-lived memory T cells, persisting at least 225 days post-infection, using a recombinant influenza virus expressing the SIY peptide, named WSN-SIY (46). Perhaps it is the dose or duration of Ag that is a crucial factor for the formation of memory 2C CD8 T cells. After WSN-SIY infection, Ag is detectable for at least 8 days post-infection (Jianzhu Chen, personal communication). By contrast, rLM-SIY likely results in a contracted Ag exposure since it exhibits greatly reduced virulence (ability to divide *in vivo*), a trait observed for multiple strains of recombinant LM (47), as compared to the wild type strain 10403S. For example, intravenous tail injection of 10,000 CFUs of wild type LM 10403S into B6 mice will yield millions of CFUs in both the spleen and liver 3 days post-infection whereas the bacterial load following the same dose of rLM-SIY will border the limit of detection (data not shown). However, rLM-SIY still induces strong anti-SIY CD8 T cell responses in naïve B6 mice despite its reduced pathogenicity (18, 39). In addition, an alternate explanation for the disappearance of 2C CD8 T cells following rLM-SIY infection could be the rejection of donor T cells by the host. Although we have bred our 2C TCR transgenic mice 10 generations onto the B6 background, it is plausible that some flanking DNA closely linked to the 2C TCR transgene could be facilitating graft rejection.

One intriguing question is why TCR signaling engages two Ras GEFs, each using intricate, independent mechanisms for their membrane recruitment and mobilization (5). Analyses on wild type and mutant 2C CD8 T cells revealed that RasGRP1 is likely crucial for sustaining TCR-induced ERK activation (Fig. 4.4*A*) and that such a prolonged signal may be required for recruitment of naïve T cells into cell cycle (Fig. 4.1*A*, 4.1*B*). Since Ras can also be activated through the IL-2 receptor (29, 30), we also sought to investigate whether IL-2 signaling induced Ras activation via RasGRP1. Consistent with the finding that cytokine receptors activate the Ras/MAPK pathway after their phosphorylation through binding of Shc adaptor protein and subsequently, the recruitment of Grb2/Sos (48), we found that IL-2 stimulations of RasGRP1$^{-/-}$ 2C T cell blasts induced Ras activation at levels resembling wild type (Fig. 4.4*B*) and restored proliferation to immobilized anti-TCR Ab (Fig. 4.1*A*, 4.1*B*). Therefore, we suspect that RasGRP1 does not directly mediate Ras activation through the IL-2 receptor. However, our findings suggest that RasGRP1 may indirectly affect IL-2 receptor

signaling by influencing CD25 expression and IL-2 secretion (Fig. 4.1C, 4.1D). Recent work in the human Jurkat T cell line suggests that an unusual type of cooperation exists between RasGRP1 and Sos whereby RasGRP1-derived Ras-GTP boosts Sos's activity by binding an allosteric pocket on the molecule, creating a positive feedback loop (49). Therefore, it has been hypothesized that RasGRP1 and Sos collaborate to induce optimal Ras activation following low-level physiological stimulation (49). Fitting this hypothesis, our results demonstrate that RasGRP1 is essential for the exquisite sensitivity of T cells in responding to a very limited number of peptide/MHC complexes on the surface of an APC.

The discovery of subcellular compartmentalization of Ras activation has garnered great attention and revealed another dimension of complexity to cellular signaling and possible cellular responses (5, 50). Although conventional wisdom had placed the site of Ras activity at the plasma membrane (PM), recent studies suggest that Ras activation also occurs at the Golgi in a PLCγ-dependent fashion by way of RasGRP1 (51-53). The rationale for alternate cellular sites of Ras activity may be to subject it to differential regulation and/or serve to pair it with a unique subset of effectors. Comparison of Golgi versus PM has found that Ras activation at the former locale appears to have delayed or sustained kinetics relative to the latter (51-53). A possible mechanism for prolonged Ras signaling at the Golgi may be through calcium signals, serving to recruit the RasGAP CAPRI to the PM to inactivate Ras at this site while at the same time mediating the translocation of RasGRP1 to the Golgi (51, 54). Recently, subcellular compartmentalization of the Ras/RasGRP1/ERK pathway has been suggested to be pivotal in developing thymocytes undergoing selection (55). Using OT-I TCR transgenic pre-selection CD4$^+$CD8$^+$ (DP) thymocytes and specific peptides spanning the boundary of positive and negative selection, it was found that negatively selecting peptides recruited Ras, Raf-1 and RasGRP1 to the PM whereas these molecules co-localized to the Golgi when thymocytes were stimulated with a peptide mediating positive selection (55). Collectively, our findings hint at the possibility that subcellular compartmentalization of Ras/RasGRP1/ERK pathway may also be critical for the commitment of naïve CD8 T cells to Ag-induced developmental programming.

4.5   Acknowledgments

4.6   Figures

Figure 4.1 RasGRP1$^{-/-}$ 2C CD8 T cells exhibit defects in TCR-induced proliferation, early activation events and IL-2 production. *A*, Purified wild type and RasGRP1$^{-/-}$ 2C CD8 T cells were cultured on either anti-TCR or anti-TCR and anti-CD28 antibody coated wells in the presence or absence of exogenous IL-2. After 3 days of in vitro culture, plates were pulsed with [^{3}H]-thymidine for a 6 h period and subsequently, incorporated radioactivity measured. *B*, CFSE-labeled RasGRP1$^{-/-}$ 2C CD8 T cells were cultured in the same manner as in *A*. Proliferation was measured 3 days post-stimulation by flow cytometry. RasGRP1-deficient 2C CD8 T cells exhibit diminished IL-2 production and decreased activation marker expression. *C*, Naïve wild type and RasGRP1$^{-/-}$ 2C CD8 T cells (Thy1.2^{+}) were cultured with either anti-TCR Ab or SIY-pulsed splenocytes (Thy1.1^{+}). After 16 h culture, induction of activation markers was assessed on stimulated T cells (CD8^{+} Thy1.2^{+}; filled histograms). Dotted, thin-line histograms represent marker expression on unstimulated (naïve) cells. *D*, Naïve wild type and RasGRP1$^{-/-}$ 2C CD8 T cells were cultured with SIY-pulsed splenocytes (Thy1.1^{+}) for the indicated times and the frequency of IL-2-positive 2C T cells (CD8^{+} Thy1.2^{+}) determined by flow cytometry.
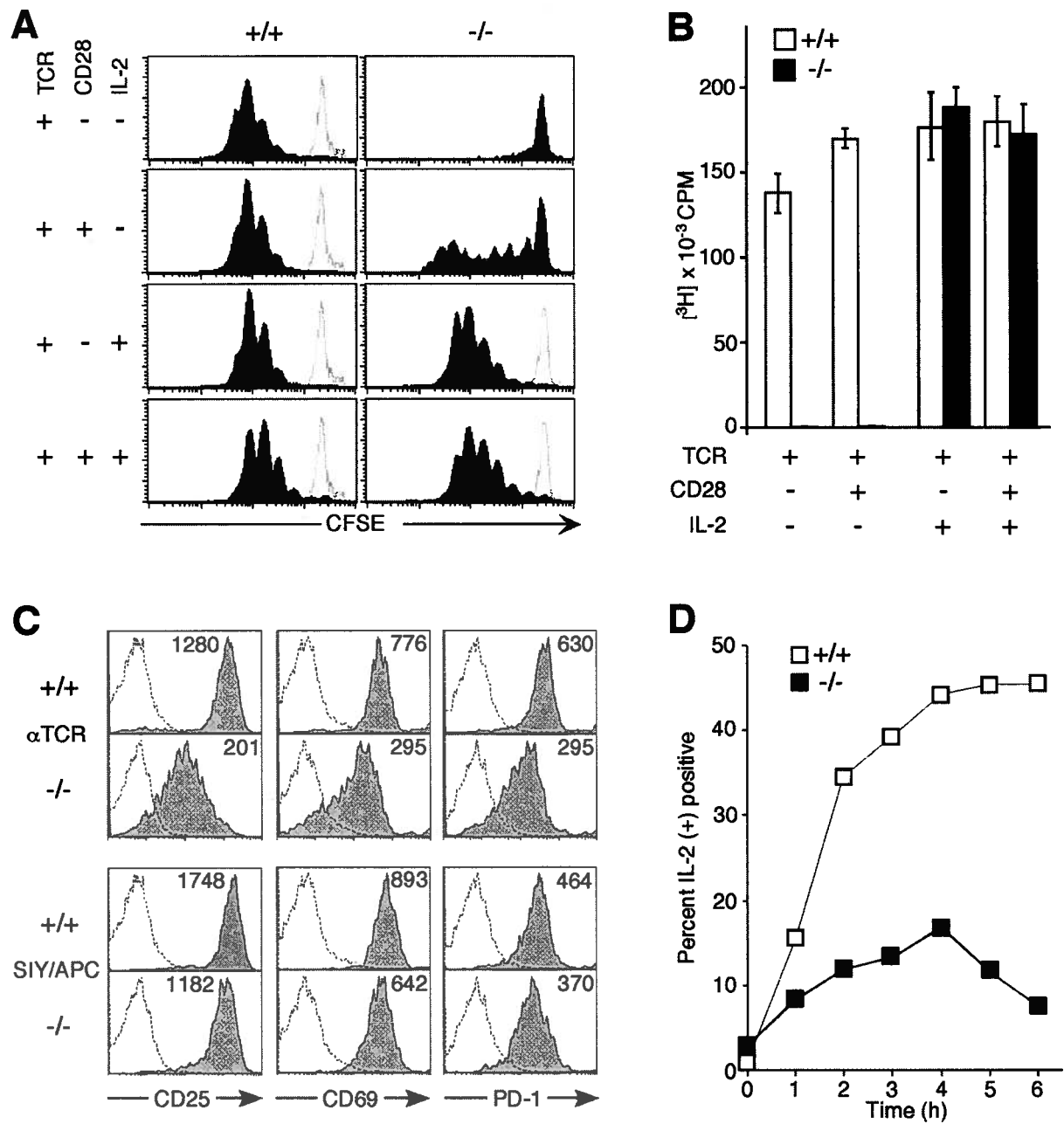
Figure 4.2 RasGRP1-deficient 2C CD8 T cells acquire an impaired effector phenotype after antigenic stimulation. Wild type and RasGRP1$^{-/-}$ peripheral 2C CD8 T cells (Thy1.2^{+}) were purified using and stimulated with the 2C TCR agonist peptide SIY and irradiated splenocytes (B6.PL, Thy1.1^{+}) either in absence (*A*) or presence of exogenous IL-2 (*B*). After 4 days of in vitro culture, 2C T cell effectors (filled histograms) were analyzed for surface markers (CD25, CD44 and CD62L) and intracellular Granzyme B expression by electronically gating on Thy1.2^{+} CD8^{+} events. For comparison sake, naïve 2C T cell profiles are also shown (thin, gray line). *C*, 2C T cell effectors generated by exposure to SIY peptide and exogenous IL-2 (as in *B*) were stimulated with SIY-coated splenocytes for 5 h and cytokine production assessed by intracellular flow cytometry.

Figure 4.3 RasGRP1 regulates the antigen dose necessary to recruit naïve T cells to initiate proliferation and effector cell formation. CFSE-labeled wild type and RasGRP1$^{-/-}$ 2C CD8 T cells (50,000; Thy1.2$^+$) were cultured in vitro for 4 days with one million (feeder; Thy1.1$^+$) CD8-depleted splenocytes and limiting numbers of SIY-coated APCs (0, 200, 1000, 2000, 5000 or 10000; shown on left). *A*, After acquisition of 150,000 total events, CFSE fluorescence of 2C T cells was determined by electronically gating on Thy1.2$^+$CD8$^+$ cells. The maximum scale for each histogram is shown vertically at the left. At the top right of each histogram, the frequency of 2C CD8 T cells that have not divided is indicated. *B*, The expression of CD44 as a function of cell division is shown for wild type and RasGRP1$^{-/-}$ 2C CD8 T cells. *C*, To assess the frequency of 2C T cells capable of producing IFN-γ, one million SIY-coated splenocytes (Thy1.1$^+$) were added to each well and cultures were incubated for an additional 4 h. Within each density plot, the frequency of cells residing within each quadrant is indicated.
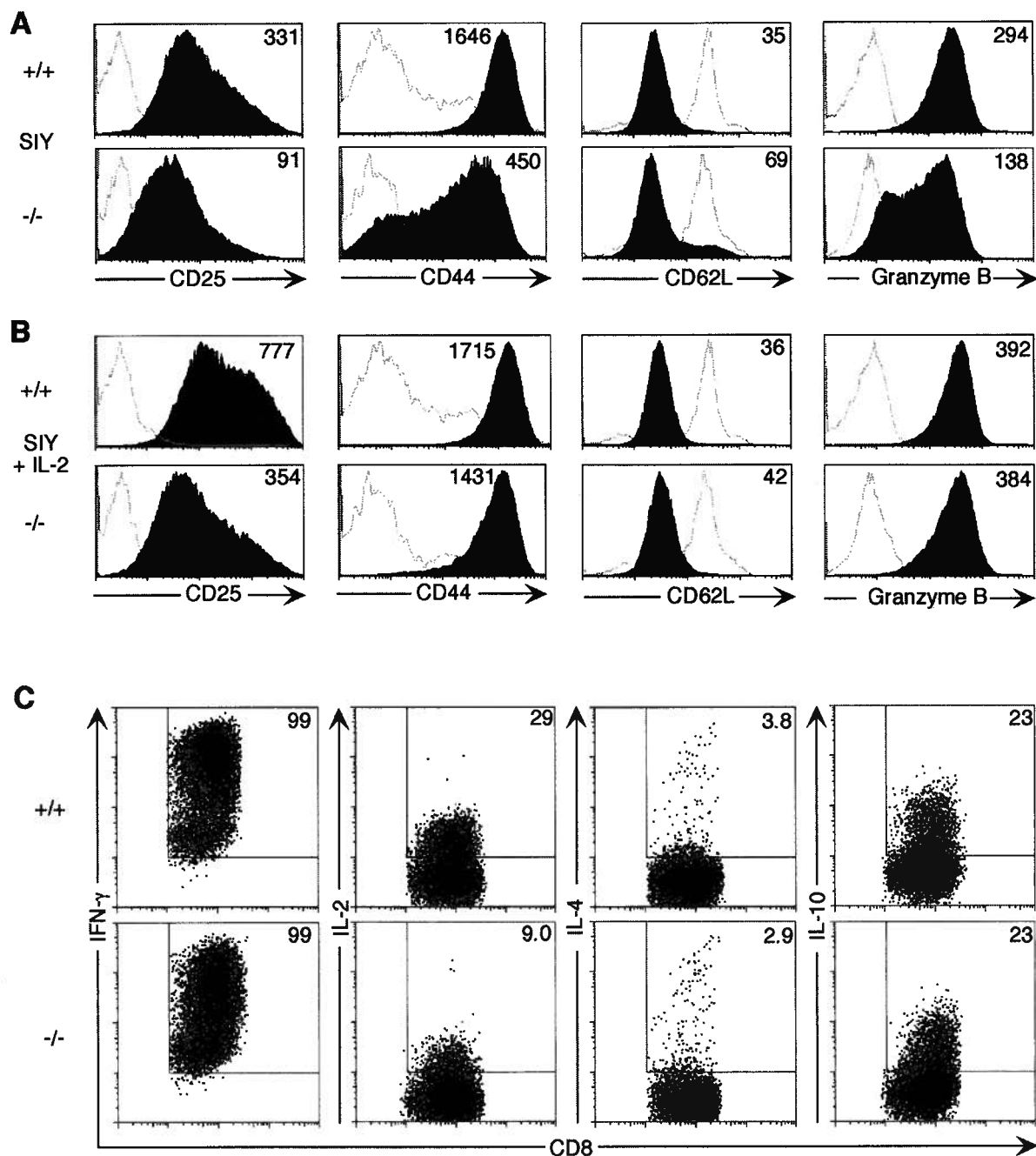
Figure 4.4 RasGRP1$^{-/-}$ 2C CD8 T cells display transient ERK activation upon TCR ligation but normal ERK phosphorylation after IL-2 stimulation. *A*, Wild type and RasGRP1$^{-/-}$ 2C CD8 T cells were stimulated with anti-TCR antibodies for the indicated times (min). Whole lysates were immunoblotted with anti-phospho-ERK, anti-ERK2, anti-phospho-LAT and anti-phospho-ZAP-70 antibodies. *B*, After 4 h serum starvation, wild type and RasGRP1$^{-/-}$ 2C CD8 T cell effectors were either left untreated (NS; non-stimulated) or incubated with IL-2 or PMA for 10 min. Ras activation was assessed by Ras-GTP pulldown assay using Raf-GST and Western blotting precipitates with an anti-Ras specific antibody. Whole lysates were probed for with anti-pan-Ras, anti-phospho-ERK and anti-ERK antibodies. Both long and short exposures of immunoblots are shown.

Figure 4.5 RasGRP1-deficient T cells exhibit a reduced rate of proliferation and diminished cytokine production upon antigen stimulation in vivo. *A*, A recombinant strain of *Listeria monocytogenes*, named rLM-SIY, was engineered to express the 2C TCR agonist peptide SIYRYYGL. To ensure proper cleavage, SIYRYYGL was flanked by 5' and 3' sequences known to be cut in murine cells, liberating naturally occurring peptides (SIINFEKL from

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ovalbumin [OVA] and LSPFPFDL from α-ketoglutaraldehyde dehydrogenase [α–KG]). *B*, CFSE-labeled wild type Thy1.2^+ 2C CD8 T cells (~1 x 10^6) were adoptively transferred into naïve B6 (Thy1.1^+) mice, which were then left untreated or infected with either rLM-SIY or wild type *Listeria monocytogenes*. One week post-infection, proliferation history of donor 2C T cells was assessed. *C-I*, An equal 50:50 mixture (10,000 cells of each genotype) of wild-type (Thy1.1^+/1.2^+) and mutant 2C T cells (Thy1.2^+/1.2^+) was generated, labeled with CFSE and co-adoptively transferred into naïve B6 (Thy1.1^+/1.1^+) mice one day prior to infection with rLM-SIY. Spleens were recovered from infected mice one-week post-infection, donor T cells (Thy1.2^+) detected with anti-Thy1.2 Ab and their genotype identified by reactivity to anti-Thy1.1 Ab. *D*, The frequency of wild type versus mutant 2C T cells are presented. *E*, Numbers of 2C T cells recovered from spleens. *F*, The majority of wild type and RasGRP1$^{-/-}$ 2C T cells lose their CFSE-endowed fluorescence seven days post-infection with rLM-SIY (filled histogram). Open histogram represents CFSE fluorescence of non-dividing 2C T cells residing in uninfected B6 mice. *G*, A reduced frequency of RasGRP1-deficient 2C T cells possesses the proliferation-associated nuclear antigen Ki-67. *H*, Wild type and RasGRP1-deficient 2C T cells (filled histogram) up-regulate CD44 during Ag-driven T cell expansion. Open histogram represents CD44 staining on naïve 2C T cells. *I*, Splenocytes were incubated for 5 h with SIY peptide and donor T cell cytokine production assessed by FACS. All error bars represent the SD.
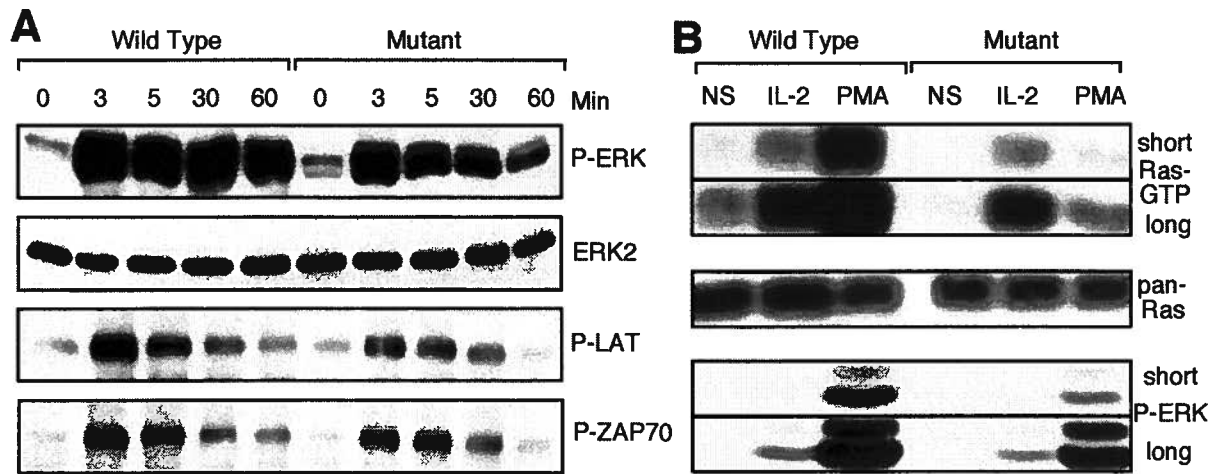
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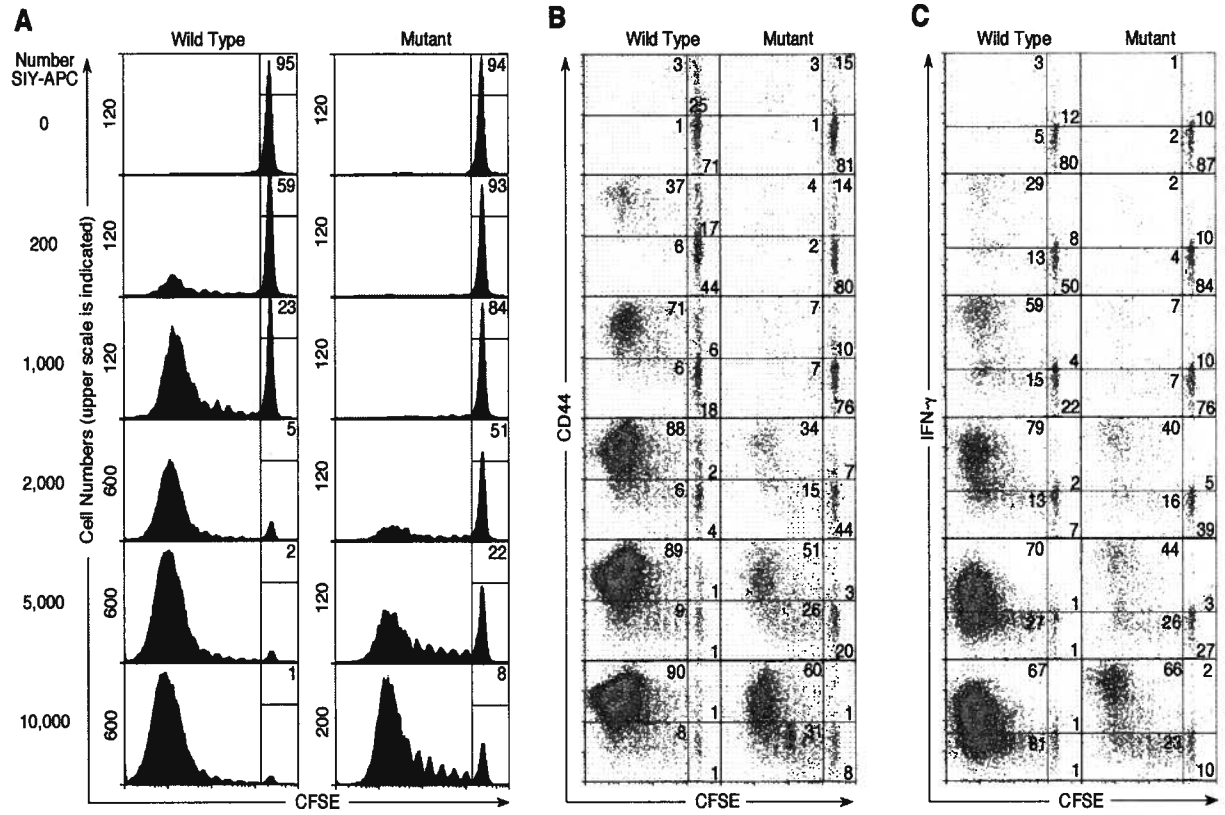
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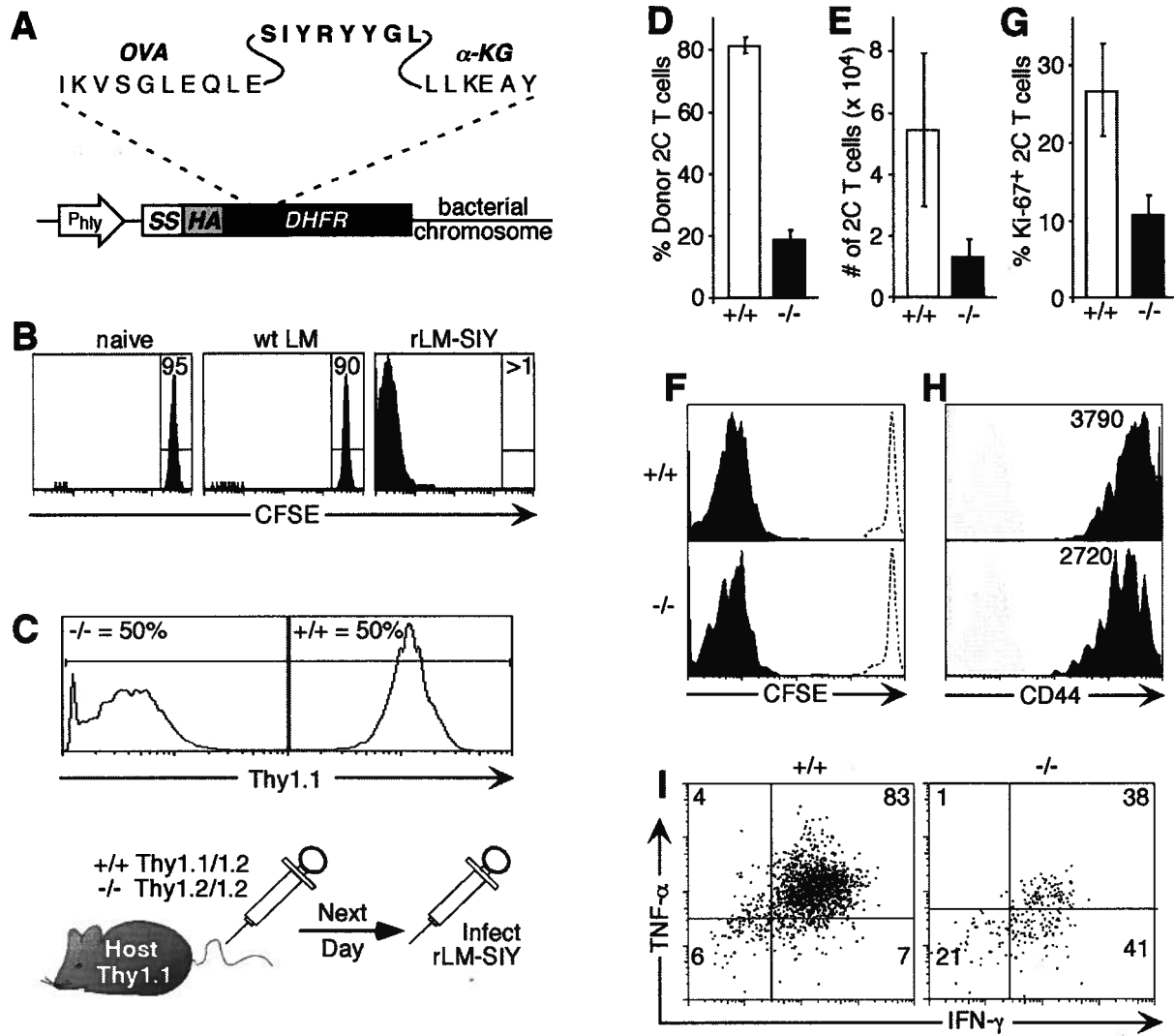
Figure 4.5 RasGRP1-deficient T cells exhibit a reduced rate of proliferation and diminished cytokine production upon antigen stimulation in vivo.

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CHAPTER 5 PREFERENTIAL DEVELOPMENT OF CD4 AND CD8 T REGULATORY CELLS IN RASGRP1-DEFICIENT MICE[1]

5.1 Introduction

During the process of thymic education, only thymocytes expressing TCRs capable of distinguishing between self and nonself are selected for survival and differentiate into mature T cells. According to the strength of signal hypothesis, thymocytes expressing TCRs with weak or modest affinity for self Ags undergo positive selection and become mature T cells. By contrast, developing T cells expressing high affinity receptors for self Ags are clonally deleted, a process referred to as negative selection. However, negative selection is imperfect because autoreactive lymphocytes escape clonal deletion and can be detected within the peripheral lymphocyte compartment (1, 2). To keep these autoreactive T cell clones in check, a dedicated lineage of T regulatory (Treg) cells functions as an important fail-safe mechanism to prevent catastrophic effect of unchecked immune responses (3, 4). Three main cell types have been considered as potential Treg cell subsets, as follows: $CD4^+CD25^+$ Treg cells (5, 6), $CD8\alpha\alpha$ intestinal epithelial lymphocytes (7), and NKT cells (3). All are thought to be induced by high affinity interactions between self peptide:MHC with the TCR on developing T cells in the thymus (1, 8). In addition to these three subsets, recent studies indicate normal mice also possess a population of $CD8^+CD44^{high}CD122^+$ T cells that function as regulatory cells and can perform roles distinct from CD4 Treg cells in suppressing T cell activation (9-12).

Of all the lineages of Treg cells, the $CD4^+CD25^+$ subset has been the most extensively characterized. $CD4^+CD25^+$ T cells develop naturally in normal individuals and are readily detectable in the thymus and secondary lymphoid organs in mice, rats, and humans, where they make up ~2–10% of the total $CD4^+$ cells (13). In addition to CD25, CD4 Treg cells also

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express high levels of CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor-related protein (14). However, the most distinguishing feature of CD4 Treg cells is their expression of Foxp3, a member of the forkhead family of transcription factor (8). The expression of Foxp3 is both necessary and sufficient for the development and function of CD4 Treg cells (5, 15). Additionally, studies have found the TCR repertoire of CD25$^+$ CD4 T cells is highly self-reactive, a conclusion supported by the observation that when T cells transduced with TCR genes derived from CD25$^+$, but CD25$^-$ CD4 T cells, they rapidly expand in lymphopenic hosts and induce autoimmune disease (16). Furthermore, studies have shown that TCR transgenic T cells can undergo conversion into Treg cells following exposure to either cognate Ag or peptide-agonist ligands on dendritic cells, respectively (17-19).

Although a vast amount of literature exists on the developmental biology, function, and TCR repertoires of CD4$^+$ Treg cells, details about CD8 Treg cells are only beginning to emerge. Studies on CD122-deficient mice have implicated the existence of CD8 Treg cells that function to preferentially regulate the immune functions of CD8 T cells. CD122-deficient mice exhibit severe hyperimmunity (9), which is associated with the expansion of abnormally activated T cells (10). However, the transfer of highly purified CD8$^+$CD122$^+$ T cells, from wild-type mice to CD122-deficient neonates, prevented the aberrant T cell phenotype from developing in the treated mice (11). Moreover, RAG-2$^{-/-}$ mice that received wild-type CD8$^+$CD122$^-$ cells die within 10 wk after cell transfer, suggesting CD8$^+$CD122$^-$ T cells become dangerously activated in the absence of CD8$^+$CD122$^+$ T cells (11). Follow-up studies indicate the suppressor activity of CD8$^+$CD122$^+$ T cells was mediated by IL-10 (12). Collectively, these results suggest CD8$^+$CD122$^+$ T cells contain novel populations that can function as Treg cells.

RasGRP1 is one of two Ras-guanyl-nucleotide exchange factors that link TCR signal transduction to Ras and MAPK activation (20, 21). Upon TCR stimulation, RasGRP1 mobilizes to the Golgi membrane by binding the phospholipase C-γ1 product diacylglycerol (DAG) through its C1 domain (22-24). Thymocytes from RasGRP1$^{-/-}$ mice are defective in TCR- and DAG-induced activation of Ras-ERK signaling (20). Furthermore, mutant mice

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exhibit a defect in positive selection, as evidenced by reduced numbers of single-positive (SP) thymocytes and T cell lymphopenia (25). By contrast, strong TCR signals responsible for negative selection and the induction of Ag-driven growth appear to be RasGRP1 independent (21). Because most Treg cells characterized to date express high affinity TCRs for self Ags, our objectives with regard to the analysis of the role of RasGRP1 in the development and function of Treg cells are 2-fold, as follows: 1) determine the role of RasGRP1 in the intrathymic development of CD4 and CD8 Treg cells, and 2) determine the role of RasGRP1 in peripheral homeostasis and function of CD4 and CD8 Treg cells. Our results indicate that although intrathymic development of $CD4^+Foxp3^+$ cells is severely impaired in the absence of RasGRP1, there exist an elevated frequency and large numbers of Foxp3-expressing CD4 Treg cells in the peripheral lymphoid tissues of mutant mice. This may be attributable to both the massive expansion of $RasGRP1^{-/-}$ $Foxp3^+CD4^+$ T cells and increased death rate of mutant $Foxp3^-CD4^+$ T cells. Additionally, $RasGRP1^{-/-}$ CD4 Treg cells were found to be functional because they could suppress the proliferation of wild-type $CD25^-$ $CD4^+$ T cells in vitro. In contrast to the CD4 Treg cells, the development of CD8 Treg cells is not affected by RasGRP1 loss. However, the suppressor function of CD8 Treg cells is dependent on RasGRP1. The implications of these findings on peripheral T cell homeostasis and the development of autoimmune diseases in $RasGRP1^{-/-}$ mice are discussed.

5.2   Materials and methods

Mice

C57BL/6J (B6) mice were obtained from The Jackson Laboratory. $RasGRP1^{-/-}$ breeder mice (20) were provided by J. Stone (University of Alberta, Edmonton, Alberta, Canada) and bred onto B6 background for more than seven generations. Mice 6–12 wk of age were used for the experiments described. All studies followed guidelines set by the Animal Care Committee at the University of British Columbia in conjunction with the Canadian Council on Animal Care.

Flow cytometry

Abs against CD4 (GK1.5), CD8α (53-6.7), CD8β (53.58), TCRβ (H57-597), CD3ε (2C11), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD94 (18d3), CD122 (5H4), CD127 (A7R34), Foxp3 (FJK-16s), NK1.1 (PK136), NKG2AB6 (16a11), NKG2D (CX5), Thy1.1 (HIS51), Thy1.2 (53-2.1), and IL-10 (JES5-16E3) were purchased from eBioscience. Annexin V-PE and Abs against 2B4, CD5 (53-7.3), Ly6C (AL-21), and Ki-67 (B56) were purchased from BD Biosciences. Foxp3 staining was performed following the protocol recommended on eBioscience web site (www.ebioscience.com/ebioscience/specs/ antibody_12/ 12-5773.htm). For anti-IL-10 staining, cells were stained for surface markers, washed, and fixed with 2% paraformaldehyde and 0.2% Tween 20 in PBS for 20 min on ice, followed by washing with PBS. Fixed cells were then stained with anti-IL-10 Ab in 0.2% Tween 20/PBS for 30 min on ice. For anti-Ki-67 staining (26), cells were fixed and permeabilized using the same protocol as for Foxp3 staining, and incubated with anti-Ki-67 Ab for 30 min at 4°C. Data were acquired using either FACScan/CellQuest software or LSRII/FACSDiva software (BD Biosciences). Data were analyzed with FlowJo (Tree Star) software.

CD4 T cell proliferation and suppression assays

Cell sorting with the FACSAria flow cytometer (BD Biosciences) was used to purify $CD4^+CD25^-$ or $CD4^+CD25^+$ cells. The purities of sorted $CD4^+CD25^+$ wild-type or mutant suppressor cells used for experimental studies are 94.2 and 95.4%, respectively. Proliferation and suppression assays were performed, as described (27). Briefly, for proliferation assays, T cells (2×10^4 cells/well) were stimulated for 72 h with titrated amounts of Con A in the presence of T cell-depleted, irradiated APCs (8×10^4 per well) in 96-well round-bottom plates, and pulsed with 1 μCi per well of $[^3H]$thymidine for the final 8 h. Suppression assays were performed under the same conditions using 2×10^4 $CD4^+CD25^-$ T cells as responders, 8×10^4 irradiated APCs, and a 1:2 titration of the indicated suppressor T cell population ($CD4^+CD25^+$) at a starting concentration of 4×10^4 cells/well in the presence of Con A at 2 μg/ml final concentration. All data are shown as mean $[^3H]$ thymidine incorporation in triplicate cultures.

Adoptive transfer experiment

Cell suspension was prepared from Thy1.1$^+$ B6 or Thy1.2$^+$ RasGRP1$^{-/-}$ animals. Cells were then stained with PE-conjugated anti-CD4 (GK1.5) and allophycocyanin-conjugated anti-CD25 (PC61.5) Abs and sorted for the CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ population (purity > 95%), respectively, using FACSAria. Sorted CD4$^+$CD25$^+$ (2 x 10^5) or CD4$^+$CD25$^-$ (1 x 10^6) Thy1.1$^+$ wild-type cells were adoptively transferred into naive B6 Thy1.2$^+$ wild-type or RasGRP1$^{-/-}$ hosts. Similarly, sorted CD4$^+$CD25$^+$ (2 x 10^5) or CD4$^+$CD25$^-$ (1 x 10^6) Thy1.2$^+$ RasGRP1$^{-/-}$ cells were adoptively transferred into naive Thy1.1$^+$ wild-type or RasGRP1$^{-/-}$ hosts. Spleens of recipients were recovered 3 wk posttransfer, and frequencies of donor cells of the indicated cell surface phenotype were quantified by flow cytometry.

CFSE labeling

Purified CD8$^+$ T cells (1 x 10^7/ml) were labeled with 1 μM CFSE (Molecular Probes) in PBS for 10 min at room temperature. After stopping the reaction with the addition of an equal volume of FCS, cells were washed four times with complete medium.

CD8$^+$ T cell purification and direct ex vivo assays

Single-cell suspensions from lymph nodes and spleens of mice were prepared and then treated with biotinylated anti-CD8 (53-6.7) mAb, followed by positive selection using MiniMACS system (Miltenyi Biotec), according to the manufacturer's specifications. The resulting cells were > 95% pure CD8$^+$ TCR$^+$ T cells. For cytokine proliferation assay, purified wild-type or RasGRP1$^{-/-}$ CD8$^+$ cells were CFSE labeled and cultured in IL-2 (200 U/ml) or IL-15 (100 ng/ml). Proliferation of gated CD8$^+$ cells was then analyzed by FACS at 72 h. For IFN-γ production assay, 2 x 10^6 purified wild-type or RasGRP1$^{-/-}$ CD8$^+$ cells were stimulated with PMA (10 ng/ml) and ionomycin (100 ng/ml) in medium containing Golgi-plug (BD Pharmingen) for 5 h at 37°C. Following stimulation, cells were stained for surface Ags and then stained intracellularly for IFN-γ.

CD8$^+$ T cell cytokine and suppression assays

CD8$^+$ T cells from B6 wild-type and RasGRP1$^{-/-}$ mice (Thy1.2$^+$) were purified, as described above. Cells were then stained with PE-conjugated anti-CD122 (5H4) and PE-Cy5-conjugated anti-CD8 (53-6.7) Abs and electronically sorted using FACSAria flow cytometer. Sorted wild-type CD8$^+$CD122$^-$, CD8$^+$CD122$^+$, or RasGRP1$^{-/-}$ CD8$^+$CD122$^+$ cells (5 x 10^4 per well) were stimulated by plate-bound anti-CD3 (10 µg/ml, 1 h incubation at 37°C, washed twice) and 10 µg/ml soluble anti-CD28 plus 100 U/ml IL-2 (final concentration) for 72 h in 24-well flat-bottom plates. For assessment of IL-10 production by the cultured CD8$^+$ cells, Golgi-plug was added to the cell cultures during the last 24 h of culture and IL-10 production was evaluated by intracellular staining. For suppression assays, sorted wild-type CD8$^+$CD122$^-$, CD8$^+$CD122$^+$, or RasGRP1$^{-/-}$ CD8$^+$CD122$^+$ cells (2 x 10^6) were stimulated using the same condition above. Three days later, activated suppressor cells from the indicated population were added at various suppressor to responder ratios to 6.5 x 10^5 CFSE-labeled sorted B6 Thy1.1 CD8$^+$CD122$^-$ cells, and cultured with anti-CD3 and anti-CD28 plus IL-2 (same stimulation condition as above) in 96-well flat-bottom plate. Two days later, the proliferation of Thy1.1$^+$ CD8$^+$CD122$^-$ wild-type cells was assessed by CFSE dilution analysis.

5.3 Results

Defective thymic differentiation of CD4$^+$Foxp3$^+$ T cells in RasGRP1$^{-/-}$ mice

Because Foxp3 is a unique marker for naturally arising CD4$^+$CD25$^+$ Treg cells, we first examined the development of Foxp3$^+$ CD4 SP thymocytes in the thymus of RasGRP1$^{-/-}$ mice. We found that although the proportion of CD4 SP thymocytes in mutant mice is 14-fold lower than in wild-type mice (0.44 vs 6.29%), the proportion of CD4 SP thymocytes that are also CD25$^+$Foxp3$^+$ in mutant mice is fairly similar to wild-type (2.02 vs 3.08%) (Fig. 5.1*A*). However, cell number comparison of Foxp3$^+$ or Foxp3$^-$ lineages in CD4 SP thymocytes between wild-type and RasGRP1$^{-/-}$ mice revealed that both CD4 Treg (Foxp3$^+$) and non-Treg (Foxp3$^-$) populations are severely affected by RasGRP1 deficiency, with both populations accounting for 4.5 and 4.2% of wild-type numbers, respectively (Fig. 5.1*B*). Next, we

examined the differentiation of Foxp3-expressing precursors in RasGRP1$^{-/-}$ thymus by evaluating the distribution of Foxp3-expressing thymocytes among the thymocyte subpopulations as defined by CD4 and CD8 expression. A recent report suggests that although Foxp3 induction can occur at the double-positive stage, it is preferentially induced at the CD4 SP stage during the development (28). We found that there is a significant increase in the proportion of CD4$^+$CD8$^+$Foxp3$^+$ thymocytes in RasGRP1$^{-/-}$ mice relative to wild type (51 vs 13%) (Fig. 5.1C). This result is consistent with the notion that there is a block in transition from Foxp3$^+$ double-positive to Foxp3$^+$ CD4 SP thymocytes in RasGRP1$^{-/-}$ mice. Alternatively, this observation could be due to a defect in Foxp3 up-regulation by RasGRP1$^{-/-}$ CD4 SP thymocytes. Interestingly, RasGRP1$^{-/-}$ Foxp3-expressing cells within the CD4 SP population display altered expression of TCRβ, CD3ε, CD5, and CD69 (Fig. 5.1D). The distribution of TCR and CD5 in the Foxp3$^+$ CD4 SP population is bimodal, with a minor population that expresses fairly normal levels of TCR and CD5 and a major population that expresses very low levels of these molecules (Fig. 5.1D). If only the TCR$^+$ population is representative of CD4 Treg cells, this would imply that the development of CD4 Treg cells is more greatly affected by the RasGRP1$^{-/-}$ mutation than implied by the analysis of Foxp3$^+$CD4 SP thymocytes. In summary, these observations indicate that naturally arising CD4$^+$Foxp3$^+$ Treg cell development in the thymus is severely impaired in RasGRP1$^{-/-}$ mice.

Favored expansion and enhanced suppressive function of peripheral CD4$^+$Foxp3$^+$ T cells in RasGRP1$^{-/-}$ mice

In contrast to the impaired development of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in the thymus, we found that the spleen of RasGRP1$^{-/-}$ mice possessed a markedly increased proportion of CD25$^+$Foxp3$^+$ cells within the CD4 population as compared with wild type (24 vs 8.6%) (Fig. 5.2A). Furthermore, there is a preferential increase in splenic CD4$^+$Foxp3$^+$ cell number relative to the CD4$^+$Foxp3$^-$ population in RasGRP1$^{-/-}$ mice (32 vs 14% of wild-type numbers; Fig. 5.2B). We noted in Fig. 5.1D that the majority of CD4$^+$Foxp3$^+$ thymocytes express very low levels of TCR and CD5. In striking contrast to the thymus, RasGRP1$^{-/-}$ peripheral CD4 Treg cells express near wild-type levels of TCRβ, CD3ε, and CD5 (Fig. 5.2C). Interestingly, both Foxp3$^+$ and Foxp3$^-$ CD4 T cells in RasGRP1$^{-/-}$ mice display signs of acute activation

(CD44^{high}, CD62L^{low}, CD69^{high}), with a higher proportion of Foxp3$^+$ CD4 cells from RasGRP1$^{-/-}$ mice expressing increased levels of acute activation markers (Fig. 5.2C).

The huge increase in peripheral Foxp3$^+$ CD4 T cell numbers in RasGRP1$^{-/-}$ mice could be due to the preferential expansion of these cells and/or the preferential death of Foxp3$^-$ CD4 T cells in mutant mice. To test this hypothesis, we performed direct ex vivo staining of Foxp3$^+$ and Foxp3$^-$ CD4 T cells to determine the proportion of proliferating and dying cells in the wild-type and mutant animals (Fig. 5.3A). We found that both CD4 Foxp3$^-$ and Foxp3$^+$ cells in RasGRP1$^{-/-}$ mice exhibit an elevated frequency of cells bearing the proliferation-associated nuclear Ag Ki-67 (26) as compared with wild-type animals (Foxp3$^-$, 24.5 vs 7.2%; Foxp3$^+$, 31.1 vs 15.6%) (Fig. 5.3A, middle panel). Costaining of the apoptotic marker annexin V and Foxp3 revealed that Foxp3$^+$ and Foxp3$^-$ CD4 T cells in RasGRP1$^{-/-}$ mice undergo different rates of cell death. It is noted that mutant Foxp3$^-$ CD4 T cells undergo a higher degree of cell death relative to their wild-type counterpart (13% annexin V$^+$ vs 4%; Fig. 5.3A). By contrast, there is a dramatic decrease in the proportion of mutant CD4$^+$Foxp3$^+$ cells that are annexin V$^+$, as compared with wild-type mice (7.4% annexin V$^+$ vs 22%; Fig. 5.3A). To provide an explanation for the increased numbers/frequency of Foxp3$^+$ T cells in the mutant periphery relative to the thymus, we performed a series of adoptive transfer experiments to distinguish between the cell intrinsic and extrinsic effects of the RasGRP1$^{-/-}$ mutation on the peripheral expansion of Foxp3$^+$ CD4 T cells (Fig. 5.3B). First, sorted wild-type CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells (Thy1.1$^+$) were transferred into either wild-type or mutant Thy1.2 hosts, respectively (Fig. 5.3B, top panel). Three weeks postadoptive transfer, significant frequencies of donor CD4 T cells were detected only in RasGRP1$^{-/-}$, but not wild-type recipients. This result suggests that it is the peripheral environment in RasGRP1$^{-/-}$ mice that favors the expansion of both wild-type CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells. This is most likely a consequence of lymphopenia associated with RasGRP1$^{-/-}$ mice. We also determined whether there is increase conversion of donor CD4$^+$CD25$^-$ into CD4$^+$CD25$^+$ T cells in RasGRP1$^{-/-}$ mice. We found that there is no conversion of either wild-type or mutant CD4$^+$CD25$^-$ into CD4$^+$CD25$^+$ T cells in wild-type hosts (Fig. 5.3C). Interestingly, 6.8% of donor wild-type CD4$^+$CD25$^-$ cells developed into CD4$^+$CD25$^+$ cells in RasGRP1$^{-/-}$ host, and these CD4$^+$ CD25$^+$ cells were also Foxp3$^+$ (Fig. 5.3C). Similarly, 4.9% of donor mutant CD4$^+$CD25$^-$ T

cells developed into CD4$^+$CD25$^+$Foxp3$^+$ cells in mutant hosts (Fig. 5.3C). Collectively, these findings suggest that the elevated frequency of CD4$^+$CD25$^+$Foxp3$^+$ T cells in RasGRP1$^{-/-}$ mice is most likely due to a combination of decreased cell death and a cellular environment that favors their peripheral expansion.

To evaluate the function of CD4$^+$Foxp3$^+$ cells in the periphery of RasGRP1$^{-/-}$ mice, we purified both wild-type and mutant peripheral CD4$^+$CD25$^+$ T cells using FACS and compared their ability to suppress the proliferation of Con A-activated wild-type CD4$^+$CD25$^-$ cells in vitro (Fig. 5.4). On a per cell basis, CD4$^+$CD25$^+$ T cells from RasGRP1$^{-/-}$ mice were more suppressive compared with wild-type CD4$^+$CD25$^+$ cells. This increased suppressor activity of CD4$^+$CD25$^+$ T cells from RasGRP1$^{-/-}$ mice correlates with the increased expression of CD44 and CD69 on these cells compared with their wild-type counterpart (Fig. 5.2C). In summary, these studies demonstrate that there is preferential accumulation of functionally active CD4$^+$Foxp3$^+$ Treg cells in the periphery of RasGRP1$^{-/-}$ mice. They also indicate that the suppressor function of CD4$^+$Foxp3$^+$ Treg cells is independent of RasGRP1.

Preferential development of memory phenotype CD8$^+$ T cells in the thymus and periphery of RasGRP1$^{-/-}$ mice

The Tec family tyrosine kinases Itk and Rlk are required for full TCR-induced activation of phospholipase C-γ1, Ca2$^+$ mobilization, and ERK activation (29, 30). Itk and Rlk perform important functions during T cell development, and, in particular, have been implicated in setting the thresholds of positive and negative selection (31-33). Interestingly, although Itk and Rlk are critical for the development of conventional CD8 T cells, Itk$^{-/-}$ and Rlk$^{-/-}$Itk$^{-/-}$ mice possess a large population of memory phenotype CD8 T cells that bear striking similarity to lineages of innate-like lymphocytes (34-36). Because RasGRP1$^{-/-}$ mice are defective in TCR- and DAG-induced activation of Ras-ERK signaling (20) and play a vital role in the positive selection of conventional T cells (20, 21), we question whether RasGRP1 is also dispensable for the development of these innate-like CD8 T cells that bear a memory phenotype. We found that there is a large increase in the proportion of CD44^{high}CD122$^+$ CD8 SP thymocytes in RasGRP1$^{-/-}$ mice relative to wild type (Fig. 5.5A, top panel). In addition, these CD122$^+$ cells in the RasGRP1$^{-/-}$ thymus are CD24^{low}, suggesting their mature status

(Fig. 5.5*A*, middle panel). There is also a slight increase in the total number of CD44^{high} CD122$^+$ cells in the thymus of RasGRP1$^{-/-}$ mice (115% of wild type) (Fig. 5.5*D*). All of these observations contrast with the greatly reduced numbers and immature phenotype of CD4$^+$Foxp3$^+$ cells found in the thymus of RasGRP1$^{-/-}$ mice (Fig. 5.1*B*). Consistent with the thymic data for this CD8 subset, there is ~5-fold increase in the proportion of CD8$^+$CD44^{high} CD122$^+$ cells in the spleen of RasGRP1$^{-/-}$ mice relative to wild type (Fig. 5.5*A*, bottom panel). We also found that the proportions of CD8 SP thymocytes and splenocytes that are NK1.1$^+$ are greatly increased in the thymus and spleen of mutant mice relative to the wild-type counterpart (Fig. 5.5*B*). In absolute numbers, there is a 1.7- and 9-fold increase in the number of CD8$^+$CD44^{high}CD122$^+$ and CD8$^+$NK1.1$^+$ cells, respectively, in the spleen of RasGRP1$^{-/-}$ mice as compared with wild type (Fig. 5.5*D*). This observation contrasts with the significantly lower numbers of CD44^{low}CD8$^+$ cells that are recovered from both the thymus and spleen of RasGRP1$^{-/-}$ mice relative to wild-type mice (Fig. 5.5*D*). However, unlike CD4 Treg cells, there is no evidence that the elevation in the numbers of CD8$^+$CD44^{high}CD122$^+$ T cells in RasGRP1$^{-/-}$ mice is due to either enhanced proliferation and/or decreased cell death of these cells relative to wild-type mice (Fig. 5.5*C*). These observations suggest that CD8$^+$ CD44^{high}CD122$^+$ cells are more resistant to the effects of RasGRP1 deficiency than CD8$^+$ CD44^{low}CD122$^-$ T cells. Collectively, they indicate that there is a preferential development of memory phenotype CD8$^+$CD44^{high}CD122$^+$ T cells in the thymus and spleen of RasGRP1$^{-/-}$ mice.

Elevated expression of NK receptors by CD8$^+$ T cells in RasGRP1$^{-/-}$ mice

Functional differences between lymphocyte populations are often accompanied by changes in receptor expression patterns. It has been reported that CD8$^+$CD44^{high} cells from normal mice expressed significant levels of NK receptor upon IL-2 activation (37). In addition, CD44^{high} CD122$^+$ and CD44^{low}CD122$^-$ CD8$^+$ T cells from Itk$^{-/-}$ and IL-15$^{-/-}$ mice, respectively, have distinct patterns of NK receptor expression (34). To distinguish the CD44^{high}CD122$^+$CD8$^+$ T cells found in RasGRP1$^{-/-}$ mice from conventional memory CD8 T cells, we compared the expression of NK receptors and memory markers on CD8+ cells from wild-type and RasGRP1$^{-/-}$ mice. We found that the proportion of CD8$^+$CD122$^+$ cells expressing high levels of Ly6C, CD94, NKG2A/C/E, NKG2D, 2B4, and NK1.1 is dramatically increased in

RasGRP1$^{-/-}$ mice compared with their wild-type counterparts (Fig. 5.6). This finding is similar to that previously reported for CD44^{high}CD122$^+$CD8$^+$ T cells in Itk$^{-/-}$ mice (34). Notably, despite this dramatic difference in the expression of NK receptors, there are only minor differences in the expression of memory makers such as CD62L and CD127 between wild-type and RasGRP1$^{-/-}$ CD8$^+$CD122$^+$ spleen cells (Fig. 5.6). Therefore, it is likely that the CD44^{high}CD122$^+$CD8$^+$ T cells present in RasGRP1$^{-/-}$ mice are similar to those described for Itk$^{-/-}$ mice and represent a cell lineage(s) that is distinct from conventional memory CD8 T cells.

Activated RasGRP1$^{-/-}$ CD122$^+$CD8$^+$ T cells are less suppressive than their wild-type counterpart

Recent studies (35, 36) have shown that CD44^{high}CD122$^+$CD8$^+$ cells in Itk$^{-/-}$ mice can proliferate in IL-2 or IL-15 without TCR stimulation and produce IFN-γ directly ex vivo. To further characterize functional similarities of CD44^{high}CD122$^+$CD8$^+$ T cells from RasGRP1$^{-/-}$ and Itk$^{-/-}$ mice, we determined whether these cells from RasGRP1$^{-/-}$ mice can proliferate in response to IL-15 or IL-2. As expected, naive conventional CD8$^+$ T cells expressing low levels of CD122 did not proliferate when cultured with either IL-15 or IL-2. By contrast, CD8$^+$CD122$^+$ T cells from either wild-type or RasGRP1$^{-/-}$ mice proliferated vigorously when cultured with either IL-15 or IL-2 (Fig. 5.7*A*). However, mutant CD8$^+$CD122$^+$ T cells proliferate less well than wild type in response to these cytokines, suggesting a role for RasGRP1 in the transmission of cytokine-dependent growth signals. Furthermore, only CD8$^+$ CD44^{high}, but not CD8$^+$CD44^{low} T cells from both thymus and spleen of wild-type or RasGRP1$^{-/-}$ mice can produce IFN-γ after PMA plus ionomycin stimulation ex vivo (Fig. 5.7*B*). These data indicate that the development of CD44^{high}CD122$^+$CD8$^+$ T cells that possess innate immune functions is independent of RasGRP1.

Recent studies show that CD8$^+$CD122$^+$ cells from normal mice can also function as Treg cells via an IL-10-dependent mechanism (11, 12). We sought to investigate whether CD44^{high} CD122$^+$CD8$^+$ T cells present in RasGRP1$^{-/-}$ mice possess similar suppressor function. To test this hypothesis, we compared the ability of CD8$^+$CD122$^+$ cells from RasGRP1$^{-/-}$ mice to

produce IL-10 using wild-type $CD8^+CD122^-$ cells as a negative control. Sorted $CD8^+CD122^+$ cells from wild-type or $RasGRP1^{-/-}$ mice were stimulated with anti-CD3 plus anti-CD28 and exogenous IL-2 for 3 days. IL-10 production was then evaluated by intracellular staining with anti-IL-10 mAb. As expected, wild-type $CD8^+CD122^-$ cells failed to produce IL-10 after in vitro activation. By contrast, $CD8^+CD122^+$ cells from either wild-type or $RasGRP1^{-/-}$ mice produced significant amounts of IL-10 compared with wild-type $CD8^+CD122^-$ cells (Fig. 5.8*A*). We then compared the ability of activated $CD8^+CD122^+$ cells from wild-type or $RasGRP1^{-/-}$ mice to suppress the proliferation of wild-type $CD8^+CD122^-$ cells (Fig. 5.8*B*). Sorted $CD8^+CD122^+$ cells from wild-type or $RasGRP1^{-/-}$ mice were stimulated with anti-CD3 plus anti-CD28 and exogenous IL-2 for 3 days and used as a source of suppressor cells. Supernatants from these cultures were also collected and assessed for their suppressor activity at 1/2 dilution. $CD8^+CD122^-$ cells and culture supernatants from wild-type mice activated in a similar manner were used as negative controls. CFSE-labeled wild-type $CD8^+$ $CD122^-$ $(Thy1.1^+)$ cells were used as responder cells. The $Thy1.2^+$ activated suppressor $(CD8^+CD122^+)$ cells and negative control $(CD8^+CD122^-)$ cells were added at various suppressor to responder ratios and stimulated with plate-bound anti-CD3, anti-CD28 (10 µg/ml), plus 100 U/ml IL-2 for another 2 days. We found that activated $CD8^+CD122^+$ cells and culture supernatants from wild-type mice completely suppressed the proliferation of wild-type $CD8^+CD122^-$ cells. By contrast, activated $CD8^+CD122^+$ cells from $RasGRP1^{-/-}$ mice were only suppressive when used at a high (1:5), but not lower ratios (1:15 and 1:45). Furthermore, the culture supernatants of activated mutant $CD8^+CD122^-$ cells were not suppressive. These data suggest that although the development of memory phenotype $CD8^+$ $CD44^{high}CD122^+$ Treg cells is independent of RasGRP1, their suppressor function is much more dependent on RasGRP1.

5.4   Discussion

We have previously concluded that RasGRP1 is especially crucial for transducing low-grade TCR signals because its absence preferentially affected the positive selection of the weakly selecting H-Y TCR (21). The development of CD4 T cells is also critically dependent on

RasGRP1 (20, 25). By contrast, the development of thymocytes expressing the more strongly selecting 2C TCR and negative selection was much less sensitive to RasGRP1 loss (21). Because recent studies have suggested that a common denominator for the development of multiple Treg lineages is their dependence on high affinity TCR/self ligand interactions for their development (1, 8), we sought to determine how the development of Treg lineages was affected in RasGRP1$^{-/-}$ mice. In this study, we found that RasGRP1$^{-/-}$ mice can support the development of both CD4 and CD8 Treg cells, albeit with differences in efficacy in the generation of CD4 and CD8 Treg cells in the thymus of RasGRP1$^{-/-}$ mice.

In the absence of RasGRP1, development of CD4 Treg cells in the thymus is severely impaired, resulting in < 5% yield relative to wild-type mice (Fig. 5.1*B*). However, there is preferential expansion of CD4 Treg cells in the periphery, and the number of splenic CD4 Treg cells is ~32% of wild type (Fig. 5.2*B*). The development of CD4 Treg cells is influenced by at least two factors: availability of IL-2 and self Ag/MHC ligands (1, 38, 39). It remains to be determined whether the poor development of CD4 Treg cells in RasGRP1$^{-/-}$ thymus reflects the paucity of one or both of these factors. We have previously shown that T cells expressing relatively high affinity TCRs for self ligands can develop via a RasGRP1-independent mechanism (21). Consistent with this hypothesis is our observation that a large proportion of peripheral CD4 T cells in RasGRP1$^{-/-}$ mice is actively cycling and undergoing apoptosis, presumably as a result of high affinity TCR/self ligand interactions (40). In this study, we further characterized the CD4 phenotype in mutant mice by discriminating between Treg and non-Treg populations. Our observations suggest that in the periphery of RasGRP1$^{-/-}$ mice, although both Treg and non-Treg subsets are undergoing massive proliferation, a smaller proportion of Treg population shows signs of apoptosis as compared with wild type (Fig. 5.3*A*), which might contribute to the high frequencies of CD4^{+}Foxp3^{+} T cells in the mutant spleens. It is also conceivable that the proliferating CD4 non-Treg cells can produce cytokines that include IL-2. Together with the lymphopenia present in RasGRP1$^{-/-}$ mice, this could provide a favorable environment for peripheral CD4 Treg cell expansion that is mediated by its high affinity IL-2R. This hypothesis is supported by our adoptive transfer experiments demonstrating that there is a preferential expansion of adoptively transferred wild-type CD4^{+}CD25^{+} cells in RasGRP1$^{-/-}$ mice relative to wild-type

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recipients (Fig. 5.3*B*, top panel). Recent studies suggest that in the presence of cognate Ag, peripheral conventional CD4$^+$CD25$^-$ T cells expressing high affinity TCRs for self ligands can be efficiently converted into Treg cells expressing Foxp3 (19). We therefore evaluated the possibility that mutant mice provide an environment niche that favors the conversion of conventional CD4$^+$CD25$^-$ T cells to CD4$^+$CD25$^+$Foxp3$^+$ Treg cells by transferring wild-type or mutant CD4$^+$CD25$^-$ cells into wild-type or mutant recipients, respectively (Fig. 5.3*B*). Interestingly, we found that the preferential expansion of transferred CD4$^+$CD25$^-$ cells (either wild type or mutant) in RasGRP1$^{-/-}$ hosts is associated with an increase in the numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells (Fig. 5.3, *B* and *C*). This result raises the possibility that the increase in Foxp3-expressing CD4 T cells in the periphery of RasGRP1$^{-/-}$ mice could at least be due in part to the conversion of CD4$^+$CD25$^-$ cells into CD4$^+$CD25$^+$Foxp3$^+$ cells. However, because a small percentage of donor CD4$^+$CD25$^-$ cells was Foxp3$^+$ before transfer (see Fig. 5.2*B*), the increase in the number of CD4$^+$CD25$^+$Foxp3$^+$ cells in RasGRP1$^{-/-}$ hosts could either be due to the conversion of CD4$^+$CD25$^-$Foxp3$^+$ cells into CD4$^+$CD25$^+$Foxp3$^+$ cells or the conversion of CD4$^+$CD25$^-$Foxp3$^-$ cells into CD4$^+$CD25$^+$Foxp3$^+$ cells. However, the fact that neither transferred mutant CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells could survive in wild-type hosts (Fig. 5.3*B*) indicates it is the mutant environment rather than cell intrinsic effects of the RasGRP1$^{-/-}$ mutation that plays a dominant role in the preferential increase of peripheral CD4 Treg cells in mutant mice.

In contrast to the development of CD4 Treg cells, intrathymic development of memory phenotype CD8 Treg cells is not affected in RasGRP1$^{-/-}$ mice. It is likely that the memory phenotype CD8 T cells that developed in RasGRP1$^{-/-}$ mice represent multiple lineages of CD8 T cells. Consistent with this hypothesis is the observation that these memory phenotype CD8 T cells are heterogeneous in terms of expression of cell surface markers such as CD44, CD122, NK1.1, Ly6C, CD94, NKG2A/C/E, NKG2D, and 2B4 (Fig. 5.6). These cells are also heterogeneous in IFN-γ production, with only 8.4 and 13.3% of total CD8 cells in the thymus and spleen, respectively, producing this cytokine upon PMA and ionomycin stimulation (Fig. 5.7*B*), whereas 15 and 50.7% of total CD8 cells (thymus and spleen, respectively) possess the memory phenotype (CD44^{high}CD122$^+$) (Fig. 5.5*A*). The preferential development of CD8 T cells that possess innate-like properties has also been observed in Tec

kinase-deficient mice (34-36). The observation that CD8 T cells of similar cell surface phenotype and function develop in both RasGRP1 and Tec-kinase-deficient mice also suggests that RasGRP1 and Tec kinases activate components of the same signaling pathway. It is also likely that a common denominator in the generation of these memory phenotype CD8 T cells is the expression of relatively high affinity TCRs for self ligands, and therefore, these cells can develop via a RasGPR1-independent mechanism. Consistent with a previous report (12), we found that activated wild-type $CD8^+CD122^+$ cells are able to produce IL-10 and efficiently suppressed the proliferation of anti-CD3-, anti-CD28-, and IL-2-activated wild-type $CD8^+CD122^-$ cells. Furthermore, culture supernatants derived from wild-type $CD8^+CD122^+$ cells activated with anti-CD3, anti-CD28, and IL-2 were also highly suppressive. However, similarly activated mutant $CD8^+CD122^+$ cells were much less suppressive at low suppressor to responder ratio. In addition, supernatants of activated mutant $CD8^+CD122^+$ cells failed to show detectable suppressive activity. Because we have previously shown that RasGRP1 plays a fundamental role in the developmental programming of Ag-activated CD8 T cells by providing signals necessary for their survival (21), it is possible that the less efficient suppressive activity of activated mutant $CD8^+CD122^+$ T cells may be due to the poorer survival of these cells. This poorer survival may also affect their ability to sustain the production of suppressor cytokines such as IL-10. Collectively, these results indicate that although RasGRP1 is not required for the development of $CD8^+CD44^{high}$ $CD122^+$ cells, the suppressive function of these cells is critically dependent on RasGRP1.

Consistent with our observation that peripheral lymphoid tissues of $RasGRP1^{-/-}$ mice harbor large numbers of both CD4 and CD8 Treg cells, we did not observe any overt autoimmune disorders in these mice, despite our hypothesis that only T cells expressing TCRs with high affinity for self ligands can develop in these mice. This observation contrasts with observations in a mouse strain that harbors a spontaneous mutation in the RasGRP1 $(RasGRP1^{lag})$ gene (41). In $RasGRP1^{lag}$ mice, there are massive lymphoproliferation and development of an autoimmune syndrome that share similarities with systemic lupus erythematosus (41). It is possible that the autoimmune phenotype that developed in $RasGRP1^{lag}$ mice might be due to contributions of the hybrid 129:B6 background in addition to disruption of RasGRP1 gene function (42). Consistent with this hypothesis, we did

observe massive lymphoadenopathy and splenomegaly in RasGRP1$^{-/-}$ mice in early backcrosses of 129 mutant mice to B6 mice. However, upon more than seven generations of backcross to the B6 background, no overt autoimmune symptoms were observed in RasGRP1$^{-/-}$ mice.

Our studies demonstrate that although the development of CD4 Treg cells in the thymus of RasGRP1$^{-/-}$ mice is very inefficient, peripheral mechanisms exist to greatly expand the numbers of CD4 Treg cells. It is likely that these CD4 Treg cells may be involved in preventing the development of overt autoimmune disease in RasGRP1$^{-/-}$ mice. By contrast, there is very efficient development of a heterogeneous population of memory phenotype CD8 T cells in the thymus, resulting in higher frequencies of these cells in the periphery of RasGRP1$^{-/-}$ mice. These memory phenotype CD8 T cells also include cells that can perform innate immune functions. To explain the contrasting functions of these memory phenotype CD8^{+} T cells that exhibit both innate functions and suppressive functions, we propose differential conditions used to activate memory phenotype CD8 T cells from either wild-type or RasGRP1$^{-/-}$ mice lead to distinct immunological functions. The development of memory phenotype CD8 T cells in RasGRP1$^{-/-}$ mice may be due to high affinity TCR interactions with self Ags, because we have previously demonstrated the high expression levels of the memory markers CD44 and CD122 found in naive mice are maintained when the self Ag is present (43, 44). These studies complement those conducted in Itk$^{-/-}$ and Itk$^{-/-}$Rlk$^{-/-}$ mice and support the hypothesis that unconventional CD8 T cells differ from conventional CD8 T cells in the requirement for Itk, Rlk, and RasGRP1 for their development. More importantly, our studies indicate RasGRP1 signaling plays a discriminative role in the intrathymic development of CD4 and CD8 Treg cells, whereas it is not required for peripheral expansion of either lineage.

5.5   Acknowledgements

5.6   Figures

Figure 5.1 Inefficient intrathymic development of CD4$^+$Foxp3$^+$ Treg cells in RasGRP1$^{-/-}$ mice. *A*, CD4/CD8 developmental profiles of wild-type and RasGRP1$^{-/-}$ mice. The expression of Foxp3 (detected by intracellular staining) and CD25 by gated CD4 SP thymocytes is indicated. *B*, Recovery of Foxp3$^+$ and Foxp3$^-$ CD4 SP thymocytes in wild-type and RasGRP1$^{-/-}$ mice. Error bars represent the SD of 3 mice per group. The yield of Foxp3$^+$ and Foxp3$^-$ cells as a percentage of wild-type numbers is as indicated. *C*, The CD4/CD8 profiles of Foxp3-expressing cells in thymus. *D*, Representative flow cytometric analysis of developmental markers on Foxp3$^+$ CD4 SP thymocytes. Wild-type and mutant subpopulations are represented in shaded and bold line histograms, respectively. Numbers shown on the plots indicate percentages of gated population.

Figure 5.2 RasGRP1$^{-/-}$ mutant mice possess a high frequency of activated peripheral CD4$^+$Foxp3$^+$ cells. *A*, Representative flow cytometric analysis of CD25 and Foxp3 expression on wild-type and RasGRP1$^{-/-}$ peripheral CD4 T cells. Plots were gated on total cells and CD4$^+$ cells, respectively. *B*, Recovery of RasGRP1$^{-/-}$ CD4$^+$Foxp3$^+$ or CD4$^+$Foxp3$^-$ spleen cells as percentages of wild type. Number of animals used is as follows: +/+, n = 5; -/-, n = 6. *C*, Flow cytometric analyses of developmental and activation markers of gated CD4$^+$Foxp3$^+$ or CD4$^+$Foxp3$^-$ spleen cells. Shaded and bold line histograms represent staining of wild-type and mutant cells, respectively. Data shown are representative of three independent analyses.

Figure 5.3 Preferential peripheral expansion of CD4 Treg cells in RasGRP1$^{-/-}$ mice. *A*, Correlation between annexin V or Ki-67 vs Foxp3 expression of wild-type or RasGRP1$^{-/-}$ CD4$^+$ T cells by intracellular staining directly ex vivo. Isotype control Ab staining was negligible. Numbers indicate percentages of annexin V- or Ki-67-positive cells within total Foxp3$^-$ or Foxp3$^+$ subpopulations. Data shown are representative of three independent experiments. *B*, RasGRP1$^{-/-}$ mice provide a favored environment for CD4 Treg cell

expansion. Sorted CD4$^+$CD25$^+$ (2 x 10^5) or CD4$^+$CD25$^-$ (1 x 10^6) Thy1.1$^+$ wild-type cells were adoptively transferred into naive B6 Thy1.2$^+$ wild-type or RasGRP1$^{-/-}$ hosts (top panel). Similarly, sorted CD4$^+$CD25$^+$ (2 x 10^5) or CD4$^+$CD25$^-$ (1 x 10^6) Thy1.2$^+$ RasGRP1$^{-/-}$ cells were adoptively transferred into naive Thy1.1$^+$ wild-type or RasGRP1$^{-/-}$ hosts (bottom panel). Recovery of donor cells was accessed 3 wk postadoptive transfer. Numbers shown are the percentages of donor cells within the recipient's total spleen. C, Same type of experiment as in B. The data indicate increased Foxp3 expression by transferred wild-type or mutant CD4$^+$CD25$^-$ donor cells that were transferred into RasGRP1$^{-/-}$ hosts. Plots shown are gated on donor CD4$^+$ cells.

Figure 5.4 Enhanced suppressive activity of peripheral RasGRP1$^{-/-}$ CD4$^+$CD25$^+$ Treg cells in vitro. Graded doses of sorted B6 wild-type (gray circles) or RasGRP1$^{-/-}$ (black squares) mutant CD4$^+$CD25$^+$ cells were cultured with 2 x 10^4 sorted B6 wild-type CD4$^+$CD25$^-$ cells for 72 h with 2 µg/ml Con A and 8 x 10^4 irradiated B6 APCs. This assay was set up in 96-well round-bottom plates, and [^{3}H] thymidine (1 µCi per well) was added to the culture for the final 8 h. Numbers on horizontal axis indicate the ratio of CD4$^+$CD25$^+$ (suppressor) cells to CD4$^+$CD25$^-$ (responder) cells. All data are shown as mean [^{3}H] thymidine incorporation in triplicate cultures. The asterisks (*) represent p values of <0.05, calculated using unpaired, two-tailed Student's t test.

Figure 5.5 Efficient development of CD44^{high}CD122$^+$CD8$^+$ cells in both thymus and periphery of RasGRP1$^{-/-}$ mice. A, Greatly increased proportion of mature memory phenotype CD8 SP T cells in the thymus of RasGRP1$^{-/-}$ mice. The thymus of RasGRP1$^{-/-}$ mice contains a greatly increased percentage of CD8$^+$CD44^{high}CD122$^+$CD24^{low} cells. B, Increased proportion of CD8$^+$ TCRβ$^+$NK1.1$^+$ T cells in both thymus and spleen of RasGRP1$^{-/-}$ mice. Numbers shown represent the percentages of gated CD8 SP cells. C, Annexin V and Ki-67 vs CD122 staining of wild-type and RasGRP1$^{-/-}$ CD8 splenocytes directly ex vivo. Numbers in the plot reflect the frequency of annexin V- or Ki-67-positive CD8 cells within the total CD122$^-$ or CD122$^+$ CD8 population. D, Increased CD44^{high}CD122$^+$CD8$^+$ and NK1.1$^+$CD8$^+$ T cells in contrast to decreased CD44^{low}CD8$^+$ T cells were observed in both thymus and spleen

of RasGRP1$^{-/-}$ mice relative to wild-type mice. Number of animals analyzed is as follows: +/+, n = 6; -/-, n = 7. Error bars, SD. Thy, Gated on CD8 SP thymocytes; Spl, gated on CD8^{+} spleen cells.

Figure 5.6 Increased expression of NK receptors by peripheral CD8^{+} T cells from RasGRP1$^{-/-}$ mice. Representative flow cytometric analysis of splenocytes from wild-type and RasGRP1$^{-/-}$ mice. The histograms denote expression of the indicated cell surface marker by gated CD8^{+} spleen cells. Numbers shown are percentages of gated cells. Number of animals analyzed is as follows: wild type, n = 4; RasGRP1$^{-/-}$, n = 5. Phenotypic data from one representative experiment are shown.

Figure 5.7 RasGRP1$^{-/-}$ CD8^{+}CD44^{high}CD122^{+} cells proliferate in response to IL-2 or IL-15 and produce IFN-γ in response to PMA plus ionomycin stimulation. *A*, CD8^{+}CD122^{-} and CD8^{+}CD122^{+} T cells were purified from spleens of wild-type or RasGRP1$^{-/-}$ mice, CFSE labeled, and cultured in IL-2 (200 U/ml) or IL-15 (100 ng/ml). After 72 h, CFSE profiles of both cell types were analyzed by FACS. *B*, IFN-γ production by wild-type or RasGRP1$^{-/-}$ CD8 SP thymocytes and CD8^{+} splenocytes was determined after PMA plus ionomycin stimulation for 5 h. Results shown are representative of three experiments.

Figure 5.8 Activated RasGRP1$^{-/-}$ CD8^{+}CD122^{+} cells are less suppressive than activated wild-type CD8^{+}CD122^{+} cells. *A*, IL-10 production is evaluated by intracellular FACS staining after activating the indicated sorted populations (+/+, wild type; -/-, RasGRP1$^{-/-}$ by anti-CD3/CD28 plus IL-2 for 3 days. *B*, Sorted Thy1.2^{+} wild-type (CD8^{+}CD122^{-}, CD8^{+}CD122^{+}) or RasGRP1$^{-/-}$ (CD8^{+}CD122^{+}) cells were stimulated with anti-CD3/CD28 plus IL-2. Three days later, cultured cells and supernatants were harvested. The indicated activated cells (suppressor) were cultured with Thy1.1^{+} CFSE-labeled wild-type CD8^{+}CD122^{-} cells (responder) at the indicated suppressor to responder ratios. The responder cells were activated with anti-CD3/CD28 plus IL-2 for 2 days. At the end of 2 days, proliferation of Thy1.1^{+} CD8^{+}CD122^{-} cells was determined by analyzing the CFSE profiles of the responder

cells. Where indicated, culture supernatant from the first culture was added to responder cells at a 1/2 dilution. The CFSE assay was done in triplicate. The CFSE profiles shown are representative of three experiments.
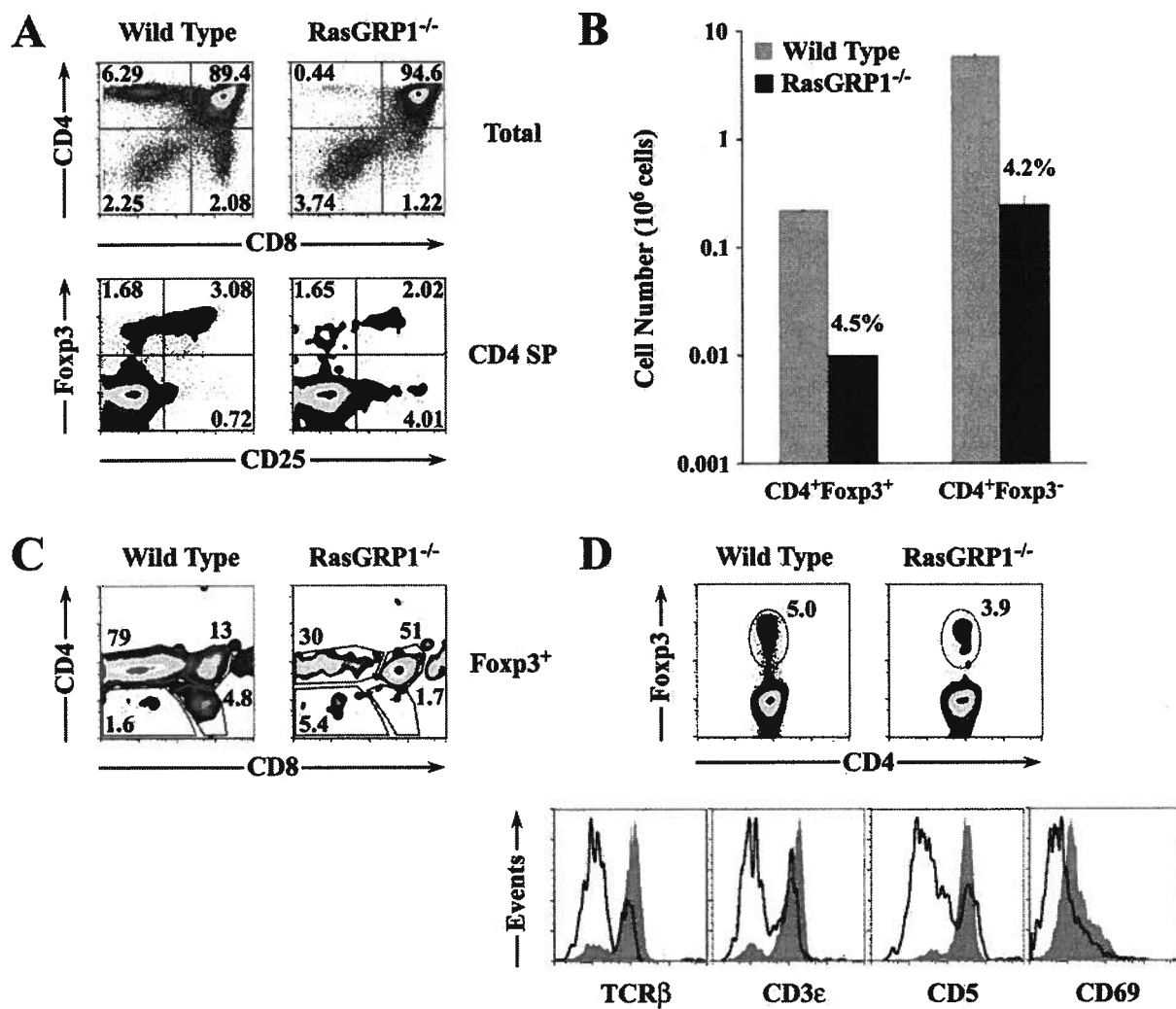
Figure 5.1 Inefficient intrathymic development of CD4$^+$Foxp3$^+$ Treg cells in RasGRP1$^{-/-}$ mice.
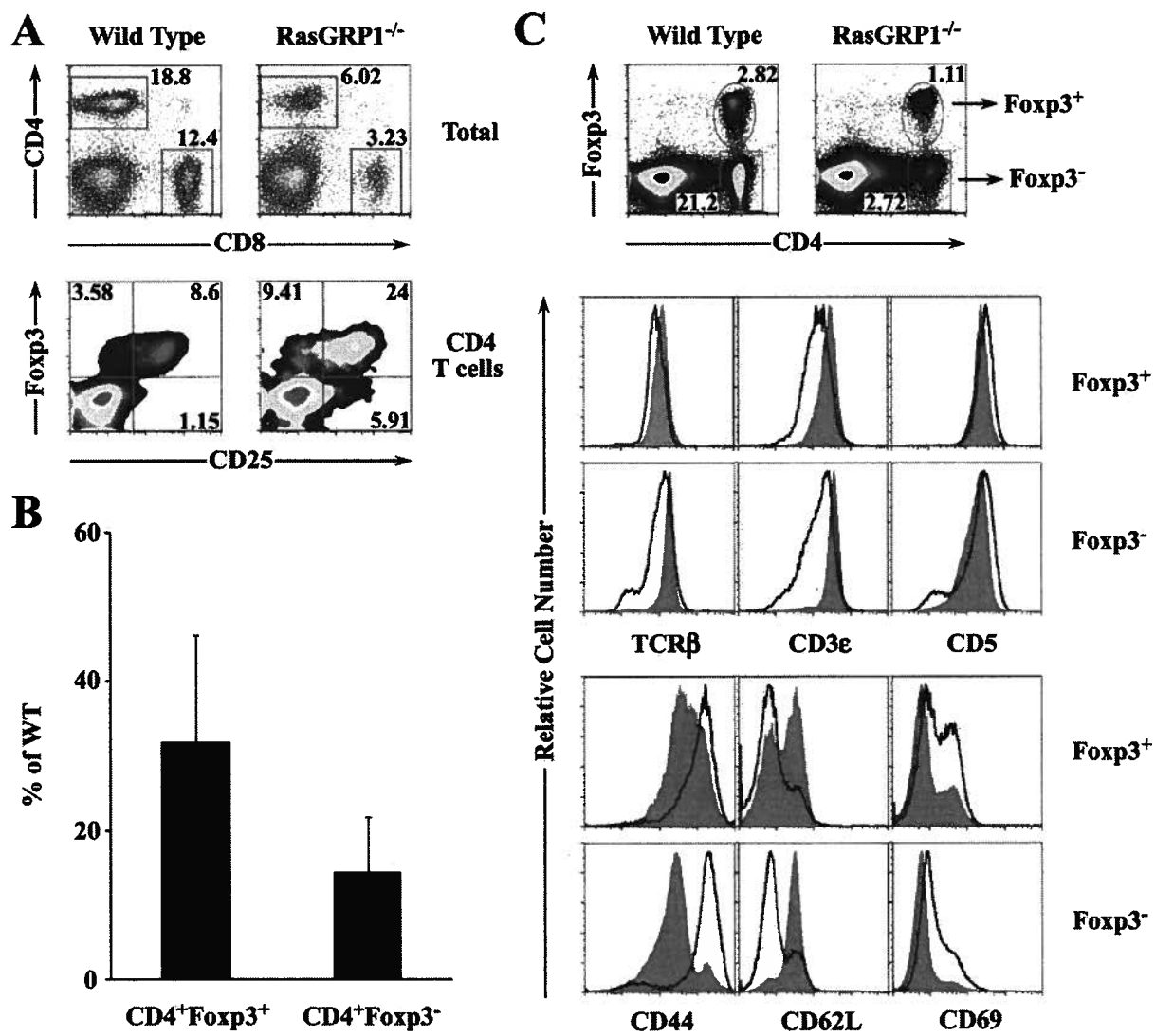
Figure 5.2 RasGRP1$^{-/-}$ mutant mice possess a high frequency of activated peripheral CD4$^+$Foxp3$^+$ cells.
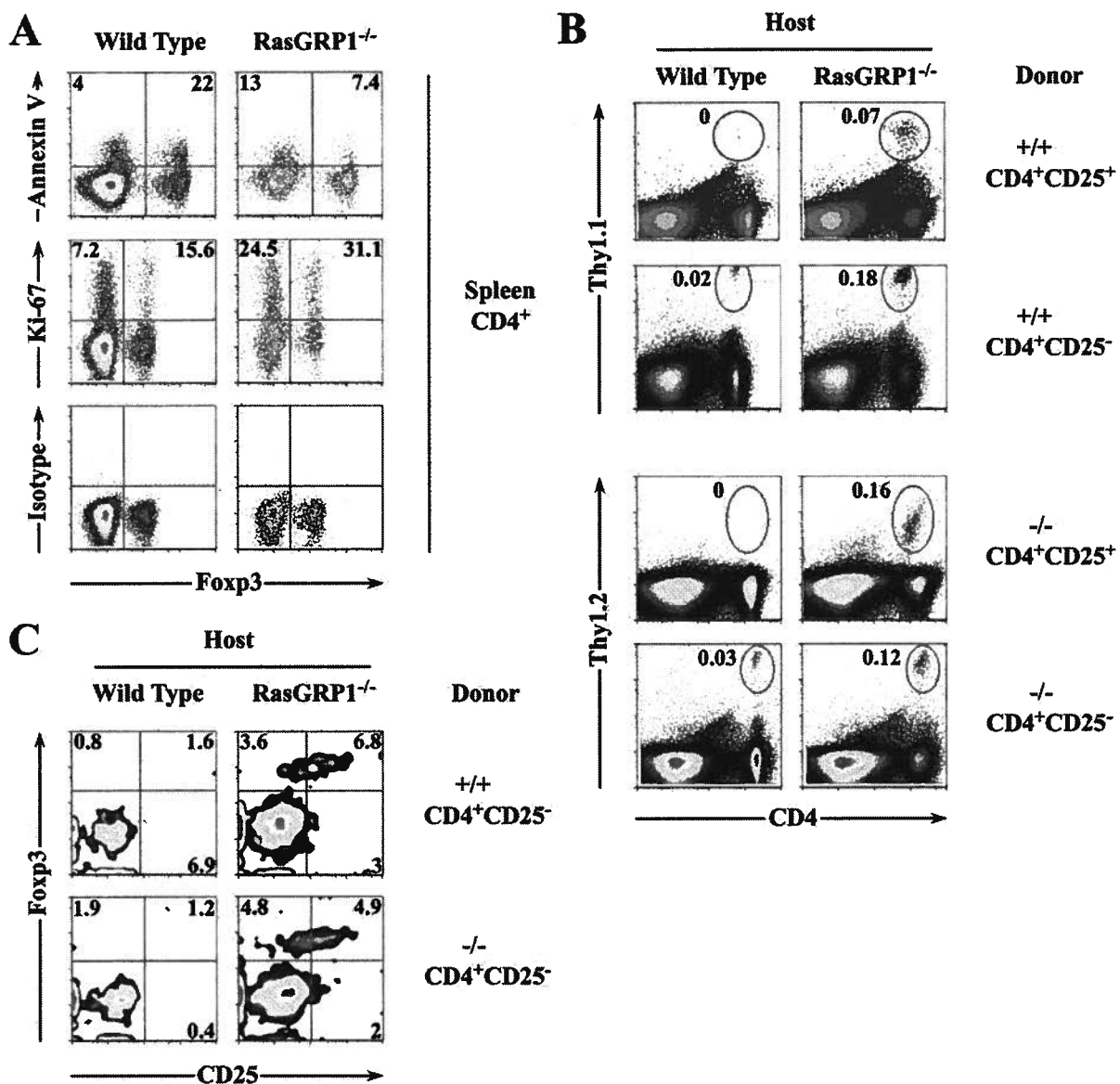
Figure 5.3 Preferential peripheral expansion of CD4 Treg cells in RasGRP1$^{-/-}$ mice.
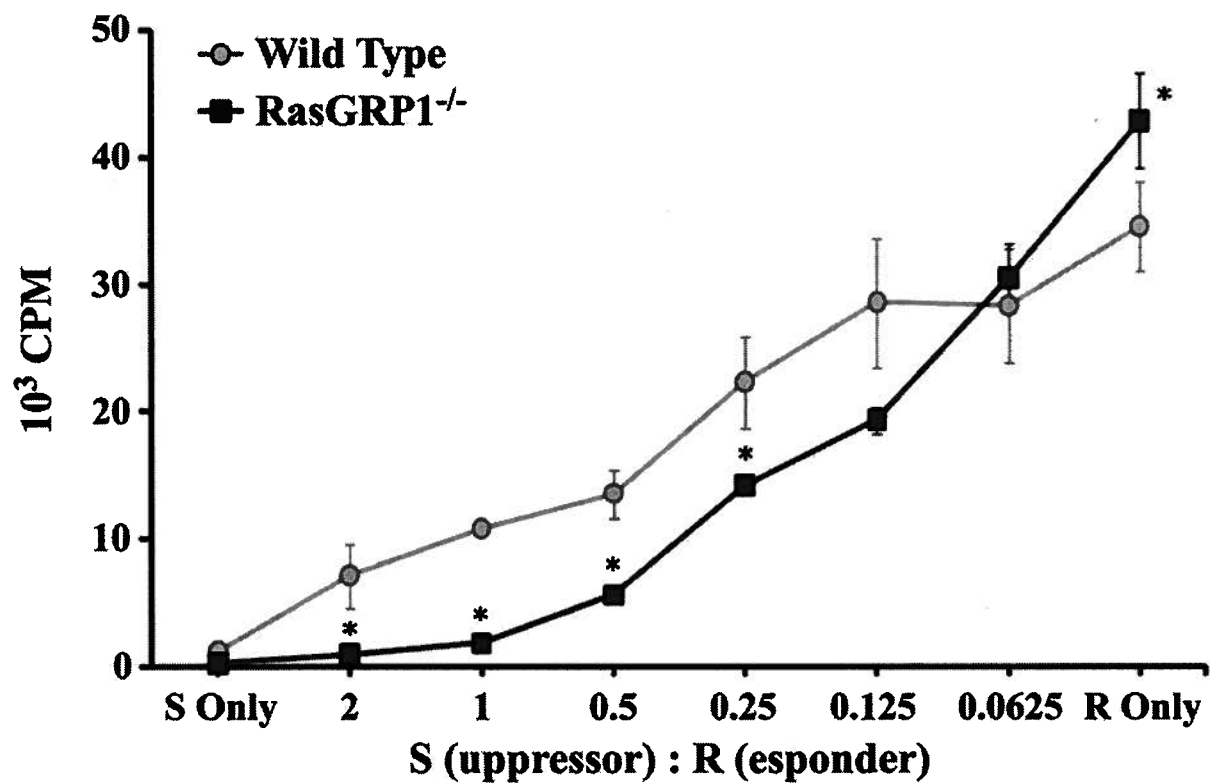
Figure 5.4 Enhanced suppressive activity of peripheral RasGRP1$^{-/-}$ CD4^{+}CD25^{+} Treg cells in vitro.
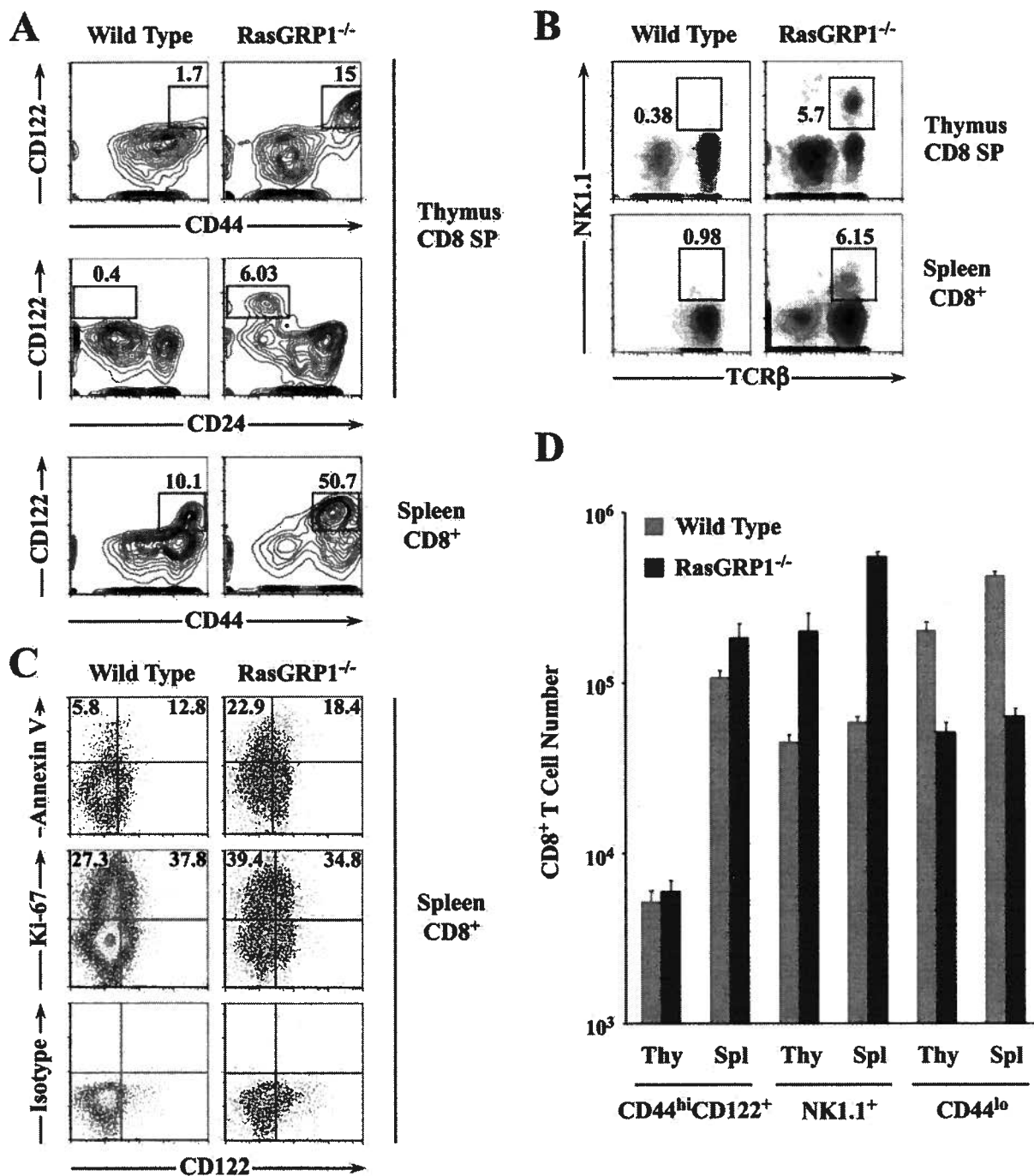
Figure 5.5 Efficient development of CD44^{high}CD122^{+}CD8^{+} cells in both thymus and periphery of RasGRP1$^{-/-}$ mice.
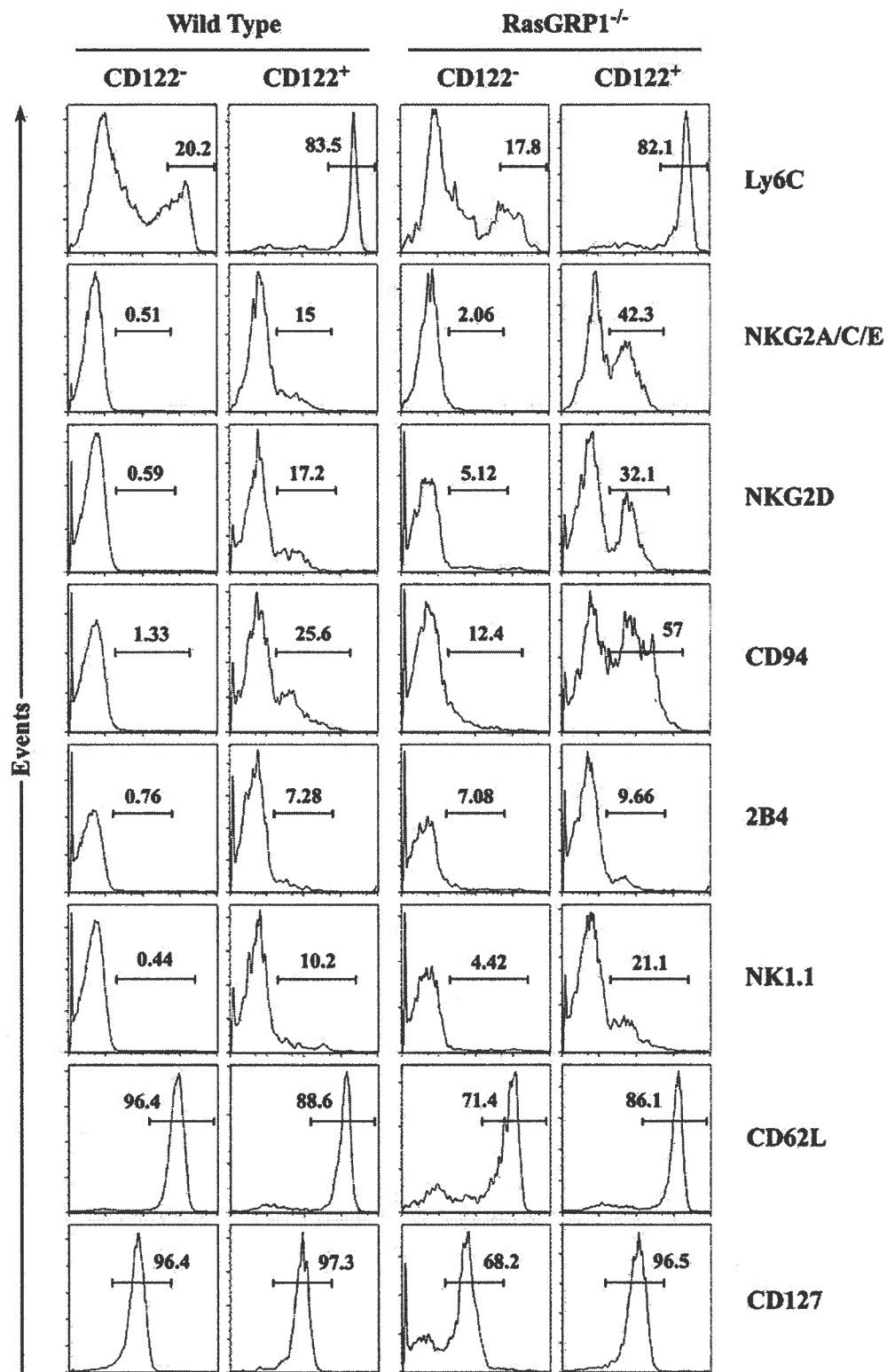
Figure 5.6 Increased expression of NK receptors by peripheral CD8$^+$ T cells from RasGRP1$^{-/-}$ mice.
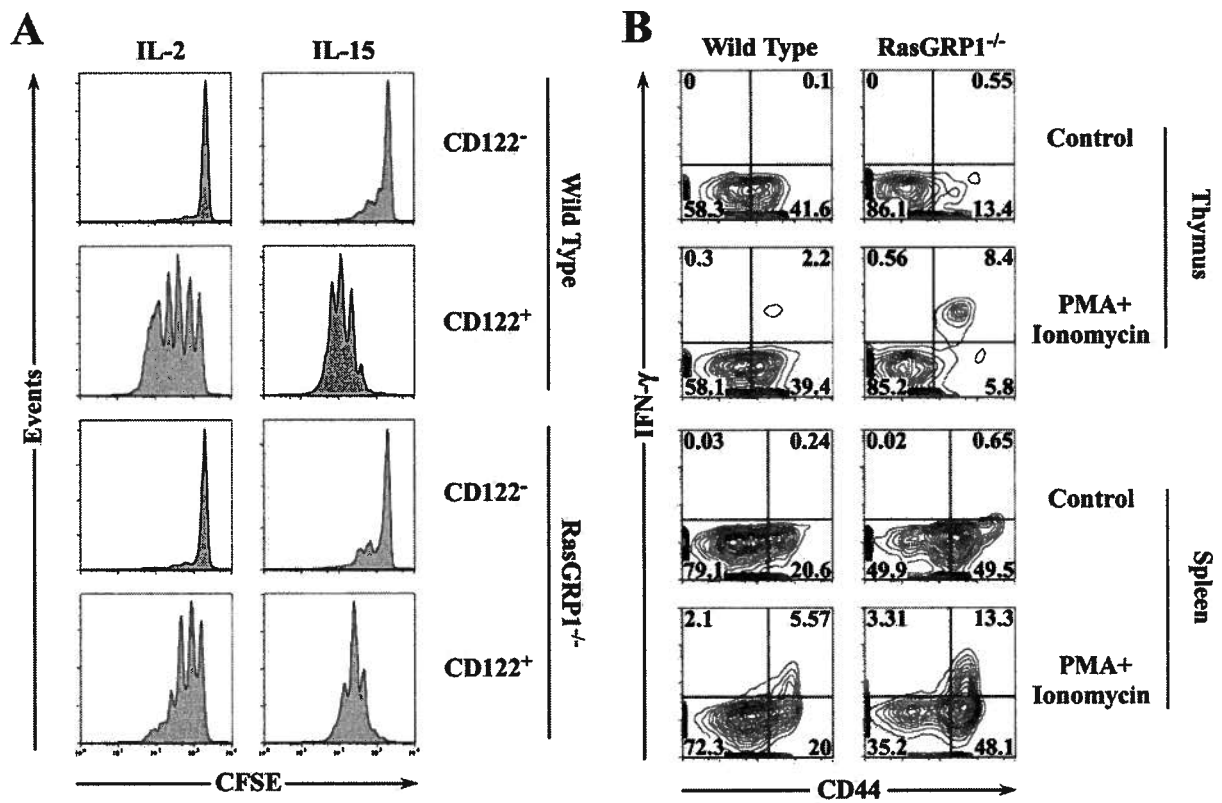
Figure 5.7 RasGRP1$^{-/-}$ CD8$^+$CD44^{high}CD122$^+$ cells proliferate in response to IL-2 or IL-15 and produce IFN-γ in response to PMA plus ionomycin stimulation.
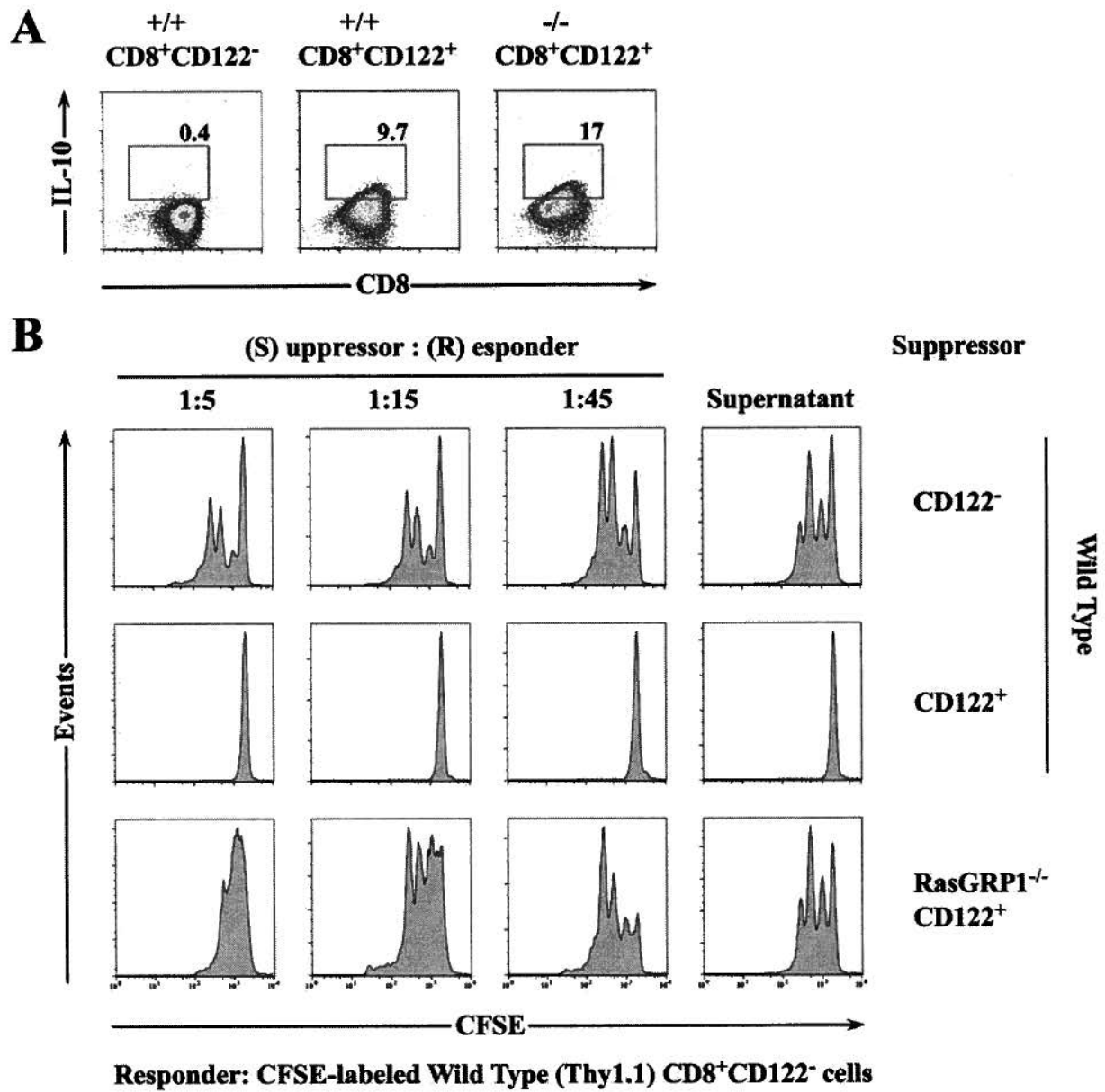
Figure 5.8 Activated RasGRP1$^{-/-}$ CD8$^+$CD122$^+$ cells are less suppressive than activated wild-type CD8$^+$CD122$^+$ cells.

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CHAPTER 6 GENERAL DISCUSSION AND PERSPECTIVE

6.1 Summarizing the role of RasGRP1 in T cell development and function

This thesis has focused on how different aspects of immune system are affected by a deficiency in RasGRP1. Novel roles of RasGRP1 in T cell development, homeostasis and peripheral function were identified. The TCR transgenic mouse models provided us with a powerful tool to study the effect of RasGRP1 null mutation in thymocyte development and T cell function. Chapter 2 was focused on the consequences of RasGRP1 null mutation in CD4 T cell development by using MHC class II-restricted AND TCR Tg mice (1). We found that the development of CD4 T cells expressing this Tg TCR is completely dependent on RasGRP1. RasGRP1$^{-/-}$ DP thymocytes exhibit marked deficits in TCR-stimulated up-regulation of the positive selection marker CD69 and the antiapoptotic protein Bcl-2. To evaluate the role of RasGRP1 in providing cellular survival signaling, we enforced Bcl-2 expression in RasGRP1$^{-/-}$ thymocytes. However, Tg Bcl-2 expression failed to restore CD4 T cell development in RasGRP1-deficient mice, indicating that RasGRP1 transmits differentiation signals independent of those necessary for Bcl-2 induction (1). Following the notion that the RasGRP1-Ras/MAPK pathway is particularly important for imparting differentiation signals that are essential for CD4 T cell development, we went on to study how the severely impaired thymic development would affect peripheral T cell homeostasis and T cell activation against pathogens (2) in Chapter 3. The results in that chapter demonstrate that despite apparently normal central tolerance, RasGRP1$^{-/-}$ mice spontaneously acquire an acutely activated and proliferating CD4 T cell population that exhibits characteristics of T cell exhaustion. To elucidate the basis for RasGRP1$^{-/-}$ CD4 T cell immune activation, we initiated a series of adoptive transfer experiment using acutely and chronically immunodeficient hosts. Remarkably, the copious amounts of cytokines and self-Ags present in hosts made lymphopenic through irradiation failed to induce the majority of RasGRP1$^{-/-}$ CD4 T cells to enter cell cycle. However, their infusion into either congenitally T cell- or T/B cell-deficient recipients resulted in robust proliferation and signs of acute activation. These findings imply that the activation and proliferation of RasGRP1$^{-/-}$ CD4 T

cells may be dependent on their residence in a chronically immunocompromised environment. Accordingly, bacterial and viral challenge experiments revealed that RasGRP1$^{-/-}$ mice possess a weakened immune system, exhibiting a T cell-autonomous defect in generating pathogen-specific T cells and delayed pathogen clearance. Therefore, our results in Chapter 3 provide a reasonable explanation of the activated phenotype of CD4 T cells in RasGRP1$^{-/-}$ mice, which suggest that chronic T cell immunodeficiency in RasGRP1$^{-/-}$ mice, resulting from impaired T cell development, may be responsible for CD4 T cell activation, proliferation, and exhaustion.

In Chapter 4, follow-up studies of the Immunity paper (Priatel et al. 2002) were performed to study the impact of RasGRP1 deficiency in naïve CD8 T cell activation by using 2C TCR Tg mouse model lacking RasGRP1. Our results suggest RasGRP1 regulates the threshold of T cell activation and Ag-induced expansion, at least partly, through the regulation of IL-2 production. Moreover, RasGRP1$^{-/-}$ 2C CD8 T cells exhibit an anergic phenotype in response to cognate Ag stimulation that is reversible upon addition of exogenous IL-2. However, the capacity of IL-2/IL-2 receptor interaction to mediate Ras-activation and CD8 T cell expansion and differentiation appears to be largely RasGRP1-independent. Although our previous studies have uncovered the significant role of RasGRP1 in the development and function of major T cell subsets, defined by the expression of coreceptor, we neglected a population that is thought to play an important role in the peripheral tolerance. Numerous studies on Treg cell suggest that this population requires high affinity TCR-MHC/self peptide engagement for development and the production of regulatory cytokines to suppress conventional T cell activation and immune responses (3). As such, the results presented in Chapter 5 (4) provide detailed analysis of CD4 and CD8 Treg cell development, homeostasis and function in the absence of RasGRP1. Despite impaired CD4 Treg development in the thymus, the periphery of RasGRP1$^{-/-}$ mice contained significantly increased frequencies of CD4 Treg cells that possessed a more activated cell surface phenotype. Interestingly, mutant CD4 Treg cells are more suppressive than their wild-type counterpart in inhibiting the proliferation of non-Treg CD4 T cells. In contrast, the intrathymic development of innate-like CD8 Treg cells, defined by CD44^{hi}CD122^{+}, is unaffected in RasGRP1$^{-/-}$ mice. Moreover, RasGRP1$^{-/-}$ mice contain greater numbers of CD8 Treg cells in the spleen, relative to wild-

type mice. Activated CD8 Treg cells from RasGRP1$^{-/-}$ mice retained their ability to synthesize IL-10 and suppress the proliferation of wild-type non-Treg CD8 T cells, albeit at a much lower efficiency than wild-type CD8 Treg cells. These results complement our previous studies and point out the crucial role of RasGRP1 in transducing optimal signal in the development and function of different T cell lineages. Overall, this thesis provides comprehensive analysis of the consequence of RasGRP1 deficiency on conventional T cell development, the regulation of T cell homeostasis, the development of Treg cells, and the regulation of immune responses. Our studies suggest that RasGRP1 is a crucial modulator downstream of TCR signaling controlling signal threshold during thymic development and peripheral function, and its presence is essential for the maintenance of peripheral tolerance and T cell homeostasis.

6.2   RasGRP1 signaling, positive selection and lineage commitment

CD8 T cells normally express MHC class I-restricted TCRs. Unexpectedly, our results in Chapter 2 (1) revealed that a small number of functional CD8 SP thymocytes expressing the Tg MHC class II-restricted TCR exists in RasGRP1 mutant mice. Furthermore, these unusual mutant CD8 SP thymocytes express markers of positive selection and are able to respond to a MHC class II-specific Ag and differentiate into cytotoxic effectors. These results raised questions regarding their nature and origin, as well as the role of RasGRP1 in lineage commitment during thymocyte development. However, the difficulty of distinguishing consequences of TCR signaling on thymocyte survival and T cell lineage commitment has hindered the assessment of the role of RasGRP1 in regulating these processes. Recent studies using mice devoid of CD4 helper T cells (HD) and Th-POK Tg mice have suggested that Th-POK is a master regulator of commitment of developing thymocytes to the CD4 T cell lineage during positive selection (5, 6). By collaborating with Bosselut group, we backcrossed Th-POK Tg mice onto the RasGRP1$^{-/-}$ background to investigate if the overexpression of Th-POK can rescue CD4 T cell development in AND RasGRP1$^{-/-}$ mice. One of key discovery in their previous paper (6) is that the up-regulation of *zbtb7b* (*thpok*) expression is tightly correlated with the intrathymic TCR signaling received by the

developing thymocytes. Only CD69-expressing DP thymocytes were able to show detectable level of *zbtb7b* expression in the RT-PCR analyses. Since our data revealed that RasGRP1$^{-/-}$ AND TCR Tg DP thymocytes possess reduced TCR expression, appear slightly immature phenotype and exhibit dramatically decreased CD69 expression (Fig. 2.2*B*), plus RasGRP1$^{-/-}$ DP thymocytes exhibit marked deficits in CD69 up-regulation against TCR stimulation (Fig. 2.3*D*), it is interesting to see if the expression of Th-POK is affected by RasGRP1 deficiency. Our preliminary data (Chen et al., manuscript in preparation) have shown that consistent with reduced CD69 expression level, RasGRP1$^{-/-}$ DP from either B6 or AND TCR Tg mice express significantly lower level of *zbtb7b* expression, compared to their wild-type counterpart. Furthermore, we have clearly observed that the enforced expression of Th-POK failed to restore CD4 T cell development within the RasGRP1$^{-/-}$ AND TCR Tg mice, which indicates RasGRP1 may play little or no role in the CD4/CD8-lineage choice while it is extremely crucial for developing thymocytes to translate the positive-selecting TCR signals into pivotal gene expressions, such as *zbtb7b*, that are required for T cell lineage commitment.

Although Th-POK was originally thought be both sufficient and necessary for the CD4 lineage during thymic development (5-7), current findings (8-10) challenged this notion and add more complexity to the mechanism of how Th-POK affects the CD4/CD8-lineage choice. The revised model proposes that Th-POK acts downstream of the TCR and GATA-3 to "lock in" the CD4$^+$ T cell fate (11). The revised model provides a more superior explanation of the latest data. The new data suggest that upon receiving positive-selecting signals through TCRs, immature DPs up-regulate the expression of GATA-3, which is required for the induction Th-POK expression. Th-POK then commits the developing thymocyte to the CD4 T cell lineage by antagonizing Runx-dependent silencing at both the *Cd4* and *zbtb7b* silencing elements, thereby "locking in" the CD4$^+$ T cell fate. As RasGRP1 acts as a signaling intermediate between TCR and crucial nuclear transcription factors such as GATA3 and Th-POK, questions remain as at which stage of this revised model does RasGRP1 fit in. Therefore, future studies will investigate the link between RasGRP1 and those major players in the CD4 lineage commitment, which will provide important novel insight into signaling pathways involved in T cell positive selection and lineage commitment.

6.3   Partial T-cell immunodeficiency and immune dysregulation

Partial T-cell immunodeficiencies constitute a heterogeneous cluster of disorders characterized by an incomplete reduction in T cell number or activity (12). The immune deficiency component of these diseases is less severe than that of the severe T-cell immunodeficiencies and therefore some ability to respond to infectious organisms is retained. Unlike severe T-cell immunodeficiencies, however, partial immunodeficiencies are commonly associated with hyper-immune dysregulation, including autoimmunity and elevated IgE production, both of which are consequences of effector T cell hyperactivity (12). Although the association of effector T cell hyperactivity symptoms with an effector T cell hypoactive disease is counter-intuitive, recent studies in mouse models have shown that hyperactivation of immune system components can be a direct outcome of a partial decrease in T cell number or activity. The 'population-dependent' model proposes that tolerance mechanisms evolve in the context of a complete peripheral T cell niche, and these mechanisms start to break down when they are forced to operate in the suboptimal context of a reduction in the functional T cell population size (12). Partial reduction in the number or function of T cells therefore disturbs the balance of tolerance, generating the unfortunate combination of immunodeficiency and immune dysregulation.

Nearly 40 different genes have been identified that cause monogenic severe T-cell immunodeficiencies in human, which can be grouped into five main categories based on the cellular function of the proteins that they encode. The five categories are: antigen-presentation, TCR signaling, cytokine signaling, V(D)J recombination and basic cellular processes (12). As in the mouse models, genetic mutations with TCR signaling defects have been well studied. Although ZAP70 deficiency blocks signaling through the TCR and typically results in severe T-cell immunodeficiency (13-15), a comprehensive range of mouse models is available for partial TCR signaling blockades, many of which combine partial immunodeficiency with elevated levels of IgE or autoimmunity (12). Loss of a TCR adaptor protein LAT or a LAT mutant (LAT^{136F}) partially impairs TCR signaling, as well as

accompanied by elevated IgE and IgG1 production and multi-organ infiltration of lymphocytes (16, 17). TCR signaling mediator, such as SH2D2A (SRC-homology-2-domain protein 2A; also known as TSAD) or RasGRP1, also belongs to this category.

In Chapter 3, we have provided detailed discussion of a mouse strain call RasGRP1^{lag}, which spontaneously developed a lymphoproliferative autoimmune syndrome exhibiting features of systemic lupus erythematosus (SLE) (18). T cells from these mice have been identified to have a spontaneous mutation for *Rasgrp1* gene, fail to activate Ras and show defects in positive selection. Peripheral RasGRP1^{lag} T cells spontaneously adopted a memory phenotype and were able to transfer disease to lymphopenic recipient mice. CD4$^+$ T cells accumulated in the lymphoid tissues of older RasGRP1^{lag} mice and were resistant to activation-induced cell death. Consistent with these results, our studies also revealed that RasGRP1$^{-/-}$ mice spontaneously acquire an acutely activated and proliferating CD4 population and possess elevated levels of serum autoantibodies (unpublished data) (2). Along with the affected homeostatic proliferation, inflammatory cytokine production, weak T cell responses against pathogenic infection, as well as altered TCR repertoire (2), the RasGRP1$^{-/-}$ mouse model seems to fit the profile of partial T-cell immunodeficiency and immune dysregulation. Since we have noticed that successive backcrossing of the targeted RasGRP1 mutation to the B6 background alleviated the autoimmune phenotype in RasGRP1^{lag} mice, it suggests that genetic factors from strain 129 mice may have contributed to the development of autoimmune disease in RasGRP1-deficient mice. Our results in Chapter 5 (4) support this explanation.

The 'population-dependent' model (12) hypothesizes that under homeostatic conditions, regulatory T cells control autoreactive T cells, through the expression of a complementary TCR repertoire specific for a particular antigen set. In the case of partial thymic immunodeficiency, a reduced number of thymocytes with restricted TCR repertoire develop and enter the periphery. This results in oligoclonal expansion and hence "holes" in the TCR repertoire for both Treg cells and autoreactive T cells, which by chance can create gaps in antigen specific tolerance. However, the lymphopenic compartment in RasGRP1$^{-/-}$ mice might also provide a beneficial environment for the homeostasis and/or expansion of Treg

cells, as evidenced from our adoptive transfer results (Fig. 5.3) (4). Favored expansion of Treg cells as well as increased conversion from non-Treg cells to Treg cells have both been observed, which suggest that the Treg population might have advantages, compared to conventional T cells, in utilizing cytokines (such as IL-2, IL-7 and IL-15) in an "empty" T cell compartment. Therefore, our data in this chapter argue that it is the Treg cells that help RasGRP1$^{-/-}$ mice fight against self-reactive and highly proliferative T cells and remain healthy until at least 1 year of age, providing a reasonable explanation for the question raised in chapter 3.

6.3   Concluding remarks

This thesis characterizes the role of RasGRP1 in T cell development, homeostasis and function. With deficiency of the RasGRP1 molecule, the development of CD4 T cells is severely impaired, arguing RasGRP1 is critically required for transmitting differentiation signaling for CD4 T cell development. As a result, the chronic T cell immunodeficiency due to the poor T cell development in RasGRP1 null-mutant mice might be responsible for the activation, proliferation and exhaustion of CD4 T cells. We also found that RasGRP1 plays a selective role in T cell signaling, controlling the initiation and duration of CD8 T cell immune responses. In addition, RasGRP1 signaling plays a discriminative role during the intrathymic development of CD4 and CD8 Treg cells, but is not required for the peripheral expansion of both lineages. Overall, the results presented in this thesis highlight the crucial role for RasGRP1 signaling pathway in various important aspects of the immune system, and provide a potential strategy of targeting RasGRP1 for therapeutics against autoimmune diseases.

6.4   References

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APPENDIX: BIOSAFETY AND ANIMAL CARE CERTIFICATES

THE UNIVERSITY OF BRITISH COLUMBIA

Department of Health, Safety and Environment

Xiaoxi Chen

has successfully completed a course in

Laboratory Biological Safety

Chair, Biosafety Committee

Biosafety Officer

Thursday, February 05, 2004

Director, Health, Safety and Environment

Certificate of Training for the Completion of a Course in

Chemical Safety Orientation

UBC

Awarded to

Xiaoxi Chen

The course content included WHMIS, chemical handling and storage, safety controls, personal protective
equipment, hazardous waste management and emergency procedures.

Presented by

Department of Health, Safety and Environment

Tuesday, March 16, 2004

David Bell

Occupational Hygiene Officer

THE UNIVERSITY OF BRITISH COLUMBIA

Department of Health, Safety and Environment

Xiaoxi Chen

has successfully completed the Canadian Nuclear Safety Commission requirement in

Radionuclide Safety and Methodology

Including receiving Class 7 Dangerous Goods

Chair, Committee on Radioisotopes and Radiation Hazards

Radiation Safety Officer

Course Date: Thursday, March 25, 2004

Director, Health, Safety and Environment

UBC

THE UNIVERSITY OF BRITISH COLUMBIA

Xiaoxi Chen

has successfully completed the online training requirements of the Canadian Council on
Animal Care (CCAC) / National Institutional Animal User Training (NIAUT) Program

_____
Chair, Animal Care Committee

_____
Veterinarian

Certificate #: 0955

Date Issued: September 08, 2005