CD5-Dependent CK2 Activation Pathway Regulates Threshold for T Cell Anergy

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CD5-Dependent CK2 Activation Pathway Regulates Threshold for T Cell Anergy

Christine M. Sestroto,* Donald J. McGuire,† Patrizia De Sarro,‡ Emily C. Brantley,§ Gloria Soldevila,† Robert C. Axtell,‖ and Chander Raman*§

CD5 activates casein kinase 2 (CK2), a serine/threonine kinase that constitutively associates with the CK2-binding domain at the end of its cytoplasmic tail. To determine the physiological significance of CD5-dependent CK2 activation in T cells, we generated a knock-in mouse that expresses a CD5 protein containing a microdeletion with selective inability to interact with CK2 (CD5ΔCK2BD). The levels of CD5 on developing and mature T cell populations from CD5ΔCK2BD mice and CD5 wild-type (WT) mice were similar. The thymus of CD5ΔCK2BD mice contained fewer double-positive thymocytes than that of both CD5WT and CD5 knockout (KO) mice, although the numbers of all other immature and mature T cell populations were unaltered. CD5ΔCK2BD T cells hypoproliferated and exhibited enhanced activation-induced cell death when stimulated with anti-CD3 or cognate peptide in comparison with CD5WT T cells. We also found that functional CD5-dependent CK2 signaling was necessary for efficient differentiation of naive CD4+ T cells into Th2 and Th17 cells, but not Th1 cells. We previously showed that anti-CD3 or cognate peptide in comparison with CD5WT T cells. We also found that functional CD5-dependent CK2 signaling was necessary for efficient differentiation of naive CD4+ T cells into Th2 and Th17 cells, but not Th1 cells. We previously showed that experimental autoimmune encephalomyelitis (EAE) in CD5KO mice was less severe and delayed in onset than in CD5WT mice. Remarkably, CD5ΔCK2BD mice recapitulated both EAE severity and disease onset of CD5KO mice. Increasing the immunization dose of myelin oligodendrocyte glycoprotein 35–55 peptide, a model that mimics high-dose tolerance, led to decreased severity of EAE in CD5WT mice but not in CD5KO or CD5ΔCK2BD mice. This property was recapitulated in in vitro restimulation assays. These results demonstrate that CD5–CK2 signaling sets the threshold for T cell responsiveness and is necessary for efficient generation of Th2 and Th17 cells. The Journal of Immunology, 2012, 189: 000–000.
CD5–CK2 SIGNALS PROMOTE DIFFERENTIATION OF TH2 AND TH17 CELLS

CD4+CD25+ T cells were purified from lymph nodes and spleens of naive mice by two-step magnetic chromatography using cell purification reagents from Invitrogen (DynaBeams) and StemCell Technologies (EasySep; Vancouver, BC, Canada). The CD4+ T cells were stimulated with anti–CD3 (1.0 μg/ml) or MOG35–55 peptide (10 μg/ml) in culture medium (IMDM, 10% FBS, 1% sodium pyruvate, 1% nonessential amino acids, and 0.5 mM 2-ME), with no added cytokines or Abs for unpolarized, 10 ng/ml IL-12 plus 5 μg/ml anti–IL-4 (11B1) for Th1-polarized, 10 ng/ml IL-12 plus 10 ng/ml IL-23 plus 10 μg/ml anti–IFN-γ (XM1G2) for Th17-polarized, or 5 μg/ml TGF-β plus 20 ng/ml IL-6 plus 10 ng/ml IL-23 plus 10 μg/ml anti–IFN-γ plus 5 μg/ml anti–IL-4 for Th1-polarized cultures in the presence of irradiated APCs. All cytokines were purchased from eBioscience or BioLegend. Irradiated Thyl.2 cell-depleted splenocytes or 6 bone marrow-derived dendritic cells were used as APCs.

Cell proliferation, apoptosis measurement, lymphocyte activation, and calcium mobilization

For these experiments “untouched” CD4+CD25+ T cells and CD8+ T cells were purified from spleens of naive mice or from mice immunized with 150 MOG35–55 peptide 7 d previously as described above. Proliferation was measured by two independent approaches, [3H]thymidine incorpora-
tion and 5-ethyl-2'-deoxyuridine (EdU) incorporation. The EdU incorporation proliferation assay was performed with CD4+CD25- T cells stimulated with varying concentrations of MOG35-55 peptide or anti-CD3 in the presence of irradiated APCs as described previously or with CD8- T cells stimulated with varying concentrations of anti-CD3 and 1 μg/ml anti-CD28 (37.51; BioLegend) (27). To measure the proportion of dividing cells, EdU (10 μM) was added for the last 1 h of the proliferation assay and its incorporation was measured using the Click-iT EdU flow cytometry assay (Invitrogen) following the manufacturer’s recommended protocol. In some experiments, EdU incorporation or CFSE dilution was determined after coculturing WT-CD5- or ΔCK2BD-CD5-expressing CD4+ T cells and stimulating with anti-CD3 mAb or MOG35-55 peptide as appropriate. To distinguish between the different CD5-expressing T cells, T cells from one of the groups was labeled with CFSE (CFDA-SE; Molecular Probes, Eugene, OR) just prior to coculturing. Dilution of CFSE in labeled, dividing CD4+ T cells was also used to track cell division.

For the measurement of apoptosis, CD4+CD25- or CD8- T cells were stimulated with varying concentrations of MOG35-55 peptide or anti-CD3 for different time periods, and in some instances under Th17 polarizing conditions. The cells were then stained with annexin V-FITC (BD Biosciences) and 7-aminoactinomycin D (7-AAD; BioLegend) to quantitate apoptotic and necrotic cell populations. The strength of this novel model is that the CD5-dependent CK2 binding-deficient mouse was generated by reconstituting the CD5KO mouse with a mutant CD5-expressing transgene under the control of the CD2 promoter (27).

The mean expression levels of CD5 on all T cell populations in the thymus, lymph nodes, and spleens of CD5WT and CD5ΔCK2BD mice were similar (Fig. 1C and data not shown). We next examined the effect of loss of CD5-dependent CK2 signaling on developing T cell populations in the thymus and in mature CD4+ and CD8+ T cells in the spleen and lymph node. As previously reported, the proportion and absolute numbers of CD4+ and CD8+-expressing double-positive (DP) and single-positive T cells in CD5WT and CD5KO mice were similar (Fig. 1D, Table I) (32). In contrast, the thymus of CD5ΔCK2BD mice contained significantly fewer DP cells compared with both CD5WT and CD5KO mice (Table I). The DP population in CD5ΔCK2BD mice contained an expanded population of cells that were CD4+CD8-, possibly representing preapoptotic cells. During negative selection, DP thymocytes go through a transitional CD4+CD8- step just before undergoing apoptosis (33). Double-negative thymocytes were higher in the thymus of CD5ΔCK2BD mice compared with that in CD5WT or CD5KO mice (Fig. 1D). The proportion and numbers of CD4+ or CD8+ single-positive cells in the thymus of CD5ΔCK2BD mice were not significantly different from those in the thymus of CD5WT or CD5KO mice in the C57BL/6 and BALB/c mice (34). In contrast, Dasu et al. (35) reported equal numbers of Tregs in CD5WT and CD5KO mice in these tissues in CD5KO mice consistently contained a greater number of Tregs than those in either CD5WT or CD5KO mice (Fig. 1D, Table I, and data not shown). We also observed significantly fewer CD4+ T cells in the lymph nodes and B220+ cells in spleens of CD5ΔCK2BD mice compared with CD5WT and CD5KO mice. The lower numbers of peripheral CD4+ T cells might be related to loss of survival as a result of inability to activate the CD5-dependent CK2 activation pathway. However, the presence of fewer follicular B cells in the spleen was unexpected, as they do not express detectable levels of CD5. We observed that the frequency of natural regulatory T cells (nTregs; CD4+CD25+Foxp3+) in lymph nodes and spleens of CD5ΔCK2BD mice were similar (Supplemental Fig. 1A). However, these tissues in CD5KO mice consistently contained a greater number of Tregs than those in either CD5WT or CD5ΔCK2BD mice. This observation is consistent with that previously reported in BALB/c mice (34). In contrast, Dasu et al. (35) reported equal numbers of nTregs in CD5WT and CD5KO mice in the C57BL/6 strain. The reason for the difference in the findings between the present study and that of Dasu et al. is currently unknown.
CD5-dependent CK2 signaling pathway regulates threshold for severity of EAE

We previously showed that both severity and incidence of MOG35–55 peptide-induced EAE in CD5KO mice reconstituted with CD5WT transgene was significantly reduced compared with CD5WT mice, CD5KO mice, and CD5KO mice reconstituted with CD5WT transgene (27). The finding demonstrated that the CD5–CK2 binding-dependent signaling pathway altered path-

CD5-dependent CK2 signaling promotes differentiation of Th2 and Th17 cells

We previously showed that both severity and incidence of MOG35–55 peptide-induced EAE in CD5KO mice reconstituted with a CD5ΔCK2BD transgene was significantly reduced compared with CD5WT mice, CD5KO mice, and CD5KO mice reconstituted with CD5WT transgene (27). The finding demonstrated that the CD5–CK2 binding-dependent signaling pathway altered path-

Table 1. Total numbers of lymphocyte populations in different tissues of CD5WT, CD5KO, and CD5ΔCK2BD mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Thymus CD4⁺CD8⁻ (DN)</th>
<th>Thymus CD4⁺CD8⁺ (DP)</th>
<th>Lymph Nodes CD4⁺</th>
<th>Lymph Nodes CD8⁺</th>
<th>Spleen B220⁺</th>
<th>Spleen CD4⁺</th>
<th>Spleen CD8⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5WT</td>
<td>43.3 ± 2.7</td>
<td>210.1</td>
<td>621.2</td>
<td>820.0</td>
<td>231.2</td>
<td>5511.0</td>
<td>772.3</td>
</tr>
<tr>
<td>CD5KO</td>
<td>39.4 ± 3.4</td>
<td>305.0</td>
<td>687.0</td>
<td>1111.1</td>
<td>361.0</td>
<td>1110.0</td>
<td>741.0</td>
</tr>
<tr>
<td>CD5ΔCK2BD</td>
<td>33.0 ± 2.7</td>
<td>210.0</td>
<td>621.0</td>
<td>820.0</td>
<td>231.0</td>
<td>5510.0</td>
<td>772.0</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM (n = 4–5 mice).

*P < 0.05; CD5ΔCK2BD versus CD5WT or CD5KO mice.

*p < 0.05; CD5KO versus CD5WT or CD5ΔCK2BD mice.
ogenesis in a disease that is T cell dependent. We now wanted to test whether this observation was recapitulated in the CD5\textsuperscript{CK2BD} knock-in mouse where the expression of CD5 is physiologically regulated. We found that EAE in CD5\textsuperscript{CK2BD} mice and CD5KO mice was significantly delayed in onset and less severe than that in CD5WT mice (Fig. 2A, Table II). The incidence of disease in both the CD5 mutant mice was also lower but the difference was not statistically significant. Because CD5 regulates the threshold of T cell activation, we asked whether the severity of EAE also correlated with increase in MOG\textsubscript{35–55} peptide immunization dose. We observed that in CD5WT mice immunized with 150 \( \mu \)g MOG\textsubscript{35–55} peptide, the onset of EAE was significantly earlier and more severe than for those immunized with 50 \( \mu \)g MOG\textsubscript{35–55} peptide (Table II). Remarkably, EAE in CD5WT mice immunized with a higher immunization dose of 450 \( \mu \)g MOG\textsubscript{35–55} peptide was significantly less severe (accumulative score) than that observed with 150 \( \mu \)g peptide, a characteristic that resembles the generation of high-dose tolerance. In contrast, the severity of EAE in both CD5KO and CD5\textsuperscript{CK2BD} mice correlated with immunization dose with no indication of high-dose anergy (Table II). Furthermore, with an increase in immunization dose the severity and time of onset of EAE in CD5KO mice and CD5\textsuperscript{CK2BD} mice approached and equaled that of CD5WT mice (Fig. 2B, 2C, Table II). A striking finding is that with respect to EAE disease severity, the mere deletion of the four amino acid CK2 binding domain within CD5 recapitulated the phenotype of the CD5KO mouse.

We analyzed the CNS cell infiltration in mice of all three CD5 strains to evaluate the correlation between disease severity and Th cell populations. The results showed that the proportion and absolute number of Th1 cells in the CNS of CD5WT, CD5KO, and CD5\textsuperscript{CK2BD} mice were similar. With respect to Th17 cells and Th-IFN\textgamma-IL17a cells (double producers), the spinal cords of CD5\textsuperscript{CK2BD} mice with EAE contained fewer cells of this population than did both CD5WT and CD5KO mice (Fig. 2D). Our results suggest that the CD5-dependent CK2 activation pathway has an impact preferentially on the generation of IL-17a–expressing Th cells, an observation also made in our previous study in the CD5\textsuperscript{CK2BD}-Tg mouse (27).

**CD5-dependent CK2 signaling pathway is necessary for efficient generation of Ag-specific Th2 and Th17 cells**

To test whether the CD5–CK2 signaling pathway has an impact on differentiation of naïve CD4\textsuperscript{+} T cells to Th1 or Th17 cells under nonpolarizing and polarizing conditions, we bred the C57BL/6 MOG\textsubscript{35–55}-specific TCR Tg mouse, 2D2TCR\textsuperscript{MOG}, with both the CD5KO mouse and CD5\textsuperscript{CK2BD} mouse. Under nonpolarizing conditions, the generation of Th1 cells following stimulation with MOG\textsubscript{35–55} peptide was 2D2.CD5WT > 2D2.CD5\textsuperscript{CK2BD} > 2D2.CD5KO (Fig. 3A). Consistently, the proportion of Th1 cells in 2D2.CD5WT CD4\textsuperscript{+} T cell cultures was ~50% greater than in 2D2.CD5\textsuperscript{CK2BD} and 3-fold greater than in 2D2.CD5KO CD4\textsuperscript{+} T cell cultures. Under Th1 polarizing conditions, the proportion of Th1 cells generated was equivalent in CD4\textsuperscript{+} T cell cultures from 2D2.CD5KO mice and 2D2.CD5\textsuperscript{CK2BD} mice but marginally greater than that in 2D2.CD5WT mice (Fig. 3A). However, under Th17 polarizing conditions, the proportion of Th17 cells in 2D2.CD5\textsuperscript{CK2BD} CD4\textsuperscript{+} T cell cultures was less than half of that in CD4\textsuperscript{+} T cell cultures from 2D2.CD5WT and 2D2.CD5KO mice (Fig. 3A). This ex vivo result largely recapitulates the observation in spinal cords of mice with EAE (Fig. 2D).

The stimulation with anti-CD3 mAb (antigen nonspecific) had a different outcome than stimulation with MOG\textsubscript{35–55} peptide (cognate peptide). The generation of Th1 cells was similar in T cell cultures under nonpolarizing conditions from all three strains of mice (Fig. 3B). Following anti-CD3 stimulation under Th1 polarizing conditions, greater proportions of Th1 cells were obtained from CD5\textsuperscript{CK2BD} CD4\textsuperscript{+} T cell cultures than from CD5WT and CD5KO CD4\textsuperscript{+} T cell cultures. The generation of Th1 cells was significantly lower in CD5\textsuperscript{CK2BD} CD4\textsuperscript{+} T cell cultures than from CD5WT and CD5KO CD4\textsuperscript{+} T cell cultures. The generation of Th1 cells was similar in T cell cultures from 2D2.CD5KO mice and 2D2.CD5\textsuperscript{CK2BD} mice but marginally greater than that in 2D2.CD5WT mice (Fig. 3A). However, under Th17 polarizing conditions, the proportion of Th17 cells in 2D2.CD5\textsuperscript{CK2BD} CD4\textsuperscript{+} T cell cultures was less than half of that in CD4\textsuperscript{+} T cell cultures from 2D2.CD5WT and 2D2.CD5KO mice (Fig. 3A). This ex vivo result largely recapitulates the observation in spinal cords of mice with EAE (Fig. 2D).

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**FIGURE 2.** CD5-dependent CK2 signaling pathway regulates the threshold for EAE severity. (A–C) Clinical course of EAE in CD5WT, CD5KO, and CD5\textsuperscript{CK2BD} mice induced with 50 \( \mu \)g/ml MOG\textsubscript{35–55} peptide (A), 150 \( \mu \)g/ml MOG\textsubscript{35–55} peptide (B), and 450 \( \mu \)g/ml MOG\textsubscript{35–55} peptide (C). Data are means ± SEM (n = 10–20 mice/group). Bars above plots denote regions of statistical significance. **p < 0.01 with Student t test. (D) Intracellular cytokine staining of infiltrating CD4\textsuperscript{+} T cell populations in spinal cords from CD5WT, CD5KO, and CD5\textsuperscript{CK2BD} mice analyzed 14 d postimmunization with 150 \( \mu \)g MOG\textsubscript{35–55} peptide. Unbracketed numbers in each quadrant represent the average frequency of CD4\textsuperscript{+} T cells expressing IFN\gamma and/or IL-17a ± SEM. Bracketed numbers represent average absolute cell counts for each subpopulation × 10\(^{4}\) ± SEM. Data are representative of one to two experiments, with two to three mice per experiment per group.
stimulated cultures was similar between CD4+ T cell cultures from the three strains of mice (Fig. 3B, Supplemental Fig. 2A). Considering that stimulation of T cells with cognate peptide rather than anti-CD3 more closely represents normal physiology, the data indicate that loss of the CD5–CK2 signaling pathway results in preferential diminution in the generation of Th17 and Th2 cells but not Th1 cells.

Table II. Analysis of disease parameters for active EAE in CD5WT, CD5KO, and CD5ΔCK2BD mice induced with different doses of MOG35-55 peptide

<table>
<thead>
<tr>
<th>MOG35-55 Peptide Dose (µg)</th>
<th>Mouse Strain</th>
<th>Day of Onset (Day)</th>
<th>Time to Peak (Day)</th>
<th>Accumulative Score</th>
<th>Incidence</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>CD5WT</td>
<td>15.0 ± 1.9*</td>
<td>18.6 ± 4.0*</td>
<td>44.5 ± 10.4*</td>
<td>20/20</td>
<td>1/20</td>
</tr>
<tr>
<td></td>
<td>CD5KO</td>
<td>19.0 ± 5.0*</td>
<td>20.2 ± 4.4</td>
<td>29.8 ± 11.7*</td>
<td>12/15</td>
<td>0/15</td>
</tr>
<tr>
<td></td>
<td>CD5ΔCK2BD</td>
<td>19.2 ± 5.2*</td>
<td>20.0 ± 4.2</td>
<td>35.1 ± 14.3*</td>
<td>14/17</td>
<td>2/17</td>
</tr>
<tr>
<td>150</td>
<td>CD5WT</td>
<td>12.5 ± 1.5</td>
<td>15.8 ± 3.6</td>
<td>59.5 ± 8.7*</td>
<td>19/19</td>
<td>4/19</td>
</tr>
<tr>
<td></td>
<td>CD5KO</td>
<td>17.2 ± 4.2*</td>
<td>18.6 ± 3.2e</td>
<td>34.6 ± 14.8e</td>
<td>15/15</td>
<td>1/15</td>
</tr>
<tr>
<td></td>
<td>CD5ΔCK2BD</td>
<td>12.9 ± 2.9</td>
<td>17.2 ± 6.9</td>
<td>48.8 ± 4.9d</td>
<td>15/15</td>
<td>2/15</td>
</tr>
<tr>
<td>450</td>
<td>CD5WT</td>
<td>13.1 ± 3.2</td>
<td>19.0 ± 6.2</td>
<td>45.4 ± 18.5</td>
<td>11/12</td>
<td>0/12</td>
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<td></td>
<td>CD5KO</td>
<td>14.7 ± 2.7</td>
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<td>44.3 ± 9.7</td>
<td>11/12</td>
<td>0/12</td>
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<tr>
<td></td>
<td>CD5ΔCK2BD</td>
<td>13.4 ± 1.7f</td>
<td>17.7 ± 2.7</td>
<td>52.5 ± 11.7f</td>
<td>9/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM (n = 10-20 mice). No scores were assigned following the day of death.

\*p < 0.05; CD5WT 50 µg dose versus CD5WT 150 µg dose or CD5WT 450 µg dose.
\*p < 0.05; CD5KO 450 µg dose versus CD5KO 50 µg dose.
\*p < 0.05; versus CD5WT mice at same dose.
\*p < 0.05; CD5WT 50 µg dose versus CD5WT 150 µg dose.
\*p < 0.05; CD5WT 50 µg dose versus CD5WT 450 µg dose.
\*p < 0.05; CD5WT 150 µg dose versus CD5WT 450 µg dose.

FIGURE 3. Contribution of the CD5–CK2 signaling pathway to the generation of Th1, Th2, and Th17 cells from naive CD4+ T cells. Naive CD4+ T cells were cocultured with irradiated APCs and stimulated with MOG 35-55 peptide (A) or anti-CD3 (B) under nonpolarizing (unpolarized) or polarizing conditions and generation of Th subpopulations was evaluated. Numbers in each quadrant represent the average frequency of CD4+ T cells expressing IFN-γ, IL-17a, and/or IL-4 ± SEM from four independent experiments performed with at least three mice per experiment. (C) Percentage 7-AAD-CD4+ T cells at 24 and 120 h and (D) RORγ+CD4+ T cells at 120 h following stimulation with MOG35-55 peptide under Th17 polarizing conditions. Data are means ± SEM of three independent experiments with at least two mice per group. (E) pSTAT1 (Ser727) and (F) pSTAT3 (Tyr705) in CD4+ T cells stimulated for 72 h with MOG35-55 peptide under Th17 polarizing conditions. The filled shaded histograms are unstimulated and the dashed histograms are isotype Ig-stained cell controls. For pSTAT1 (E), the mean fluorescence intensity for 2D2.CD5ΔCK2BD is 52 ± 4.8 and for 2D2.CD5WT is 30 ± 4.3. For pSTAT3 (F), the mean fluorescence intensity for 2D2.CD5ΔCK2BD is 32 ± 2.9 and for 2D2.CD5WT is 21 ± 3.5. Histograms represent data from one representative mouse per group from two mice per experiment (n = 3 experiments). *p < 0.05, **p < 0.01, ***p < 0.001.
Cell death, RORγt expression, and activation of STAT1 and STAT3 in CD5ΔCK2BD T cells during Th17 polarization

The CD5–CK2 signaling pathway regulates survival of activated T cells and could represent a mechanism for the diminished Th17 cells in Th17-polarized cultures containing T cells from 2D2.ACD5ΔCK2BD mice (27). Consistent with this possibility we observed a greater proportion of dying T cells in Th17 polarization cultures of 2D2.ACD5ΔCK2BD mice than that in 2D2. CD5WT mice (Fig. 3C). The increased cell death was not compensated by greater cell division. The proportion of Th17 cells in cell cycle as measured by uptake of EdU during a 1-h pulse labeling was similar for both 2D2. CD5WT and 2D2. ΔCK2BD T cells (Supplemental Fig. 2D). The diminished Th17 generation with naive 2D2 ΔCD5ΔCK2BD T cells was also associated with fewer T cells that have upregulated RORγt than that in 2D2. CD5WT cultures (Fig. 3D). However, the expression of Rorc (gene for RORγt) at a per-cell level was almost identical between 2D2. CD5WT and 2D2. ΔCK2BD T cells, indicating that loss of CD5–CK2 signaling pathway did not alter intrinsic RORγt expression (Supplemental Fig. 2C).

A recent report showed that CD5 costimulation enhanced differentiation of naive human T cells to Th17 cells that was associated with upregulation of IL-23R and elevated activation of STAT3 (38). We observed that loss of the CD5–CK2 signaling pathway had no effect on IL-23R expression on Th17 cells (Supplemental Fig. 2B, 2C). However, 2D2.ΔCK2BD Th17 cells did contain greater levels of activated STAT1 and STAT3 compared with 2D2. CD5WT Th17 cells (Fig. 3E, 3F). Overall, the data indicate that the loss of CD5–CK2 signaling attenuates Th17 generation by enhancing cell death and enhanced activation of STAT1 and STAT3.

CD5ΔCK2BD T cells hypoproliferate in response to Ag receptor activation

CD5KO T cells hyperproliferate following stimulation of the Ag receptor, a property associated with the loss of CD5-dependent regulation (9). The inhibitory activity of CD5 is ascribed to the ITAM/ITIM domain of CD5, a domain that is intact in the CD5ΔCK2BD mouse. This mouse model now offers the opportunity to selectively determine the contribution of the CD5–CK2 signaling pathway to T cell activation. Ca2+ mobilization and upregulation of CD25/CD69 are parameters of early T cell activation. In response to TCR stimulation, CD4+ T cells from CD5WT mice and CD5ΔCK2BD mice behaved similarly for both of these parameters (Supplemental Fig. 1B, 1C, and data not shown). In contrast, CD5KO CD4+ T cells exhibited a lower threshold for Ca2+ flux and more accelerated kinetics for upregulation of CD25 and CD69 (Supplemental Fig. 1B, 1C). This observation is similar to that previously reported for thymic T cells (1). The results indicate that the CD5–CK2 signaling pathway does not have an impact on proximal events of TCR signaling.

CK2 is a major promoter of cell proliferation and cell survival, and inhibitors of this kinase attenuate cell cycle progression (26). We therefore tested the prediction that loss of CD5-dependent CK2 signals attenuates cell proliferation. Consistent with our prediction, we observed that at all concentrations of anti-CD3, CD4+ T cells from CD5ΔΔCK2BD mice significantly hypoproliferated in comparison with CD4+ T cells from CD5WT mice (Fig. 4A). As expected, CD4+ T cells from CD5KO mice hyperproliferated following stimulation with anti-CD3. A similar observation was noted when Ag-specific T cells were stimulated with MOG35–55 peptide (Fig. 4B). 2D2. CD5ΔCK2BD T cells significantly hypoproliferated and 2D2. CD5KO T cells hyperproliferated in comparison with 2D2. CD5WT T cells following stimulation with cognate peptide. Because CK2 also promotes cell survival, we wanted to test whether the apparent diminished proliferation of CD5–CK2 signaling-deficient T cells reflected enhanced AICD. When anti-CD3 was used for activation, we found that the proportion of CD4+ T cells undergoing apoptosis (annexin V+7-AAD−) was similar in cultures from CD5ΔCK2BD mice and CD5WT mice (Fig. 4C). However, in MOG35–55 peptide-stimulated cultures, apoptosis was significantly greater when CK2 binding to CD5 was selectively abrogated (Fig. 4D, Table III). CD5KO T cells consistently exhibited enhanced AICD following stimulation with anti-CD3 or cognate peptide (Fig. 4C, 4D). Taken together, the above results indicate that decreased survival in CD5–CK2 signaling-deficient T cells contributes only partially to the diminished proliferation that follows T cell activation.

We directly interrogated whether loss of CD5–CK2 signaling impairs entry into the cell cycle. To address this question we used EdU to pulse label cells for the last 1 h following 72 h stimulation with anti-CD3 or cognate peptide. We then quantitated the amount of EdU uptake by a flow cytometric assay. This approach enumerates the proportion of cells progressing through the cell cycle during the short pulse labeling and therefore overcomes confounders such as cell death. We found that the proportion of EdU+ CD4+ CD5–CK2 signaling-deficient T cells stimulated with either anti-CD3 or MOG35–55 peptide was lower than that for CD5WT T cells stimulated similarly (Fig. 4E, 4F). In contrast, the proportion of EdU+ CD4+ CD5-deficient T cells was always greater than that in stimulation cultures containing CD5WT T cells. The hypoproliferation of CD5ΔCK2BD or 2D2. ΔCK2BD T cells may not be intrinsic to the activation threshold but rather influenced by the differences in the cytokine environment. To address this possibility, we cocultured WT–CD5–expressing CD4+ T cells with ΔCK2BD–CD5–expressing CD4+ T cells and stimulated them with anti-CD3 or MOG35–55 peptide for 48 or 72 h. To distinguish between the two T cell populations, for analysis WT–CD5 T cells were labeled with CFSE. Proliferation was assessed by measuring EdU uptake. We observed that the proportion of dividing CD5–CK2 signaling-deficient CD4+ T cells was significantly less than total dividing WT–CD5–expressing CD4+ T cells even when cultured under the same environmental conditions (Fig. 4G, 4H). Similar results were obtained with cocultures containing labeled ΔCK2BD–CD5–expressing T cells with CFSE and unlabeled WT–CD5–expressing T cells, demonstrating that CFSE labeling did not introduce any artifact (data not shown). These results demonstrate that CD5–CK2 signaling-deficient CD4+ T cells are intrinsically hypoactive to Ag receptor-induced proliferation.

We next asked whether the CD5–CK2 signaling pathway also promotes TCR stimulation-initiated proliferation and cell cycle entry in CD8+ T cells. We found that the extent of CD8+ T cell proliferation following anti-CD3 stimulation recapitulated the observations made with CD4+ T cells (Fig. 4I). The frequencies of CD8+ T cells in cell cycle from CD5WT, CD5KO, or CD5ΔCK2BD mice were not significantly different from each other (Fig. 4J). This result suggests that differences in proliferation probably reflect variances in susceptibility to AICD. Overall, our results indicate that the CD5–CK2 signaling pathway promotes cell cycle progression in CD4+ T cells following TCR activation.

The CD5–CK2 signaling pathway regulates T cell conversion to nonresponsiveness

CD5WT mice immunized with 450 μg MOG35–55 peptide developed less severe EAE than did mice immunized with a lower dose of 150 μg peptide, a characteristic similar to high-dose tolerance (Fig. 2A, Table II). However, in both CD5KO and CD5ΔΔCK2BD mice, the severity of EAE directly correlated with Ag dose. This suggested that the CD5–CK2 signaling pathway...
may be involved in regulating T cell response to Ag re-exposure. To directly test this, we performed an in vitro recall assay where CD4+ T cells were activated with anti-CD3 or cognate peptide, rested, and then restimulated with anti-CD3 or MOG35–55, respectively. We measured proliferation and cell cycle entry. We observed that CD4+ T cells from CD5WT, CD5KO, and CD5ΔCK2BD

Table III. CD5–CK2 signaling pathway regulates TCR-induced apoptosis in T cells

<table>
<thead>
<tr>
<th>Anti-CD3 (µg/ml)</th>
<th>CD5WT</th>
<th>CD5KO</th>
<th>CD5ΔCK2BD</th>
<th>MOG35–55 (µg/ml)</th>
<th>2D2.CD5WT</th>
<th>2D2.CD5KO</th>
<th>2D2.CD5ΔCK2BD</th>
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<tr>
<td>0</td>
<td>9.7 ± 1.3</td>
<td>10.7 ± 1.8</td>
<td>9.8 ± 0.3</td>
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<td>15.0 ± 1.1</td>
<td>27.0 ± 3.6</td>
<td>26.2 ± 2.8^d</td>
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<td>0.1</td>
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<td>10.9 ± 0.1</td>
<td>9.5 ± 0.7</td>
<td>1</td>
<td>19.6 ± 0.6</td>
<td>39.7 ± 2.1^d</td>
<td>35.9 ± 1.5^e</td>
</tr>
<tr>
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<td>14.0 ± 1.3</td>
<td>9.6 ± 0.7^a</td>
<td>10</td>
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<td>43.1 ± 2.6</td>
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<td>100</td>
<td>31.4 ± 0.5</td>
<td>39.0 ± 2.2</td>
<td>71.4 ± 4.4</td>
</tr>
</tbody>
</table>

Cells were stimulated with varying concentrations of anti-CD3 or MOG35–55 peptide in the presence of irradiated APCs for 24 h. Apoptosis was evaluated by staining cells with annexin V and 7-AAD. Data are presented as percentages of events ± SEM.

*p < 0.01; versus CD5KO mice.

*p < 0.05; 2D2.CD5ΔCK2BD versus 2D2.CD5WT mice.

*p < 0.001; versus 2D2.CD5WT mice.

*p < 0.05; 2D2.CD5KO mice versus 2D2.CD5ΔCK2BD mice.

*p < 0.01; 2D2.CD5WT mice versus 2D2.CD5ΔCK2BD mice.

*p < 0.05; 2D2.CD5KO mice versus 2D2.CD5ΔCK2BD mice.

*p < 0.001; versus 2D2.CD5ΔCK2BD mice.

*p < 0.01; 2D2.CD5KO mice versus 2D2.CD5WT mice.
mice proliferated equally when restimulated with 0.1 μg/ml anti-CD3 (Fig. 5A). In contrast, at 1 μg/ml anti-CD3, CD5KO and CD5ΔACK2BD T cells proliferated more vigorously than did CD5WT T cells. We next wanted to determine whether this property of CD5 was active when T cells were stimulated with cognate peptide. MOG35–55 peptide-activated and rested CD4+ T cells from 2D2.CD5WT, 2D2.CD5KO, or 2D2.CD5ΔACK2BD mice were restimulated with 1 and 10 μg/ml MOG35–55 peptide and proliferation was measured. The proliferation of CD4+ T cells from 2D2.CD5ΔACK2BD mice was several fold greater than CD4+ T cells from either 2D2.CD5WT and 2D2.CD5KO mice at both MOG35–55 concentrations (Fig. 5B). We then evaluated cell cycle entry using the EdU incorporation assay. We observed that fewer CD4+ T cells from CD5WT mice stimulated with anti-CD3 or MOG35–55 peptide entered cell cycle than did similarly stimulated CD4+ T cells from CD5KO or CD5ΔACK2BD mice (Fig. 5C, 5D). Consistently, CD5ΔACK2BD CD4+ T cells responded more vigorously to restimulation than did CD5KO CD4+ T cells. Similar to CD4+ T cells, CD8+ T cells from CD5KO mice or CD5ΔACK2BD mice responded more vigorously when restimulated than did CD5WT CD8+ T cells (Fig. 5E).

Considering that WT-CD5-expressing T cells and ΔCK2BD-CD5-expressing T cells produce different levels of cytokines, we evaluated their proliferation in cocultures following restimulation with anti-CD3 or MOG35–55 peptide. At all time points of measurement, we observed significantly greater proportions of ΔCK2BD-CD5-expressing T cells in cell cycles compared with WT-CD5-expressing T cells (Fig. 5F, 5G). The CFSE dilution assay showed that CD5–CK2 signaling-deficient T cells went through one or more generations of cell division than did WT T cells (Supplemental Fig. 3A). The decreased proliferation in WT-CD5-expressing T cells was not associated with enhanced cell death (Supplemental Fig. 3B).

We next evaluated whether the CD5–CK2 signaling pathway also regulated re-exposure to Ag when the initial priming was performed in vivo. CD5WT or CD5ΔACK2BD mice were immunized with MOG35–55 peptide and 7 d later, CD4+ T cells from spleens and lymph nodes were isolated from each group of mice and cocultured with MOG35–55 peptide. We again observed that a significantly greater proportion of CD5ΔACK2BD T cells incorporated EdU than did CD5WT T cells (Fig. 5H). There was no difference in T cell death between the two groups of mice (Supplemental Fig. 3C). Overall, our results show that the CD5–CK2 signaling pathway regulates response to re-exposure to Ag.

**CD5 regulates cytokine production in CD4+ T cells**

2D2.CD5KO and 2D2.CD5ΔACK2BD CD4+ T cells cultured under nonpolarizing conditions contain fewer IFN-γ-expressing T cells than do their 2D2.CD5WT counterparts (Fig. 3A). Therefore, we examined whether the CD5–CK2 signaling pathway regulated Ag
receptor-induced cytokine production. To address this question, naive CD4+ T cells from 2D2.CD5WT, 2D2.CD5KO, and 2D2.CD5ΔCK2BD mice were stimulated with MOG35–55 peptide (10 and 100 μg/ml) and supernatants collected on days 1, 2, 3, and 5 were analyzed for levels of IFN-γ, IL-17a, IL-6, and IL-2 and IL-10. At each time point, the levels of IFN-γ, IL-17a, and IL-6 in 2D2.CD5ΔCK2BD T cell cultures were dramatically greater than those in CD4+ T cell cultures from 2D2.CD5WT mice (Fig. 6A–C, 2D2.CD5KO, and 2D2.CD5ΔCK2BD T cell cultures were dramatically greater than those in CD4+ T cell cultures from 2D2.CD5WT mice (Fig. 6A–C, Supplemental Fig. 3A–C). Although T cell cultures from 2D2.CD5KO mice contained higher levels of these three cytokines than did 2D2.CD5WT T cell cultures (day 2–day 5), they were consistently lower than 2D2.CD5ΔCK2BD T cell cultures. The levels of IL-2 and IL-10, cytokines associated with Treg function, followed different kinetics. Although IL-2 levels in T cell cultures from 2D2.CD5WT and 2D2.CD5KO increased rapidly, the cytokine persisted longer than when the T cells were obtained from 2D2.CD5WT mice (Fig. 6D, Supplemental Fig. 3D). In contrast, detectable levels of IL-2 in T cell cultures from 2D2.CD5ΔCK2BD mice did not increase beyond day 1 of stimulation. The level of this cytokine had contracted to basal levels by day 3 in 2D2.CD5KO and 2D2.CD5ΔCK2BD T cell cultures and by day 5 in 2D2.CD5WT T cell cultures. This contraction is probably associated with T cell exhaustion. IL-10 levels rose and contracted in 2D2.CD5ΔCK2BD T cell cultures (Fig. 6E, Supplemental Fig. 3E). In contrast, IL-10 levels continuously increased in T cell cultures from 2D2.CD5WT and 2D2.CD5KO mice (Fig. 6E). A striking finding is that although MOG35–55 peptide-stimulated unpolarized and Th17-polarized 2D2.CD5ΔCK2BD T cell cultures contain fewer Th1 and Th17 cells, respectively (Fig 3A), the levels of IFN-γ and IL-17a produced were the greatest. This would suggest that the CD5−CK2 signaling pathway is needed for the expansion of these subsets rather than production of these cytokines.

We also determined the cytokine levels in cultures of anti-CD3-stimulated T cells. Cytokine levels in CD4+ T cell cultures stimulated with anti-CD3 in the presence of irradiated APCs reflected the general trend of MOG35–55-stimulated cultures. However, the main difference was that the CD5KO T cell cultures contained greater levels of all cytokines than those in the T cell cultures from CD5WT or CD5ΔCK2BD mice (Supplemental Fig. 4).

**CD5 signaling differentially regulates Th1 versus Th17 disease**

Th1 and Th17 T cells are the major effector T cell populations in multiple sclerosis and EAE that cross-regulate each other (39). Furthermore, we recently showed that multiple sclerosis broadly stratifies into a Th1- or Th17-predominant disease (31). We therefore wanted to determine whether loss of the CD5−CK2 signaling pathway differentially affects the outcome of EAE disease induced by encephalitogenic Th1 cells versus Th17 cells. To address this question, encephalitogenic Th1 or Th17 cells generated from MOG35–55-peptide-immunized CD5WT, CD5KO, or CD5ΔCK2BD mice were adoptively transferred into CD5WT mice and development of EAE was monitored. For Th1 disease, we found that encephalitogenic CD5ΔCK2BD Th1 cells induced the most severe EAE mean maximum disease score based on accumulative score and mortality (Fig. 7A, Table IV). Although EAE induced by encephalitogenic CD5KO Th1 cells was more severe than that induced by CD5WT Th1 cells, the difference was not statistically significant. Unlike Th1 disease, encephalitogenic Th17 cells from CD5ΔCK2BD mice induced slightly less severe EAE than did CD5WT Th17 cells; however, the difference was not significant (Fig. 7B, Table IV). Th17 cells from CD5KO mice induced the least severe disease.

**Discussion**

The development of the CD5KO mouse more than 15 y ago led to a major revision in our understanding of CD5 biology (1). That study reversed the existing dogma that CD5 was a costimulator to that of an attenuator in T cell activation. Using the CD5KO mouse, we determined that CD5 had an important role in promoting survival of activated T cells (28). This discovery resolved the puzzle as to why loss of negative regulation in the CD5KO mice did not lead to the development of spontaneous autoimmunity. We subsequently demonstrated that the prosurvival activity required the CK2 binding domain located within the distal portion of CD5 cytoplasmic tail (27). In this study, we developed a knock-in mouse with a 12-nucleotide deletion in exon 10 of the CD5 gene encoding the codons for the four amino acids (S458–S461) necessary for CK2 to interact with CD5. This mouse gave us the opportunity to examine the consequence of loss of the CD5-dependent CK2 signaling pathway in a context that maintains physiological regulation of CD5 expression. We found that the expression of CD5 on all T and B lymphoid populations in this CD5-dependent CK2 signaling-deficient mouse was identical to that in the CD5WT mouse. This observation suggests that the CD5−CK2 signaling pathway does not alter TCR or BCR signaling events that directly affect CD5 expression (11, 14, 40, 41). In fact, we found that Ca2+ mobilization and upregulation of CD25 or CD69 following TCR stimulation was unaltered in both CD4+ and CD8+ T cells from the CD5ΔCK2BD mouse. Both of these proximal parameters of Ag receptor signaling are tyrosine kinase dependent and were elevated in T cells from the CD5KO mouse as previously reported (1). Although the loss of CD5Δ−CK2 signaling did not dramatically alter mature T cell populations in the C57BL/6 mouse, the absolute numbers of CD4+CD8− DP cells were significantly lower than

![FIGURE 6.](http://www.jimmunol.org/)

CD5−CK2 signaling pathway regulates Ag receptor-induced cytokine production. Naive 2D2.CD5WT, 2D2.CD5KO, and 2D2.CD5ΔCK2BD CD4+ T cells were cocultured with irradiated APCs and stimulated with 100 μg/ml MOG35–55 peptide for 1–5 d. Supernatants were collected on days 1, 2, 3, and 5 to quantify secreted levels of IFN-γ (A), IL-17a (B), IL-6 (C), IL-2 (D), and IL-10 (E) by ELISA. Data ± SEM represent readings from one mouse of two to three mice per group per experiment, from at least four experiments.
immunization dose CD5WT mice developed less severe EAE, conditions with MOG 35–55 peptide for 72 h. Cells were transferred to

** denotes region of statistical significance. * from two separate experiments with 6–11 mice per group. Bar above plot

we do not understand the underlying mechanism of this pheno-

those seen in both the CD5WT and CD5KO counterparts. At present,

do we not understand the underlying mechanism of this pheno-

type, and we are developing appropriate animal models to address

this question.

We previously reported that EAE in CD5KO mice was significantly

less severe and delayed in onset compared with that in CD5WT mice

(28). Remarkably, we found that EAE in CD5ΔCK2BD knock-in

mice essentially recapitulated the CD5KO mice in severity and

onset. This is an intriguing observation because the CD5KO

mouse, unlike CD5ΔCK2BD mouse, lacks the CD5-ITIM–mediated

regulation in addition to the CD5–CK2 signaling pathway. CD5ΔCK2BD mice and CD5WT mice have similar numbers of

nTregs. Furthermore, nTregs from CD5ΔCK2BD mice are less

efficient than those from CD5WT mice (D. McGuire and C. Raman,

unpublished observations). Thus, it is unlikely that nTregs in

CD5ΔCK2BD mice are responsible for the lower severity of EAE

than in CD5WT mice. With an increase in MOG35,55 peptide

immunization dose CD5WT mouse developed less severe EAE,

whereas CD5ΔCK2BD and CD5KO mice developed disease earlier

in onset and with greater severity. The outcome of this study

slightly differs from our previous findings that showed EAE in

CD5KO mice reconstituted with a CD5 transgene expressing CD5

with the same microdeletion was significantly less severe in both

severity and incidence than that in CD5KO mice (27). In our

previous study, the CD5ΔCK2BD transgene was expressed at

lower levels than the endogenous CD5 in CD5WT mice and was under

the control of the CD2 promoter, with no dynamic regulation of

expression by TCR signals. These significant distinctions could

account for the differences between these studies. Nevertheless,

the overall findings show that the CD5 signaling pathway is an

important regulator for setting threshold for response to immunization

and represents a critical signaling pathway in T cells.

We determined that the CD5–CK2 signaling pathway was nec-

essary for the differentiation of naive CD4+ T cells to Th17 or Th2

cells stimulated with cognate peptide under polarizing conditions.

Similarly, the CNS of CD5–CK2 signaling-deficient mice with

EAE also contained fewer IL-17–expressing Th cells but not Th1

cells than that in CD5WT mice. A recent report showed that CD5

is an important costimulator for Th17 differentiation; however, the

mechanism was not resolved (38). Another recent study made

the elegant finding that CK2 is recruited to JAK1 and JAK2 and

participates in the activation of STAT3 and STAT5 (37). STAT3

is required for Th17 differentiation, and STAT5 is required for Th2

differentiation (42, 43). Additional studies still need to be per-

formed to elucidate the precise CD5–CK2 signaling pathway that

promotes Th2 and Th17 differentiation. However, one mechanism

for promoting Th17 generation might be by regulating STAT1

activation (44). In fact, we find that the loss of the CD5–CK2

signaling pathway leads to hyperactivation of STAT1. We also

observed enhanced activation of STAT3 in CD5–CK2 signaling-
deficient Th17 cells. This enhanced STAT3 activation may be a

consequence of gain of STAT1 activity, which has been recently

observed in humans (45). Although STAT3 phosphorylation in

response to STAT3 activating cytokines such as IL-6 and IL-21

was augmented, the study showed that the enhanced STAT1 activity

inhibited Th17 differentiation. This cross-regulation by STAT1 on

STAT3-induced genes may be one explanation for our finding that

in the absence of CD5–CK2 signaling pathway Th17 differentia-
	
	

tion was inhibited, but greater levels of both IL-17A and IL-6 were

produced by activated CD5ΔCK2BD T cells. We also found that

the differentiation of naive CD4+ T cells to Th1 cells stimulated

with cognate peptide under nonpolarizing conditions was dimin-

ished when the CD5–CK2 signaling pathway was ablated. This

finding perhaps explains the enhanced passive EAE observed when encephalitogenic Th1 cells


**FIGURE 7.** The CD5–CK2 signaling pathway differentially affects Th1 and Th17 EAE. Th1 and Th17 EAE were induced by passive transfer of encephalitogenic CD5WT, CD5KO, or CD5ΔCK2BD T cells. Splenocytes from immunized donor mice of each CD5 genotype were depleted of CD8+ and CD25+ cells and restimulated under Th1 (A) and Th17 (B) polarizing conditions with MOG35,55 peptide for 72 h. Cells were transferred to sublethally irradiated CD5WT mice to induce disease. Data ± SEM are from two separate experiments with 6–11 mice per group. Bar above plot denotes region of statistical significance. *p < 0.05; **p < 0.01 with Student t test.

<table>
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<th>Cell Source (CD8+CD25−)</th>
<th>Splenocytes</th>
<th>Day of Onset</th>
<th>Max. Score</th>
<th>Time to Peak</th>
<th>Accumulative Score</th>
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<td>3.4 ± 0.8</td>
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<tr>
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<td>4.4 ± 0.7a</td>
<td>23.8 ± 1.9</td>
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<tr>
<td><strong>CD5WT</strong></td>
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<td>3.3 ± 1.4</td>
<td>20.1 ± 3.3</td>
<td>30.4 ± 13.3</td>
<td>8/8</td>
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</table>

Data are presented as means ± SEM (n = 6–11 mice).

*p < 0.05; disease induced with CD5ΔCK2BD Th1 cells versus disease induced with CD5WT Th1 cells.

*p < 0.05; disease induced with CD5KO Th17 cells versus disease induced with CD5WT Th17 cells.
are generated from CD5ΔCK2BD donor mice. Overall, our results now support a growing paradigm for a Th differentiation that involves cross-talk of cytokine-dependent JAK/STAT signaling and the CD5–CK2 activation pathway. CK2 is an important participant in various stages of cell division, including cell cycle entry (26). Therefore, our finding that CD4+ and CD8+ T cells hypoproliferate in response to TCR stimulation with anti-CD3 or cognate peptide was predictable. Because CK2 is also an important regulator of cell survival, and we showed in this and a previous study that ablation of CD5–dependent CK2 activation leads to enhanced AICD, it was important to determine whether the decreased proliferation merely reflects enhanced death (27). Using the thymidine analog EdU we found that T cells from the CD5ΔCK2BD mouse exhibited diminished cell cycle entry, demonstrating that the hypoproliferation was independent of cell death.

The in vitro recall response following a short rest after primary stimulation is an effective assay for determining threshold for development of anergy in T cells. We found that CD5–CK2 signaling-deficient T cells proliferated more efficiently, with a greater proportion entering into cell cycle than in CD5WT T cells. This in vitro result suggests that the CD5–CK2 signaling pathway plays an essential role in setting threshold for the development of anergy and thus recapitulates the enhanced severity of EAE with increasing dose of MOG35–55 peptide in CD5KO mice and CD5ΔCK2BD mice.

This study has revealed that the CD5–CK2 signaling pathway has a unique role in promoting differentiation of Th2 and Th17 cells and in regulating the threshold for development of anergy. We suggest that manipulation of the CD5–CK2 signaling pathway might be useful for the improvement of immune responses to vaccines, or conversely for attenuating dysregulated T cells in autoimmunity.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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