[ORIGINAL CONTRIBUTIONS]Impaired Th2 differentiation of CD4+ T cells from Rap2b knockout mice

Impaired Th2 differentiation of CD4$^+$ T cells from Rap2b knockout mice

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ABSTRACT

Rap1 and Rap2 are Ras-like small G proteins. Rap1 plays major roles in embryogenesis and in hematopoietic systems, as demonstrated by gene knockout studies. On the other hand, Rap2 functions had been unclear until we identified its effectors, protein kinases upstream of c-Jun N-terminal kinase (JNK), the stress-activated mitogen-activated kinase (MAPK). Herein, we report the first gene knockout study of Rap2. Rap2b-null mutant (*Rap2b*$^{-/-}$) mice were viable and showed no overt abnormalities in the development or homing of hematopoietic cells. *Rap2b*$^{-/-}$ CD4$^+$ T cells exhibited MAPK activation patterns comparable to those of *Rap2b*$^{+/+}$ counterparts upon acute T-cell receptor (TCR) stimulation. Both *Rap2b*$^{-/-}$ CD4$^+$ T cells and *Rap2b*$^{+/+}$ counterparts secreted similar amounts of IFN-γ upon TCR restimulation after growth under T helper 1 (Th1) polarizing condition. However, secretion of IL-4, IL-5 and IL-13 from Th2-conditioned *Rap2b*$^{-/-}$ cells was half that of *Rap2b*$^{+/+}$ counterparts, suggesting impaired Th2 polarization. Notably, sorted *Rap2b*$^{-/-}$ CD62L^{high} CD4$^+$ T cells, consisting mainly of naïve cells, showed normal Th2 polarization. By contrast, *Rap2b*$^{-/-}$ CD62L^{low} CD4$^+$ T cells showed impaired Th2 polarization, potentially accounting for the abnormality of unsorted *Rap2b*$^{-/-}$ CD4$^+$ T cells. Impaired Th2 polarization has not previously been reported in Rap-related genetic models and requires further investigation. *Ryukyu Med. J., 34* (*1, 2*) *45~52, 2015*

Key words: Rap2b, small G protein, knockout mouse, Th2 polarization

INTRODUCTION

Rap small G proteins (Rap1a, Rap1b, Rap2a, Rap2b, and Rap2c) are closely related to Ras proteins (H-Ras, N-Ras, and K-Ras). Rap1 regulates levels of activity of integrin and extracellular signal-regulated kinase (ERK). Rap2 function had been unclear until we identified Traf2- and Nck-interacting kinase (TNIK) and its homologs as Rap2-specific effectors[1, 2-5)]. These kinases activate c-Jun N-terminal kinase (JNK), and Rap2a knockdown suppresses glutamate receptor-induced TNIK-mediated JNK activation in neurons[6)]. In addition, Rap2b, but not Rap2a or Rap2c, activates phospholipase Cε (PLCε), a dual enzyme[1, 7, 8)]. The Rap2b-PLCε-Ras-ERK pathway is activated by Gs-coupled receptors[8)].

Rap proteins are activated and inactivated by GDP/GTP exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Rap GEFs include C3G, CalDAG-GEFI, RA-GEF-1/PDZ-GEF-1/RapGEF2, RA-GEF-2/PDZ-GEF-2, and PLCε, which activates Rap1 only[1, 9-11)]. Rap GAPs include SPA-1, a

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primary hematopoietic tissue Rap GAP[1].

Rap signaling plays key roles during embryogenesis and in the hematopoietic systems. Rap suppression causes embryonic lethality in *C3G*$^{-/-}$ and *RA-GEF-1/PDZ-GEF-I/RapGEF2*$^{-/-}$ mice, neutrophilia and platelet dysfunction in *CalDAG-GEFI*$^{-/-}$ mice[12], and B-cell dysfunction and splenomegaly in *RA-GEF-2/PDZ-GEF-2*$^{-/-}$ mice[13]. Rap suppression through *SPA-1* transgenic overexpression reduces thymic cellularity due to defective β-selection of $\alpha\beta$ T cells but not $\gamma\delta$ T cells: CD4$^-$ CD8$^-$ double-negative (DN) thymocytes accumulate, and double-positive (DP) cells are diminished. On the other hand, excessive Rap signaling in *SPA-1*$^{-/-}$ mice makes CD44^{high} CD4$^+$ memory T cells nonresponsive to T-cell receptor (TCR) stimulation due to ERK suppression[14].

Both *Rap1a*$^{-/-}$ [15] and *Rap1b*$^{-/-}$ [16] mice are partially embryonic lethal, and *Rap1a*$^{-/-}$;*Rap1b*$^{-/-}$ compound mice are completely embryonic lethal[15]. T cells from *Rap1a*$^{-/-}$ mice proliferate poorly after stimulation with immobilized anti-CD3ε monoclonal antibody (mAb) or anti-CD3ε plus anti-CD28 mAbs[17], and neutrophils from these mice produce lower levels of superoxide[15]. On the other hand, *Rap1b*$^{-/-}$ mice exhibit reduced lymph node (LN) B cell counts[18], impaired T cell-dependent humoral immunity[19], B cell maturation defects, and natural killer (NK) cell dysfunctions[20].

Rap2$^{-/-}$ mice have not been available. However, a Rap2 effector, Misshapen/NIKs-related kinase (MINK)[4], is implicated in negative selection in the thymus[21]. Mice reconstituted with MINK-knockdown hematopoietic stem cells exhibit CD4$^+$ single-positive (SP) cell accumulation due to DP cell defects in activation of JNK and induction of pro-apoptotic proteins following TCR stimulation by self-antigens. Thus, we speculated that Rap2 might affect negative selection by regulating MINK and JNK. Rap2 might also affect CD4$^+$ Th cell subsets because the differentiation of CD4$^+$ T cells from JNK-deficient mice into Th1 cells is defective but differentiation into Th2 cells is normal[22]. To gain insights into the roles of Rap2 in embryogenesis and the hematopoietic system, we generated *Rap2b*$^{-/-}$ mice.

MATERIALS and METHODS

Targeted disruption of the *Rap2b* gene

Rap2b$^{-/-}$ mice were generated as described previously[23] (Fig. 1). *Rap2b* gene contains a single exon with an open reading frame (ORF) encoding 183 amino acids. A C57BL/6J genomic library (BACPAC Resource Center, Oakland, CA, USA) was screened with a cDNA probe[5, 23]. The 11.8-kbp *Ase*I-*Sac*I fragment containing the entire *Rap2b* exon was subcloned into pBluescript. The targeting vector contained two *lox*P sites flanking the ORF (floxed allele, *Rap2b*^{flox}). The vector was linearized and electroporated into C57BL/6J-derived Bruce4 embryonic stem cells. Clones were selected in the presence of G418, and homologous recombination was confirmed by Southern blotting. Correctly targeted cells were injected into Balb/c blastocysts, which were then transplanted into pseudopregnant females. Chimeric offspring were mated to C57BL/6J mice, and germ-line transmission of the *Rap2b*^{flox} allele was confirmed. The *Rap2b*$^{flox/+}$ mice were then crossed with CAG-Cre transgenic mice (C57BL/6 background)[24] to delete the *lox*P-flanked region (deletion allele, *Rap2b*$^-$). All alleles were confirmed by Southern blotting (AlkPhos Direct Labelling and Detection System, GE Healthcare, Buckinghamshire, UK). *Rap2b*$^{-/+}$ mice were intercrossed to generate *Rap2b*$^{-/-}$ and *Rap2b*$^{+/+}$ littermates and genotyped by multiplex PCR. Wild-type allele primers were 5'-TGA GCT ATT CGC AGA GAT CGT G-3' and 5'-ACC GTC TGA ACC AAA ATC AAC GA-3'. Deletion allele primers were 5'-GAA GAG CTC CAG CCG TTG TC-3' and 5'-TCC CCT TCA CTT CCT CTG CTT C-3'. Detection of Rap2b mRNA expression by reverse transcription PCR (RT-PCR) was performed with primers 5'-CAC AGG TTC CTG AGG CAA A-3' and 5'-ATG AGA AGA GGG CCA CCA TA-3' using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as the internal control, as described previously[25]. RT-PCR primers for GATA3 were 5'-GAA ACC GGA AGA TGT CTA GCA AAT CG-3' and 5'-ATG TGG CTG GAG TGG CTG AAG-3'. Mice were maintained under specific pathogen-free conditions and used at 8-12 weeks of age. All experiments were approved by the institutional Animal Experiments Safety and Ethics Committee and the Living Modified Organism Experiments Safety Committee (University of the Ryukyus, Okinawa Institute of Science and Technology Graduate University, and Tokyo Metropolitan Institute of Gerontology).

Flow cytometry

Flow cytometry (FCM) and magnetic cell isolation were performed as described[26]. Single-cell leu-

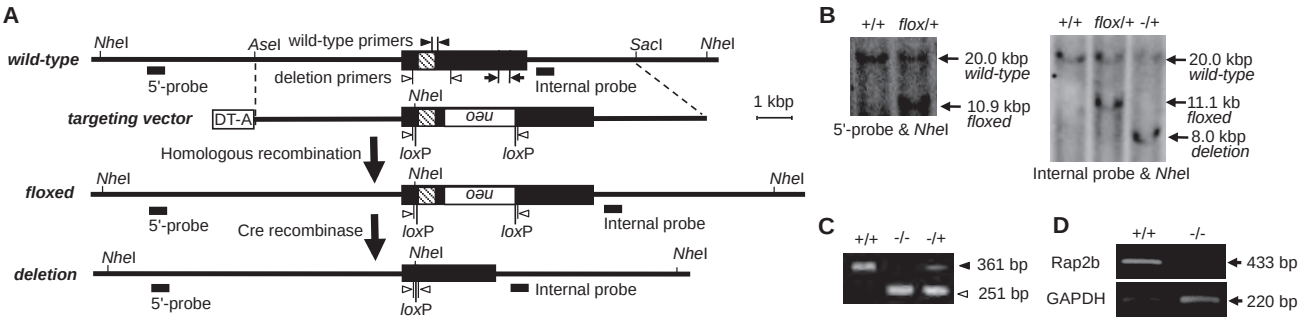


Fig. 1 **Generation of *Rap2b*$^{-/-}$ mice** (A) *Rap2b* knockout strategy. In the wild-type allele, hatched and filled boxes represent ORF and untranslated regions, respectively. Thick lines represent Southern blotting probes. Arrowheads represent primer pairs for PCR genotyping. Arrows represent primers for RT-PCR. The targeting vector contained a diphtheria toxin A cassette (DT-A), an artificial *Nhe*I site, and a neomycin resistance cassette PGK-neo (neo) in a reverse orientation. (B) Southern blotting of *Nhe*I-digested tail DNA from mice carrying various alleles with indicated probes. The floxed allele was confirmed by the detection of 10.9- and 11.1-kbp bands. The deletion allele was confirmed by the detection of an 8.0-kbp band. (C) Multiplex PCR genotyping with tail DNA. The reaction contained both primer pairs represented by filled and open arrowheads in (A) for the amplification of 361- and 251-bp fragments from wild-type and deletion alleles, respectively. (D) RT-PCR analysis of *Rap2b* mRNA in LN CD4$^+$ T cells.

kocyte suspensions were prepared from pooled axial and inguinal LNs, spleen, and thymus. For surface staining, cells pretreated with Fc blocker (BD Biosciences, San Jose, CA, USA) were incubated with fluorochrome-conjugated mAbs. mAbs were from BD Biosciences, except for anti-CD19 (Caltag, Burlingame, CA, USA) and anti-CD44 (eBioscience, San Diego, CA, USA). Samples were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). For isolation of CD4$^+$ T cells, Fc blocker-pretreated LN cells were incubated with anti-CD4-phycoerythrin followed by anti-phycoerythrin magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and sorted with an AutoMACS sorter (Miltenyi Biotec). CD4$^+$ CD62L^{high} and CD4$^+$ CD62L^{low} LN cells were isolated with the CD4$^+$ CD62L$^+$ T Cell Isolation Kit II (Miltenyi Biotec) after depletion of CD4$^+$ CD25$^+$ regulatory T cells (Treg) with the kit-supplied anti-CD25 mAb.

Cell stimulation

For activation of mitogen-activated protein kinases (MAPKs), cells in RPMI-1640 medium containing 0.5% bovine serum albumin were incubated with hamster mAbs against CD3ε and CD28 (BD Biosciences) at 4℃ for 30 min, washed, and then incubated with a goat anti-hamster IgG antibody (Jackson Immunoresearch, West Grove, PA, USA) at 37℃. Stimulation was stopped with ice-cold phosphate-buffered saline, and cell lysates were subjected to western blotting as described[2]. All MAPK antibodies were from Cell Signaling Technology (Danvers, MA, USA).

For Th1/Th2 polarization assays, cells were generally plated in 24-well plates pre-coated with anti-CD3ε and anti-CD28 mAbs (5μg/ml each; eBioscience) at an initial density of 2.5×10^5 cells/well in 2 ml of RPMI-1640 containing 10% fetal calf serum, 50μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, pH 7.4, and 1 mM sodium pyruvate (primary stimulation, day 0). Either 20 ng/ml recombinant mouse IL-12 (rIL-12, PeproTech, Rocky Hill, NJ, USA), 10μg/ml anti-IL-4 mAb (BioLegend, San Diego, CA, USA), and 10 ng/ml rIL-2 (PeproTech) (Th1 condition), or 20 ng/ml rIL-4 (PeproTech), 10μg/ml anti-IFN-γ mAb (BioLegend), and 10 ng/ml rIL-2 (Th2 condition) were included. On day 3, before reaching high density, cells were split into two fresh antibody-coated plates and refed with fresh polarization media. For restimulation, cells were harvested on day 5, washed, and replated on fresh antibody-coated 96-well plates (1μg/ml each mAb) at the initial density in the presence of 10 ng/ml rIL-2 alone (neutral condition). Cytokines in culture supernatants were quantified with enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). Cell proliferation was examined by using the carboxyfluorescein diacetate succinimidyl ester dye method with a Guava EasyCyte Plus flow cytometer and Guava Ex-

press Pro software (MerckMillipore, Darmstadt, Germany).

Statistical analysis

A two-tailed Student's t test assuming equal variance was performed using Microsoft Excel software.

RESULTS

Normal embryogenesis of *Rap2b*$^{-/-}$ mice

Using the Cre/*lox*P recombination system (Fig. 1A) and CAG-Cre transgene acting at the zygote stage, we generated global heterozygous *Rap2b*$^{-}$ mice (*Rap2b*$^{-/+}$; Fig. 1B). Intercrossing yielded offspring at the expected Mendelian frequency (24%:53%:23% ratio of *Rap2b*$^{+/+}$:*Rap2b*$^{-/+}$:*Rap2b*$^{-/-}$, n = 152), as assessed by PCR (Fig. 1C). The *Rap2b*$^{-}$ allele lacked the ORF, as verified by sequencing of the PCR product (the 251-bp fragment, not shown). Loss of *Rap2b* mRNA expression in *Rap2b*$^{-/-}$ mice was confirmed by RT-PCR in LN CD4^{+} T cells (Fig. 1D). Thus, embryogenesis was normal in *Rap2b*$^{-/-}$ mice.

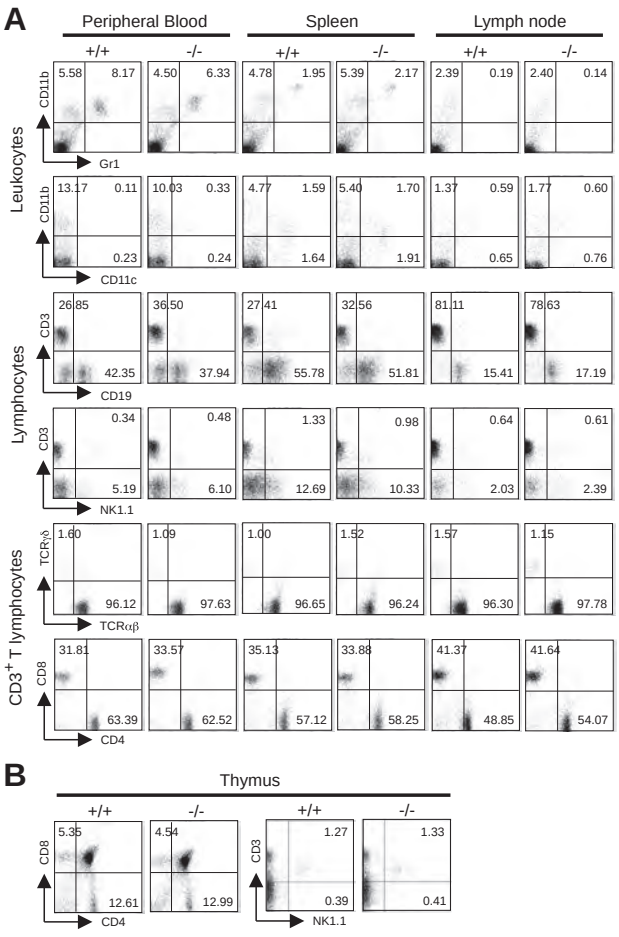


Fig. 2 **Leukocyte profiles** (A) Leukocytes from peripheral blood, spleen and lymph nodes were analyzed for the indicated markers by FCM. Each panel displays results for a pair of markers as a bivariate dot plot on four-decade logarithmic scales (10^0–10^4). The analysis gate for T lymphocytes was set based on CD3. Numbers represent percentages of cells in quadrants of interest. Data are representative of two experiments. (B) Thymocytes were analyzed and the data presented as in (A). Live lymphocytes were gated based on forward and side scatter.

Normal leukocyte development in *Rap2b*$^{-/-}$ mice

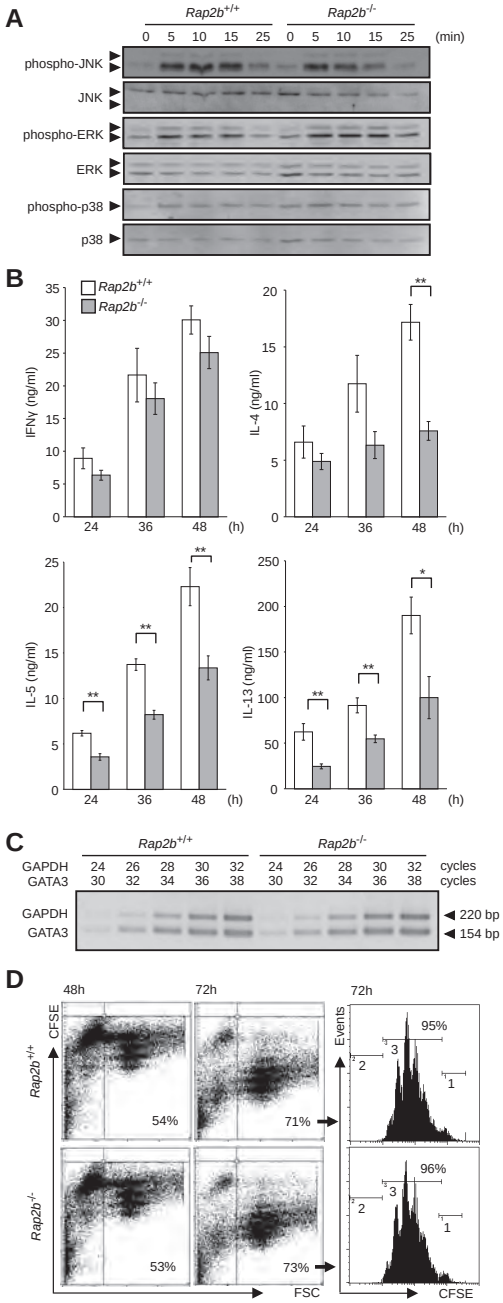
Splenic, LN, and thymic cellularity was similar in *Rap2b*$^{-/-}$ and *Rap2b*$^{+/+}$ littermates (not shown). FCM analyses of leukocytes from peripheral blood, spleen, and LNs found normal proportions of granulocytes, dendritic cells/monocytes/macrophages, B cells, NK cells, $\alpha\beta$ T cells, $\gamma\delta$ T cells, CD4^{+} T cells, and CD8^{+} T cells (Fig. 2A). Furthermore, *Rap2b*$^{-/-}$ thymocytes showed normal percentages of DN, DP, CD4^{+} SP, and CD8^{+} SP cells (Fig. 2B). Thus, β-selection and positive selection of self-MHC-restricted T cells were normal, as in *Rap1a*$^{-/-}$ and *Rap1b*$^{-/-}$ mice[15, 20]. Because *Rap2b*$^{-/-}$ mice showed no sign of autoimmune disorder (not shown), negative selection of self-reactive lymphocytes occurred normally. Percentages of NK1.1^{+} CD3^{+} NKT cells were also normal, as in *Rap1a*$^{-/-}$ and *Rap1b*$^{-/-}$ mice[20]. Thus, leukocyte development and homing appeared to be normal in *Rap2b*$^{-/-}$ mice.

Normal acute MAPK activation but impaired Th2 polarization of *Rap2b*$^{-/-}$ CD4^{+} T cells

When LN CD4^{+} T cells from *Rap2b*$^{+/+}$ and *Rap2b*$^{-/-}$ littermates were stimulated in suspension via antibody-mediated crosslinking of CD3 and CD28, they showed similar time courses and extents of activation of JNK, ERK, and p38 MAPKs (Fig. 3A). Thus, we did not detect any effects of *Rap2* deletion on MAPK activation in this acute TCR stimulation assay.

The Th1/Th2 polarization of LN CD4^{+} T cells was examined next. After primary stimulation with plate-bound CD3 and CD28 mAbs under Th1 or Th2 conditions, cells were restimulated under the neutral condition (Fig. 3B). Levels of IFN-γ secretion from Th1-conditioned *Rap2b*$^{+/+}$ and *Rap2b*$^{-/-}$ CD4^{+} T cells during restimulation were similar, which suggested normal Th1 polarization had occurred. By contrast, IL-4 secretion from Th2-conditioned

Rap2b$^{-/-}$ CD4$^+$ T cells was roughly half that of *Rap2b*$^{+/+}$ cells. Similar results were obtained for other Th2 cytokines IL-13 and IL-5, suggesting impaired Th2 polarization. The relative level of mRNA encoding GATA3, the key transcriptional regulator of Th2 polarization, was not reduced in *Rap2b*$^{-/-}$ CD4$^+$ T cells (Fig. 3C). The reduced Th2 cytokine secretion appeared not to be due to perturbed cellular fitness during primary stimulation, because *Rap2b*$^{+/+}$ and *Rap2b*$^{-/-}$ cells proliferated similarly (Fig. 3D).



Defective Th2-polarization of *Rap2b*$^{-/-}$ CD62L^{low} CD4$^+$ T cells

To understand the underlying mechanisms of impaired Th2 polarization, we examined the relative proportions of CD4$^+$ T cell subpopulations within the "total" CD4$^+$ T cell population used above. Total CD4$^+$ T cells from naïve mice contain CD62L^{high} CD44^{low} "naïve" cells, CD62L^{high} CD44^{high} "central memory" (CM) cells, and CD62L^{low} CD44^{high} "effector memory" (EM) cells[27]. CD62L^{low} cells were implicated in efficient Th2 polarization of total cells by earlier studies[28]. However, the relative proportions of CD62L^{low} cells in *Rap2b*$^{+/+}$ and *Rap2b*$^{-/-}$ cells were comparable (Fig. 4A).

CD62L^{high} and CD62L^{low} CD4$^+$ T cells were then sorted after depletion of Treg cells and Th2-polarized in the same way as described above (Fig 4B). In marked contrast to the total cells, *Rap2b*$^{-/-}$ and *Rap2b*$^{+/+}$ CD62L^{high} cells secreted IL-13 equally upon restimulation. Consistent with a previous report[28], *Rap2b*$^{+/+}$ CD62L^{low} cells secreted more IL-13 than CD62L^{high} cells did. Notably, however, *Rap2b*$^{-/-}$ CD62L^{low} cells secreted only half the IL-13 secreted by their *Rap2b*$^{+/+}$ counterparts, recapitulating the situation in total cells. It is again unlikely to be due to differences in cellular fitness conditions between *Rap2b*$^{-/-}$ and *Rap2b*$^{+/+}$ CD62L^{low} cells (*Rap2b*$^{-/-}$/*Rap2b*$^{+/+}$ ratio of cell numbers after restimulation for 48 h = 1.07 $\pm$ 0.11, two independent experiments).

Fig. 3 **MAPK activation and cytokine production** (A) CD4$^+$ T cells were subjected to antibody-mediated CD3/CD28 crosslinking for the indicated time. Western blotting of cell lysates was performed with the indicated antibodies. (B) CD4$^+$ T cells were stimulated with plate-bound mAbs against CD3 and CD28 for 5 days under Th1 or Th2 conditions. Cells were then restimulated under neutral condition, and the culture supernatant cytokines were quantified. Bars represent mean $\pm$ SD from triplicate culture wells. Data are representative of two independent experiments (*$p < 0.05$, **$p < 0.01$). (C) RT-PCR analyses of LN CD4$^+$ T cells. GAPDH and GATA3 fragments were amplified separately, and products at indicated numbers of amplification cycles were subjected to co-electrophoresis. (D) Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cells were stimulated under Th2 condition and harvested at 48 and 72 h. CFSE/forward scatter (FSC) dot plots were compared to set a gate to exclude cells that are dead (FSC^{int} CFSE^{int}) or still non-blasting (FSC^{int} CFSE^{high}) at 72 h. In histograms of gated cells at 72 h (roughly 70% of total cells), six division peaks are present within region 3 (dividing cells, roughly 95% of gated cells) in addition to a single peak within region 1 (non-dividing cells). Region 2 includes unlabeled cells.

DISCUSSION

We have generated and characterized mice lacking a Rap2 gene for the first time. The global null mutation (*Rap2b*$^{-/-}$) did not affect embryogenesis, and the development and homing of various leukocytes were unaffected as well. Rap2b may not function in these processes, or Rap2a, Rap2c or both may play redundant roles. *Rap2b*$^{-/-}$ CD4^{+} T cells showed normal TCR-mediated activation of MAPKs, including JNK. However, their MAPK activation pattern may be different from that of *Rap2b*$^{+/+}$ cells under different TCR stimulation conditions. For instance, different TCR stimulation conditions result in distinct types of ERK activation, which are related to differential crosstalk with the phosphatidylinositol-3 kinase pathway[29].

The impaired Th2 polarization of total *Rap2b*$^{-/-}$ CD4^{+} T cells (Fig. 3B), and specifically of CD62L^{low} cells (Fig 4B) has not been observed in other *Rap*-related models[15]. This CD62L^{low} cell defect could account for the impairment of total CD4^{+} T cells (Fig. 3B). In a study on which our culture conditions are largely based[28], CD4^{+} T cells (total or CD62L^{high} cells) were stimulated with plate-bound anti-CD3 mAb in the presence of rIL-2 plus rIL-4. After restimulation in the presence of rIL-2 alone, CD62L^{high} cells, depleted of CD62L^{low} cells, secreted much less IL-4 than the total cells did.

The reason for the *Rap2b*$^{-/-}$ CD62L^{low} cell defect remains unknown. The CD62L^{high} cell subpopulation consisted mainly of naïve cells and a smaller number of CM cells, and showed normal Th2 polarization. On the other hand, CD62L^{low} cells consisted of EM cells, and their defect might reflect poor conversion of Th1-committed EM cells into Th2 cells, which could occur *in vitro*[30] . Rap2b might facilitate such difficult processes even though it is dispensable for easy Th2 polarization of CD62L^{high} cells. Rap2 could undergo activation after TCR stimulation[31]. The Rap2 effector TNIK participates in Wnt/β-catenin signaling, which augments Th2 polarization through GATA3 mRNA upregulation after TCR stimulation[32, 33]. The Rap2b-PLCε-Ras-ERK pathway[8] may also augment Th2 polarization by stabilizing GATA3 at the protein level without affecting GATA3 mRNA levels. Continued ERK activation at a certain level appears to be necessary for GATA3 protein stability, because treatment of CD4^{+} T cells with an ERK pathway inhibitor for 24 h under Th2-polarizing conditions decreases GATA3 protein levels without affecting GATA3 mRNA levels[34]. As Treg cells were depleted before sorting, they are not involved in the *Rap2b*$^{-/-}$ CD62L^{low} cell defect. They also do not appear to be involved in impaired Th2 polarization of total CD4^{+} T cells, because their relative proportions in *Rap2b*$^{+/+}$ and *Rap2b*$^{-/-}$ cells were comparable as assessed by FCM analysis of Treg intracellular marker Foxp3 (unpublished observation). Further studies are needed to clarify the role of Rap2b in Th2 polarization of CD4^{+} T cells.

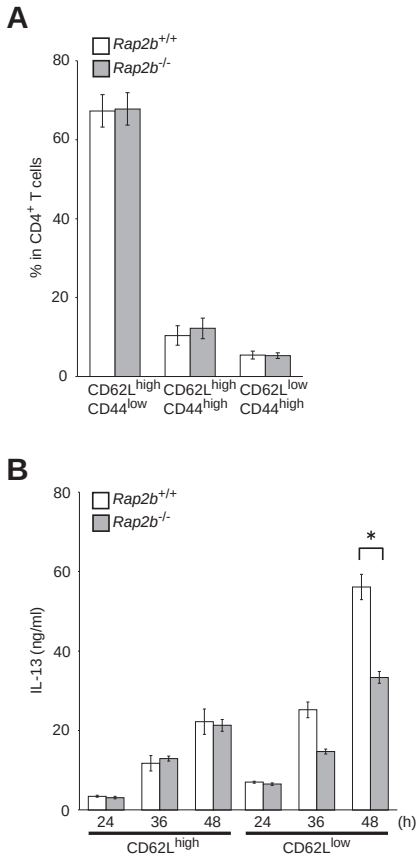


Fig. 4 **Quantitative and functional comparisons of CD4^{+} T cell subpopulations** (A) LN cells from *Rap2b*$^{+/+}$and *Rap2b*$^{-/-}$ littermates were analyzed for the indicated markers by FCM. Analysis gates were set on live CD4^{+} T cells. Bars represent the percentage of cells with the indicated marker profiles (mean ± SD from five independent experiments). (B) CD62L^{high} and CD62L^{low} CD4^{+} T cells from *Rap2b*$^{-/-}$ and *Rap2b*$^{+/+}$ mice were stimulated under Th2 condition, restimulated under neutral condition, and IL-13 in culture supernatants was quantified (mean ± SD from three culture wells, representative of two independent experiments, *$p < 0.05$).

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