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The combined loss of Gads and CD127 reveals a novel function of Gads prior to TCR β expression

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Abstract

The Gads adaptor protein is an essential component of the T cell signaling complex critical for T cell receptor-mediated calcium mobilization. After expression of TCR β in T cell precursors, Gads is required for optimal Bcl-2 expression and cell survival. Similarly, the IL-7 receptor chain CD127 is also necessary for optimal Bcl-2 expression and cell survival in TCR β -expressing thymocytes. Based on these observations, we tested whether Gads and CD127 might regulate convergent or linear signaling pathways by crossing Gads^{-/-} mice with CD127^{-/-} mice. Thymi from Gads^{-/-}CD127^{-/-} mice were barely detectable and many of the thymocytes were within the DN1 population. By contrast, B cell development in the Gads^{-/-}CD127^{-/-} mice was comparable to that of CD127^{-/-} mice, indicating that the combined loss of Gads and CD127 did not lead to a global deficit in hematopoiesis. Analysis of Lin⁻Sca-1⁺c-kit⁺ bone marrow cells and bone marrow chimera experiments indicated that Gads^{-/-}CD127^{-/-} T cell precursors either failed to migrate into the thymus or survive in the thymus. These data demonstrate that Gads functions at a stage of T cell development that had not been previously described.

Keywords

T cell development; Signal transduction; Adaptor proteins; Gads; IL-7 receptor

Introduction

T cell development proceeds through an ordered series of steps separated by checkpoints. When cells enter the thymus from the bone marrow, they lack CD4 and CD8 and are called

CD4⁺CD8⁻ double-negative (DN) thymocytes. The murine DN population can be divided into at least four major subsets based on their expression of CD44 and CD25 [1]. At the DN1 (CD44^{hi}CD25⁻) and DN2 (CD44^{hi}CD25⁺) stages, thymocytes receive signals through CD117 (c-kit), CD127 (IL-7R α), and the Notch receptor. Notch is required for commitment to the T cell lineage [2–11]. The importance of IL-7 receptor signaling in the progression beyond the DN1 stage was demonstrated using mice lacking CD127, γ c, or IL-7; T cell development in these mice lines was significantly, but partially, blocked at the DN1 stage [12–16]. Similarly, mice lacking CD117 signaling had fewer thymocytes than wild-type mice [17, 18] and T cell development was partially blocked at the DN1 stage [19]. While loss of either IL-7 receptor or CD117 signaling led to partial blocks in T cell development at the DN1 stage, the combined loss of these receptors led to a nearly complete absence of thymocytes [18]. These observations indicate that CD117 and the IL-7 receptor trigger pathways that converge to promote the progression of T cell development beyond the DN1 stage.

Later in development, at the CD44^{lo}CD25⁺ DN3 stage, thymocytes express T cell receptor (TCR) β and the pre-TCR. Thymocytes unable to express TCR β or activate signaling pathways downstream of the pre-TCR cannot progress beyond the DN3 stage of development [20, 21]. For example, mice lacking the LAT or SLP-76 adaptor proteins had a complete block in T cell development at the DN3 developmental stage [22–24]. In addition, Gads deficiency resulted in a partial block in T cell development at the DN3 stage caused by a reduction in Bcl-2 expression [25–27]. The similarities in mice lacking LAT, Gads, and SLP-76 support the biochemical signaling model first described in T cell lines in which ligation or expression of the TCR or pre-TCR activates kinases that lead to the phosphorylation of the membrane-bound adaptor protein LAT and the recruitment of the Gads and SLP-76 adaptor proteins [28, 29]. The formation of the LAT/Gads/SLP-76 complex is required for optimal TCR-induced calcium mobilization and T cell development.

In addition to pre-TCR-mediated signals, CD127 is required for optimal progression beyond the DN3 stage of development; CD127^{-/-} DN3 and DN4 thymocytes have reduced Bcl-2 expression [16]. Because Bcl-2 expression was impaired in DN3 and DN4 thymocytes lacking Gads or CD127, we crossed Gads^{-/-} mice with CD127^{-/-} mice to test whether Gads and CD127 regulate convergent or linear signaling pathways. Surprisingly, this double knockout mouse line revealed a function for Gads at a stage of T cell development that precedes the expression of the pre-TCR or TCR complex and was previously not known to require Gads. These data support a function for Gads in thymocytes through a receptor other than the pre-TCR or TCR.

Materials and methods

Mice

The generation of Gads^{-/-} C57BL/6 mice was described previously [26]. CD127^{-/-} C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CD127^{-/-} mice were backcrossed onto the Gads^{-/-} background until CD127^{+/-}Gads^{-/-} mice were identified and bred to generate CD127^{-/-}Gads^{-/-} mice. Mice were housed under specific pathogen-free conditions, and all experiments were performed in compliance with the

University of Kansas Medical Center Institutional Animal Care and Use Committee. For phenotypic assays, mice were used between the ages of three and 5 weeks. Thymi from four to six *Gads*^{-/-}*CD127*^{-/-} mice were pooled into a single sample. For other mouse lines, each sample was generated from individual mice. The number of replicates indicated for each experiment reflects the number of samples rather than the number of mice. Bone marrow chimera assays were performed when the recipients were 6–8 weeks of age.

Cell staining and flow cytometry

Anti-CD4–Horizon V450, anti-CD4–FITC, anti-CD8–FITC, anti-CD8–Alexa647, anti-CD24–PE, anti-CD25–Alexa700, anti-CD25–APC–Cy7, anti-CD44–Horizon V450, anti-CD44–PE–Cy7, anti-CD45.1–Pacific Blue, anti-CD45.2–PE–Cy5, anti-CD117–PE–Cy7, anti-Sca-1–Alexa700, anti-TCR β –PE, anti-TCR β –PE–Cy5, and anti-Ter119–FITC were purchased from BioLegend (San Diego, CA) or BD Biosciences (San Jose, CA). Lineage markers were identified using biotinylated anti-Ter119, anti-CD11b, anti-Gr-1, anti-CD3 ϵ , and anti-B220 followed by streptavidin-APC–Cy7 (Biolegend). Cell labeling was performed in PBS containing 2 % FCS. To quantify the number of thymocytes and mast cells, CountBright™ beads (Life Technologies, Grand Island, NY) were added.

Flow cytometry studies were performed using a BD LSR II (BD Immunocytometry Systems, San Jose, CA). Data were analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA). Thymocytes were gated on the live lymphocyte gate, and doublet discrimination was performed. Thymocytes were gated on the Ter119⁻CD45⁺ population.

Bone marrow chimera

CD45.1⁺ or CD45.2⁺ recipient mice were irradiated using 700 rad. Approximately 4 h later, mice were injected IV with 2×10^6 bone marrow cells from congenic donor mice. Mice were euthanized 6 weeks after the transfer, and the number and phenotype of the thymocytes were determined.

Statistics

All data are presented as mean $\pm$ SD. Data comparing wild-type, *Gads*^{-/-}, *CD127*^{-/-}, and *Gads*^{-/-}*CD127*^{-/-} mice were analyzed using a one-way ANOVA followed by a Tukey's post hoc test.

Results

Gads^{-/-}*CD127*^{-/-} mice have few CD117⁺ DN1 thymocytes

Because the genes encoding *Gads* and *CD127* are located on the same chromosome, *CD127*^{-/-} mice were back-crossed onto the *Gads*^{-/-} genetic background until we identified *Gads*^{-/-}*CD127*^{+/-} mice. *Gads*^{-/-}*CD127*^{+/-} mice were interbred to generate the *Gads*^{-/-}*CD127*^{-/-} mouse line. Consistent with previous reports [13, 25, 26, 30], thymi from wild-type, *Gads*^{-/-}, and *CD127*^{-/-} mice, were readily detectable (Fig. 1a). However, thymi from *Gads*^{-/-}*CD127*^{-/-} mice were typically only visible under microscopy.

For each genotype, the average number of thymocytes per mouse was calculated (Fig. 1b). Wild-type, *Gads*^{-/-}, and *CD127*^{-/-} mice contained, on average, $10^8 \pm 1.8 \times 10^7$ cells, $1.2 \times 10^7 \pm 1.9 \times 10^6$ cells, and $1.5 \times 10^6 \pm 6.5 \times 10^5$ cells per mouse, respectively (Fig. 1a). By contrast, thymi from *Gads*^{-/-}*CD127*^{-/-} mice contained 450 ± 120 thymocytes per mouse. The remarkably small thymus seen in *Gads*^{-/-}*CD127*^{-/-} mice indicates that *Gads* and *CD127* likely regulate convergent signaling pathways. Because of the paucity of thymocytes in *Gads*^{-/-}*CD127*^{-/-} mice, all phenotypic analyses of *Gads*^{-/-}*CD127*^{-/-} thymocytes were performed using four to six thymi pooled into a single sample, whereas samples from the other mouse lines were derived from separate mice. The number of experimental replicates indicated throughout this report reflects the number of samples, not the number of mice.

To define the stage of T cell development at which *Gads* and *CD127* converge, we examined CD4 and CD8 expression of thymocytes from each genotype (Fig. 2a). As previously reported, a greater percentage of *Gads*^{-/-} thymocytes were DN thymocytes ($16 \pm 5.7\%$) than wild-type thymocytes ($4.6 \pm 1.7\%$, $p < 0.01$) [25, 26]. The percentage of DN thymocytes in wild-type and *CD127*^{-/-} ($2.7 \pm 1.6\%$) mice were comparable [13, 30]. In *Gads*^{-/-}*CD127*^{-/-} mice, the percentage of thymocytes in the DN stage was greater than the other genotypes; $56 \pm 29\%$ of *Gads*^{-/-}*CD127*^{-/-} thymocytes were DN ($p < 0.01$, comparing *Gads*^{-/-}*CD127*^{-/-} mice to all other genotypes). In all genotypes tested, DP and SP thymocytes could be generated, indicating that the developmental blocks are incomplete.

Because the primary block in T cell development in *Gads*^{-/-}*CD127*^{-/-} mice was within the DN population, we gated on cells that lacked surface TCR β expression and subdivided the TCR β ⁻ DN thymocytes using CD44 and CD25 (Fig. 2b). Most TCR β ⁻ DN thymocytes ($76 \pm 6.7\%$) in *Gads*^{-/-}*CD127*^{-/-} mice were in the CD44^{hi}CD25⁻ DN1 subset. This percentage of *Gads*^{-/-}*CD127*^{-/-} DN thymocytes that were DN1 cells was greater than that in wild-type mice ($9.5 \pm 3.5\%$), *Gads*^{-/-} mice ($8.5 \pm 3.3\%$), and *CD127*^{-/-} mice ($45 \pm 18\%$) ($p < 0.001$ for *Gads*^{-/-}*CD127*^{-/-} mice compared to all other phenotypes).

The DN1 developmental stage can be further subdivided based on CD24 and CD117 expression [31]. The earliest stages of T cell development in the thymus are CD117⁺ TCR β ⁻ DN1 thymocytes [31–34]. There were no differences in the percentages of TCR β ⁻ DN1 thymocytes that expressed CD117 among wild-type, *Gads*^{-/-}, and *CD127*^{-/-} mice (Fig. 2c). However, a smaller percentage of *Gads*^{-/-}*CD127*^{-/-} DN1 thymocytes expressed CD117 than wild-type DN1 cells (6.2 ± 3.1 vs. $17 \pm 5.6\%$, $p = 0.027$). The largest population of TCR β ⁻ DN1 thymocytes in *Gads*^{-/-}*CD127*^{-/-} mice was the CD24⁻CD117⁻ subset; $82 \pm 7.5\%$ of *Gads*^{-/-}*CD127*^{-/-} DN1 thymocytes were CD24⁻CD117⁻. This percentage was significantly greater than in wild-type mice ($35 \pm 18\%$, $p < 0.05$) and *Gads*^{-/-} mice ($56 \pm 8.1\%$, $p < 0.05$), and trended higher than *CD127*^{-/-} mice ($70 \pm 14\%$), although the difference between *CD127*^{-/-} mice and *Gads*^{-/-}*CD127*^{-/-} mice did not reach the level of statistical significance.

Collectively, these data demonstrate that *Gads* and *CD127* are required for T cell development beyond the DN1 stage.

The block in T cell development in $Gads^{-/-}CD127^{-/-}$ mice is intrinsic to the T cell lineage

To examine the T cell lineage in more detail, we examined the early thymic progenitors in the bone marrow. The numbers of total bone marrow cells were comparable across the four mouse strains (Fig. 3a), suggesting that there is no global defect in hematopoiesis in $Gads^{-/-}CD127^{-/-}$ mice. However, the percentage of bone marrow cells that were $Lin^{-}Sca-1^{+}CD117^{+}$ and the absolute number of these cells were greater in $Gads^{-/-}CD127^{-/-}$ mice than in wild-type mice ($p < 0.05$) (Fig. 3b, c). No other statistically significant differences were observed across the mouse lines. These data suggest that loss of *Gads* and *CD127* did not impair the generation of the $Lin^{-}Sca-1^{+}CD117^{+}$ cells.

LSK cells retain multi-lineage potential, so we tested whether B cell development was more impaired in $Gads^{-/-}CD127^{-/-}$ mice than the single knock-out mouse lines (Fig. 3d, e). Previous studies showed that *Gads* was not expressed in developing B cells and *Gads* deficiency did not impair B cell development [35]. By contrast, loss of *IL-7* or *CD127* resulted in impaired B cell development [12, 13]. Our observations here were consistent with these previous reports. The number and phenotype of the B cell precursors in $Gads^{-/-}CD127^{-/-}$ mice was similar to that of $CD127^{-/-}$ mice, suggesting that loss of *Gads* did not exacerbate the defect seen in $CD127^{-/-}$ mice. These data suggest the defect in lymphocyte development seen in $Gads^{-/-}CD127^{-/-}$ mice is likely restricted to the T cell lineage. Further, $Gads^{-/-}CD127^{-/-}$ early thymic precursors may fail to emigrate from the bone marrow or the production of these precursors may be enhanced.

To test whether the impaired T cell development seen in $Gads^{-/-}CD127^{-/-}$ mice is due to a defect in the generation of the thymus, we performed a bone marrow chimera experiment in which wild-type or $Gads^{-/-}CD127^{-/-}$ bone marrow cells were injected into irradiated wild-type recipients (Fig. 4). Thymi from recipient mice injected with wild-type bone marrow cells contained $5.4 \times 10^7 \pm 1.8 \times 10^7$ cells and the cells were nearly exclusively donor-derived. The donor-derived cells had a distribution of DN, DP, and SP thymocytes that resembled the thymic populations of non-irradiated mice, while the distribution of host-derived cells was skewed toward SP cells. These SP thymocytes might reflect incomplete destruction of host-derived cells. By contrast, thymi from wild-type mice injected with $Gads^{-/-}CD127^{-/-}$ bone marrow cells contained $9.4 \times 10^5 \pm 10^5$ cells, and only 1.0 ± 0.011 % of the cells were donor-derived. The wild-type thymocytes were mostly found within the DP gate, and there were too few $Gads^{-/-}CD127^{-/-}$ to accurately and reproducibly discern the proportion of cells in each gate.

The number of $Gads^{-/-}CD127^{-/-}$ cells found in irradiated wild-type thymi was greater than the number of cells we found in $Gads^{-/-}CD127^{-/-}$ mice (Fig. 1b), suggesting that $Gads^{-/-}CD127^{-/-}$ bone marrow cells could populate the thymus. Further, the presence of wild-type thymocytes might provide a more hospitable microenvironment for the $Gads^{-/-}CD127^{-/-}$ thymocytes than in $Gads^{-/-}CD127^{-/-}$ mice.

To determine whether *Gads* and *CD127* are required for thymic function, we injected wild-type bone marrow cells into irradiated $Gads^{-/-}CD127^{-/-}$ mice. Unfortunately, $Gads^{-/-}CD127^{-/-}$ mice were highly susceptible to the toxic effects of the irradiation and only one out of twenty irradiated $Gads^{-/-}CD127^{-/-}$ mice survived the procedure. The

surviving mouse had 2.6×10^7 donor-derived thymocytes consisting of the normal pattern of DN, DP, and SP thymocytes. These data suggest that the thymic tissue in $Gads^{-/-}CD127^{-/-}$ mice has the capacity to promote T cell development, but the $Gads^{-/-}CD127^{-/-}$ thymocytes are impaired.

The impaired survival of irradiated $Gads^{-/-}CD127^{-/-}$ mice was consistent with our overall experience with this mouse line. Few mice survived beyond 4–6 months and most breeding females only had 1–2 l.

Discussion

The phenotype of mice lacking both *Gads* and *CD127* revealed a function of *Gads* that was not previously known. Studies in T cell lines showed that *Gads* associates with LAT and SLP-76 and is required for TCR-mediated PLC γ 1 activation (reviewed in [28, 29]). Consistent with this function, overexpression of *Gads* induces the activities of the NF-AT and IL-2 promoters when cells are stimulated through the TCR [36–38]. Data from mouse lines lacking *Gads* or expressing a dominant-negative form of *Gads* supported this model for the function of *Gads* [25, 26, 39]; *Gads*-deficient T cells and thymocytes had reduced TCR-mediated calcium mobilization. Consistent with this biochemical function of *Gads*, mice lacking *Gads* function had impaired generation of mature T cells [25, 26, 39]. The primary blocks in T cell development correlated with the stages of T cell development in which TCR β and TCR α are expressed for the first time.

The scant number of thymocytes seen in $Gads^{-/-}CD127^{-/-}$ mice suggested that *Gads* and *CD127* regulate pathways that converge to promote T cell development. Based on prior observations that *Gads* and *CD127* promote the expression of Bcl-2 in TCR β^+ DN thymocytes [16, 27], we predicted that mice lacking both *Gads* and *CD127* would have a developmental block at the DN3 stage of T cell development. However, the data shown here indicate that the primary block in T cell development is earlier than the DN3 stage, providing the first evidence that *Gads* is required prior to the expression of the pre-TCR.

Although previous analyses of *Gads*-deficient mice did not reveal any obvious role for *Gads* prior to the expression of TCR β , we analyzed early T cell development in $Gads^{-/-}$ mice more thoroughly than reported in prior publications. While the percentage of DN thymocytes that were DN1 (CD44^{hi}CD25⁻) were comparable between wild-type and $Gads^{-/-}$ mice, the distribution of cells within the DN1 gate varied between the two genotypes (Fig. 2). Fewer $Gads^{-/-}$ DN1 thymocytes expressed CD24 or CD117 than wild-type DN1 cells. CD117⁺CD24⁻, CD117⁺CD24⁺, and CD117⁻CD24⁺ cells represent DN1 subsets that can differentiate into DP and SP thymocytes [31]. The CD117⁻CD24⁻ cell population is likely more diverse than the other DN1 subsets, but also includes cells that can differentiate into DP thymocytes. These CD24⁻CD117⁻ DN thymocytes were overrepresented among $Gads^{-/-}$ DN1 thymocytes and most of these cells expressed CD90 (data not shown).

While the analysis of CD24 and CD117 expression on $Gads^{-/-}$ DN1 thymocytes suggested that *Gads* might have an important function within the DN1 developmental stage, this effect became more apparent when $Gads^{-/-}$ mice were crossed with $CD127^{-/-}$ mice. In

$Gads^{-/-}CD127^{-/-}$ mice, nearly all of the DN1 thymocytes lacked CD24 or CD117 expression (Fig. 2c).

The small number of thymocytes in $Gads^{-/-}CD127^{-/-}$ mice resembled the thymic phenotype reported previously for $\gamma c^{-/-}CD117^{-/-}$ mice [18]. This similarity suggested that *Gads* and CD127 might be functionally linked to γc and CD117. The IL-7 receptor consists of CD127 and γc , providing a clear link. However, the data presented here suggest that *Gads* might be linked to CD117. This putative link was demonstrated by the similarity of impaired T cell development at the DN1 stage in mice lacking *Gads* (Fig. 2) and CD117 [19]. Previously published data demonstrated that *Gads* could interact with CD117 in a manner dependent on the SH2 domain of *Gads* [40]. In addition to binding *Gads*, CD117 also binds Grb2, an adaptor protein closely related to *Gads* [41, 42]. The phosphotyrosine residues of CD117 that bind Grb2 (Tyr-703 and Tyr-936) are contained within the same consensus sequence, YxN, found within the Grb2-binding sites and *Gads*-binding sites of LAT (Tyr-171 and Tyr-191) [43–47]. Whereas LAT provides a scaffold for *Gads* and Grb2 to bind, it is possible that CD117 fulfills a comparable function prior to the expression of the pre-TCR. In further support of this model, both LAT and CD117 can associate with phospholipase C γ (PLC γ) [42]. Thus, CD117 and LAT may both associate with the *Gads*/SLP-76/PLC γ complex. More studies are needed to test this model.

In conclusion, analysis of T cell development in $Gads^{-/-}CD127^{-/-}$ mice revealed a previously unknown function of *Gads*. While *Gads* clearly functions downstream of the pre-TCR and TCR, these receptors are not expressed until the DN3 and DP developmental stages, respectively. Here, we demonstrated that *Gads* is also required at an earlier stage, a stage that precedes pre-TCR expression.

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Fig. 1.

Loss of *Gads* and *CD127* resulted in a small thymus with few thymocytes. **a** Representative photographs of the thoracic cavities of wild-type, $Gads^{-/-}$, $CD127^{-/-}$, and $Gads^{-/-}CD127^{-/-}$ mice. **b** The absolute number of $Ter119^{-}CD45^{+}$ thymocytes (mean $\pm$ S.D.) is shown ($p < 0.001$ between all genotypes, $n = 8$ samples)

Fig. 2.

Gads^{-/-}CD127^{-/-} mice had a block within the DN1 developmental stage. Wild-type, Gads^{-/-}, CD127^{-/-}, or Gads^{-/-}CD127^{-/-} thymocytes were isolated and analyzed by flow cytometry using antibodies against Ter119, CD45, CD4, CD8, TCR β , CD44, CD25, CD24, and CD117. **a** Thymocytes from each genotype were analyzed for CD4 and CD8 expression. Shown are the percentages of DN, DP, and DP thymocytes from a representative sample out of at least 13 for each genotype. **b** Ter119⁻CD45⁺TCR β ⁻DN thymocytes were analyzed for CD44 and CD25 expression. The percentages of DN thymocytes within the DN1 gate are shown from a representative sample out of at least seven for each genotype. **c** DN1 thymocytes were analyzed for CD24 and CD117 expression. Shown are the percentages of DN1 thymocytes in each quadrant for a representative sample out of at least four of each genotype

Fig. 3.

Gads and CD127 were not required for the production of early thymic progenitors in the bone marrow. **a** The absolute numbers of total bone marrow cells from each genotype are shown ($n = 4$, mean $\pm$ SD). Shown are data from at least four mice of each genotype. **b** Cells from (**a**) were gated on Lin⁻ cells and analyzed for Sca-1 and CD117 expression. Shown are the percentages of bone marrow cells that are Lin⁻Sca-1⁺CD117⁺. **c** The absolute numbers of Lin⁻Sca-1⁺CD117⁺ cells identified in (**b**) are shown (mean $\pm$ SD). **d** The absolute numbers of B220⁺ cells in the bone marrow are shown (mean $\pm$ SD). **e** Bone marrow cells from mice of each genotype were labeled with anti-B220 and anti-IgM. Representative data are shown

Fig. 4.

$Gads^{-/-}CD127^{-/-}$ bone marrow cells failed to populate a wild-type thymus. *Upper panels*— $CD45.2^{+}$ wild-type mice (*left panel*) or $CD45.1^{+}$ wild-type mice (*right panel*) were irradiated and injected with bone marrow cells from $CD45.1^{+}$ wild-type ($n = 9$) (*left panel*) or $CD45.2^{+}$ $Gads^{-/-}CD127^{-/-}$ ($n = 6$) (*right panel*) mice. Six weeks later, the percentages of donor-derived and recipient-derived thymocytes were determined and representative data are shown. *Middle and lower panels*—the CD4/CD8 distribution of donor-derived (*middle panels*) and host-derived (*lower panels*) are shown