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Transcriptional Regulator Id2 Is Required for the CD4 T Cell Immune Response in the Development of Experimental Autoimmune Encephalomyelitis

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An effective immune response to Ag challenge is critically dependent on the size of the effector cell population generated from clonal activation of Ag-specific T cells. The transcription network involved in regulating the size of the effector population, particularly for CD4 Th cells, is poorly understood. In this study, we investigate the role of Id2, an inhibitor of E protein transcription factors, in the generation of CD4 effectors. Using a T cell-specific conditional Id2 knockout mouse model, we show that inhibitor of DNA binding (Id)2 is essential for the development of experimental autoimmune encephalomyelitis. Although Ag-specific and IL-17–producing CD4 T cells are produced in these mice, the activated CD4 T cells form a smaller pool of effector cells in the peripheral lymphoid organs, exhibit reduced proliferation and increased cell death, and are largely absent in the CNS. In the absence of Id2, E protein targets, including the proapoptotic protein Bim and SOCS3, are expressed at higher levels among activated CD4 T cells. This study reveals a critical role of Id2 in the control of effector CD4 T cell population size and the development of a Th17-mediated autoimmune disease. *The Journal of Immunology, 2012, 189: 000–000.

The magnitude of a CD4 T cell response is a tightly controlled feature of the adaptive immune system. Upon activation through TCR and costimulatory molecule signaling, a few Ag-specific CD4 T cells can proliferate to form a large pool of effector cells capable of performing immune functions. The expansion phase is then followed by contraction of the population to leave a small number of long-lived memory cells. Various intrinsic and extrinsic factors, including transcription factors and cytokines, have been implicated in regulating the T cell population size at each stage of this response (1). For example, expression of the transcription factors T-bet (2) and Blimp-1 (3) has been shown to be associated with reduced T cell survival upon contraction, whereas IL-7 has been shown to promote T cell survival through this phase by promoting the expression of antiapoptotic protein Bcl-2 (4). However, why each individual T cell expresses different levels of these intrinsic factors, why each individual T cell responds differently to extrinsic factors, and how these intrinsic–extrinsic factors cross-regulate each other are still not well understood.

Recently, the inhibitor of DNA binding (Id) proteins, a family of helix–loop–helix transcriptional regulators, including Id2 and Id3, have been identified to be important in the control of many aspects of T cell responses, including the T cell population size. In CD8 T cells, Id2 and Id3 have been shown to control the numbers of effector and memory cells, at least partially through promoting T cell survival (5–7). In CD4 T cells, Id3 deficiency is also associated with reduced regulatory T cell differentiation (8). In addition, Id3 has been shown to be important for the enforcement of naive T cell state (9), and Id3-deficient mice spontaneously develop a T cell-mediated autoimmune disease similar to human Sjögren’s syndrome (10). However, relatively little is known about the role of Id2 in CD4 T cell responses. One previous report has shown that Id2-deficient mice have increased Th2 dominance, but this difference was largely caused by the lack of a CD8+ dendritic cell subset and therefore was not necessarily related to Id2 function in CD4 T cells (11). Nevertheless, two other studies with double-positive thymocytes (12) and pro-T cell lines (13) showed that E protein transcription factors, the direct target proteins of Id2, may regulate genes important for CD4 T cell responses, including apoptosis-related genes Bcl-2 and Bim, cell cycle-related genes Rb and Cdk6, and cytokine signal regulators SOCS1 and SOCS3 (12, 13). SOCS1 and SOCS3 directly control the CD4 T cell response to multiple cytokines regulating effector and/or memory function and population size, such as IL-7, IL-6, IL-12, and IL-15 (14). Many of those cytokines can also regulate the expression of Id2 (6). Thus, Id2 may be involved in the cross-regulation of intrinsic and extrinsic factors for CD4 T cell population control. To date, studies based on Id2 knockout mice cannot resolve these possibilities because the mice do not have normal development of lymph nodes (15), and the model cannot separate the CD4 T cell-intrinsic role of Id2 from extrinsic ones.

To investigate how Id2 is involved in CD4 T cell responses, we studied Id2 conditional knockout mice (16) with the experimental autoimmune encephalomyelitis (EAE) model, a CD4 T cell–dominant autoimmune disease model. EAE is a rodent model of human multiple sclerosis. By administering exogenous neuro-

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; EAE, experimental autoimmune encephalomyelitis; ICDS, human CD5; Id, inhibitor of DNA binding; MOG, myelin oligodendrocyte glycoprotein.

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autoantigens, a small number of preexisting autoreactive CD4 T cells in the mice can be activated and induce CNS inflammation, demyelination, and paralytic symptoms. The EAE model is an ideal tool to reveal potential roles of Id2 in many aspects of CD4 T cell responses, including T cell activation, differentiation, migration, and population maintenance. With an Id2 reporter mouse model, we found that Id2 is dynamically regulated in the process of CD4 T cell activation. More strikingly, mice with T cell-specific Id2 deficiency are resistant to EAE, developing a smaller effector population in their peripheral lymphoid organs that fails to infiltrate the CNS. This defect is at least in part due to reduced percentage of proliferating cells and increased death of effector CD4 T cells, and analysis of genes dysregulated in the absence of Id2 showed higher expression of Bim and SOCS3 in these cells. These results establish the importance of Id2 in effector CD4 T cell population size control.

Materials and Methods

Animals

The Id2Cre/CD4Cre reporter mouse model has been reported previously (16). Briefly, an IRES-driven, truncated human CD5 (hCD5) cDNA, without the untranslated region of the Id2 gene. The Id2Cre/Cre mouse was used to generate conditional Id2 knockout mice and has been described (17). The entire protein coding region of the Id2 gene was flanked by loxP sites. These mice were crossed with CD4-Cre transgenic mice (Tacnicson, Hudson, NY) to generate T cell-specific Id2-deficient mice (ΔΔ, Id2Cre/ΔCre). The CD4-Cre transgene-negative littermates were used as wild-type controls (ΔΔ, Id2Cre/Cre). All animal work was reviewed and approved by the Duke Institutional Animal Care and Use Committee.

T cell culture and stimulation

Splenic CD44hiCD62L+ naive CD4 T cells were sorted and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μ/ml penicillin, 100 μ/g/ml streptomycin, and 55 μg/ml 2-mercaptoethanol. For TCR stimulation, cells were treated with plate-bound anti-CD3e (1 μg/ml) and anti-CD28 (2.5 μg/ml) (both from BioLegend, San Diego, CA) plus IL-2 (2 ng/ml; Peprotech, Rocky Hill, NJ). For unstimulated wells, cells were treated with IL-2 only.

Induction of EAE

EAE was induced in 6-wk-old male mice by s.c. injection with 100 μg myelin oligodendrocyte glycoprotein (MOG)35-55 peptide (MEVGWYRSPFSRVHLYRNGK; United Peptide, Rockville, MD) emulsified in complete Freund’s adjuvant. One week later, mice were boosted with the same emulsion. Lymph nodes were harvested 9 d after EAE induction. CD4 T cells from the same lymph nodes as (left, Id2Cre/Cre) and CD4 T cells isolated from the interface.

Cell preparation and flow cytometry analysis

CD4 T cells were harvested from spleens and draining lymph nodes (inguinal and axillary). Mononuclear cells from the CNS were obtained as described (18). For intracellular staining, 1 mg BrdU in D-PBS was injected i.p. 15 h before sacrificing the animal, and the staining was performed with the BD Biosciences BrdU Flow Kit according to the manufacturer’s protocols. Cells were analyzed with a FACSCantoII flow cytometer (BD Biosciences) or sorted with a MoFlo cell sorter (Beckman Coulter, Indianapolis, IN).

Real-time PCR

Splenocytes were harvested from mice 9 d after EAE induction. CD4 T cells were enriched with the EasySep mouse CD4+ T cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada). MOG I-A(b)+CD4+ T cells were sorted, and total RNA from the cells was extracted followed by DNase I treatment using the RNAqueous micro kit (Life Technologies). Reverse transcription was performed with M-MLV reverse transcriptase (Life Technologies). The cDNA was used for real-time PCR with a Mastercycler ep realflex (Eppendorf, Hamburg, Germany). The primer sequences are as follows: Bcl2F, 5′-GGACTTGAAGTGCCATTGGTA-3′; Bcl2R, 5′-GGGAGCCTC/ZEN/GCGGTAATCATTTGC/3IABkFQ/-3′; BimF, 5′-GGAGATCCAGGATTGCAACAGG-3′; BimR, 5′-CAGAGATATAGGCTGTGTCG-3′; SOCS1F, 5′-CTGCAGGAGCTGTGTCG-3′; SOCS1R, 5′-CCCCACTTAAATCTGCGG-3′; SOCS1probe, 5′-56-FAM/GCATCCTC/ZEN/CTTAACCCGATC/3IABkFQ-3′. Abs against CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14), IL-17A (Tc1-118H10.1), and IFN-γ (XM01.2) were from BioLegend. The Abs against IdCD5 (UCHT2) was from BD Biosciences (San Jose, CA). 7-Aminactinomycin D (7-AAD) was from Life Technologies (Grand Island, NY). The mouse MOG38-49 I-A(b) tetramer was supplied by the National Institutes of Health Tetramer Core Facility to identify the MOG-specific CD4 T cells. The Ab against CD69 (H10D1F5) was from BD Biosciences (San Jose, CA). 7-Aminactinomycin D (7-AAD) was from Life Technologies (Grand Island, NY). Abs against CD69 (H10D1F5) were from BD Biosciences (San Jose, CA). 7-Aminactinomycin D (7-AAD) was from Life Technologies (Grand Island, NY).

FIGURE 1. Activated CD4 T cells express higher levels of Id2 than naive CD4 T cells. (A) The expression of hCD5 by subsets of splenic CD4 T cells from Id2Cre/CD4Cre reporter mice was analyzed. Left, Gating of the subsets. Right, Histograms showing the hCD5 expression of CD44hiCD62L+ (orange), CD44hiCD62L− (red), and CD44hiCD62L+ (blue) CD4 T cells. (B) Splenic CD44hiCD62L+ naive CD4 T cells from Id2Cre/CD4Cre reporter mice were sorted and cultured with anti-CD3/anti-CD28 Abs plus IL-2 (red) or with IL-2 only (blue). Histograms show their hCD5 expression before and 24 h after culture. (C) Expression of hCD5 by MOG-I-A(b)+ (red) or MOG-I-A(b)− (blue) CD4 T cells isolated from draining lymph nodes of Id2Cre/CD4Cre reporter mice 9 d after EAE induction. (D) Expression of hCD5 by IL-17A+IFN-γ+ (blue), IL-17A−IFN-γ− (black), IL-17A+IFN-γ− (red), and IL-17A−IFN-γ+ (orange) CD4 T cells from the same lymph nodes as (C). Numbers above histograms indicate mean fluorescence intensity. Shaded histograms in each plot show hCD5 background of comparable populations of wild-type CD4 T cells. All plots are representative of three independent experiments.
We used an Id2\textsuperscript{hCD5/hCD5} reporter mouse model, in which surface hCD5 expression can be used as a marker for cellular Id2 expression (16), to first investigate the expression of Id2 in different subsets of CD4 T cells. In the spleen of naive mice, we found that CD4\textsuperscript{hCD5} effector memory-like CD4 T cells expressed higher levels of hCD5 than the CD4\textsuperscript{low}CD62L\textsuperscript{+} central memory-like or the CD4\textsuperscript{low}CD62L\textsuperscript{+} naive CD4 T cells (Fig. 1A), suggesting that Id2 expression is correlated with previous T cell activation. Because the exact activation history of the preexisting T cells is not defined in these mice, we next sorted CD4\textsuperscript{high}CD62L\textsuperscript{low} or CD4\textsuperscript{low}CD62L\textsuperscript{+} naive CD4 T cells (Fig. 1A), showing that Id2 expression is correlated with previous T cell activation. These differences of Id2 expression have been verified by quantitative PCR (Supplemental Fig. 1). To confirm this finding in vivo, we induced EAE, a CD4 T cell-mediated autoimmune disease, in the Id2\textsuperscript{hCD5/hCD5} reporter mice. Nine days after s.c. immunization with MOG peptide, we analyzed the MOG-specific CD4 T cells, as well as IL-17A and/or IFN-\(\gamma\)-producing CD4 T cells from the draining lymph nodes. We again found that these CD4 T cells expressed higher levels of hCD5 than the non-MOG-specific or non-cytokine-producing CD4 T cells (Fig. 1C, 1D). The in vitro and in vivo findings of Id2 upregulation after CD4 T cell activation suggest that Id2 may play a role during the activation process and/or in the maintenance of the activated T cells.

**Mice with T cell-specific Id2 deficiency do not develop EAE**

To test the functional significance of the upregulation of Id2 expression in CD4 T cells during immune responses in vivo, we used the EAE model to compare the control (ff, Id2\textsuperscript{ff}CD4Cre\textsuperscript{−}) mice versus T cell-specific Id2-deficient (\(\Delta/\Delta\), Id2\textsuperscript{ff}CD4Cre\textsuperscript{+}) mice. Both mice have grossly normal inguinal (Fig. 2A) and axillary (data not shown) lymph node development. In the naive mice, despite a small reduction in total thymocyte numbers, the T cell-specific Id2-deficient mice have similar thymocyte development and splenic CD4 T cell composition compared with the control mice (Supplemental Fig. 2A–H). Similarly, no difference was observed with inguinal and axillary lymph node cell analysis (data not shown). The Id2-deficient CD4 T cells are also able to differentiate into IL-17–producing Th17 cells in vitro, with ability to survive and proliferate comparable to that of the control cells (Supplemental Fig. 2I). After immunization with MOG peptide, all control mice developed paralytic symptoms characteristic of EAE; however, none of the T cell-specific Id2-deficient mice developed the disease (Fig. 2B). Because the development of disease symptoms in the EAE model is critically dependent on CD4 T cell infiltration into the CNS, we analyzed the CNS tissue from the mice 15 d after EAE induction, when the disease severity was at its peak in control mice. Although we found a significant number of CD4 T cells in the brain and spinal cord of control mice, nearly no CD4 T cell infiltration was observed in the CNS of T cell-specific Id2-deficient mice (Fig. 3A, 3B). The CD4 T cells found in the CNS of control mice consist of a significant proportion of...
MOG-specific cells, as well as IL-17- and/or IFN-γ-producing cells, whereas the few CD4 T cells found in the CNS of T cell-specific Id2-deficient mice do not contain these populations (Fig. 3C, 3D). Together, the complete resistance to the disease and the absence of T cell infiltration in the CNS showed that Id2 expression is indeed functionally relevant to CD4 T cell responses in vivo, and Id2 deficiency may cause a significant defect in CD4 T cell response to MOG immunization, possibly early in the initiation phase of the disease.

**EAE induction generates a smaller pool of effector CD4 T cells in mice with T cell-specific Id2 deficiency**

Two possibilities may lead to the absence of CD4 T cell infiltration to the CNS in mice with Id2 deficiency: the effector CD4 T cells may develop in the periphery normally but fail to migrate to the CNS or the development of the effector T cells may be defective. To distinguish these possibilities, we next examined the CD4 T cell response in peripheral lymphoid organs of the mice 9 d after immunization, when control mice started to show paralytic symptoms. Compared to control mice, the percentage and number of MOG-specific CD4 T cells was lower in the T cell-specific Id2-deficient mice both in the spleen and draining lymph nodes (Fig. 4A). Intracellular cytokine staining also revealed a decrease of splenic cytokine-producing CD4 T cell populations in the T cell-specific Id2-deficient mice (Fig. 4B), especially the IL-17A+IFN-γ+ cells, which have been reported to be especially encephalitogenic (19). These results correlate with the higher expression of Id2 seen in these populations in the Id2hCD5/hCD5 model (Fig. 1C, 1D). It is noteworthy that residual MOG-specific and cytokine-producing CD4 T cells can be detected in the T cell-specific Id2-deficient mice, suggesting that these T cells have not totally lost their capability of activation and differentiation; presence of these cells in the spleen but not in the CNS also suggests that they have entered the circulation but possibly failed to enter the CNS.

**Id2-deficient CD4 T cells show reduced percentage of proliferating cells and increased cell death**

A smaller population of effector T cells may be the result of reduced cell proliferation or increased cell death. We examined the proliferation of MOG-specific CD4 T cells in the expansion phase of the immune response (6 d after EAE induction) by in vivo BrdU labeling. We found a significant decrease of percentage of BrdU+ cells from the draining lymph nodes of T cell-specific Id2-deficient mice compared with the control mice (Fig. 5A: f/f, 18.4 ± 2.7%; Δ/Δ, 11.3 ± 3.1%; p = 0.002). We next compared cell death between control and Id2-deficient CD4 T cells with 7-AAD staining. Corresponding to their reduced population size, we found increased cell death in MOG-specific CD4 T cells as well as IL-17A+IFN-γ+ CD4 T cells from T cell-specific Id2-deficient mice.}

**Figure 4.** Mice with T cell-specific Id2 deficiency have a smaller pool of effector CD4 T cells in the secondary lymphoid organs 9 d after EAE induction. (A) Representative plots and bar graphs showing percentages and numbers of MOG-specific cells among CD4 T cells from spleens of control (f/f) and Id2 conditional knockout (Δ/Δ) mice. Similar changes were observed in the lymph nodes as shown in the bar graphs. (B) Representative plots and bar graphs of cytokine-producing CD4 T cells from spleens of control (f/f) and Id2 conditional knockout (Δ/Δ) mice. n ≥ 3 for each group. Error bars indicate SD. *p < 0.05.
mice 9 d after EAE induction (Fig. 5B, 5C). The percentage of 7-AAD− cells among MOG I-A(b)+ cells in the spleen were as follows: Δf/f, 49.9 ± 2.7%; ΔΔ, 60.1 ± 4.1%; p = 0.0023. The percentage of 7-AAD− cells among IL-17A+ IFN-γ+ cells in the spleen were as follows: Δf/f, 20.6 ± 13.4%; ΔΔ, 60.5 ± 23.6%; p = 0.001. Repeating the experiments with a fixable Live/Dead stain (Life Technologies) generated similar results (data not shown). These findings indicate that Id2-deficient CD4 T cells, after activation, suffer from reduced proliferation, increased cell death, form a smaller effector cell population, and are unable to induce EAE.

**Id2-deficient CD4 T cells express higher levels of Bim and SOCS3**

Two possible mechanisms may explain how Id2 affects the CD4 T cell population size: direct control of apoptosis or inhibition of cytokine signaling. Altered expression of both antiapoptotic and proapoptotic genes, such as Bcl-2 and Bim, has been observed in Id2-deficient CD8 T cells (7). We used real-time PCR to examine their expressions in splenic MOG-specific CD4 T cells from animals 9 d after EAE induction, and we found that the expression of the proapoptotic Bim was increased in Id2-deficient CD4 T cells but not Bcl-2 (Fig. 6A). This result correlates with the increased cell death of this population and is compatible with a previous report that E proteins can upregulate Bim expression (13).

In addition to direct regulation of apoptosis, Id2 may also regulate cell survival and other aspects of T cell responses through regulation of cytokine signaling. Two important suppressors of cytokine signaling in T cells, SOCS1 and SOCS3, have been reported to be positively regulated by E proteins (12, 13). In particular, increased SOCS3 can inhibit cytokine signaling through STAT3, an important signal transduction mediator of proinflammatory cytokines such as IL-6 (22). T cell-specific STAT3 knockout mice are also resistant to EAE and also demonstrate a reduced size of IL-17-producing effector CD4 T cell population (23). We examined the expression of SOCS1 and SOCS3 in Id2-deficient MOG-specific CD4 T cells by real-time PCR and found that SOCS3 expression was profoundly upregulated compared with control CD4 T cells; there was also a trend toward increased SOCS1 expression in these cells (Fig. 6B). These results indicate that Id2 is required for the maintenance of the effector CD4 T cell population size through regulating the expression of genes related to apoptosis control and cytokine signaling.

**Discussion**

Once CD4 T cells get activated in an immune response, both intrinsic and extrinsic factors contribute to the maintenance and regulation of the effector T cells, controlling the size of the population and the function of individual cells. In this study, we demonstrate that transcriptional regulator Id2, previously best known for its role in the development of various lineages of hematopoietic cells (24), is also an important regulator of CD4 T cell responses, especially in the Th17-mediated EAE disease model.

In the absence of Id2, the population size of MOG-specific CD4 T cells is reduced. The cytokine-producing CD4 T cells are also reduced; specifically, we found that IL-17A+IFN-γ CD4 T cells almost completely disappeared in the T cell-specific Id2-deficient mice. The findings implied that this population is particularly dependent on Id2 for its formation and/or survival. Previous studies reported that this population is more encephalitogenic and has a stronger propensity to migrate into the CNS (19). In a tumor model, the IFN-γ production capacity of Th17 cells has also been shown to be crucial for their anti-tumor activity (25). These reports suggest that the IL-17A+IFN-γ+ CD4 T cells may be important effector cells of a Th17-mediated immune response. They also help explain that despite relatively comparable development of IL-17A+ or IFN-γ+ CD4 T cells in the peripheral lymphoid organs of the T cell-specific Id2-deficient mice and a reduced but still significant population of MOG-specific CD4 T cells, no CNS infiltration or disease symptoms developed in these mice. Why this population is particularly dependent on Id2 and whether the population can be specifically targeted for controlling Th17-mediated autoimmune diseases remain important questions to be addressed.

One possible explanation lies in Id2-mediated regulation of SOCS3. By removing the inhibitor Id2, SOCS3 expression increases.
SOCS3 can suppress signaling of many Th17-related cytokines, including IL-21 and IL-23, through its association with the cytokine receptor and inhibition of JAKs, reducing the phosphorylation and activation of the downstream signal transduction molecule STAT3 (22). IL-21 production is upregulated in Th17 cells upon IL-6 stimulation, and it can upregulate the expression of IL-23 receptor by Th17 cells, increasing the cellular responsiveness to IL-23 and thus “stabilize” Th17 cells (26). IL-23 signaling is critical for the development of IL-17A*IFN-γ*CD4 T cells in the periphery. The phenotype similarity between STAT3-deficient CD4 T cells and Id2-deficient CD4 T cells, and the upregulation of SOCS3 in the latter, strongly suggests that Id2 influences CD4 T cells through its regulation of cellular responses to cytokines. However, because E proteins and Id proteins regulate many genes, it is possible that other important pathways are also affected in the Id2-deficient CD4 T cells.

The current study focuses on the role of Id2 in the effector stage of the CD4 T cell immune response. Whether Id2 plays a role in the formation of CD4 T cell memory is also an important question. Yang et al. (6) previously showed that Id2 is important for the formation of short-lived effector memory CD8 T cells in a Listeria infection model. The possibility that Id2 contributes to the survival of effector memory CD4 T cells can be tested in the future with a variety of prime-boost immunization models or infection-rechallenge models. In addition, we have not completely ruled out a potential role of Id2 in regulating T cell migration and effector functions; a comprehensive examination in these aspects may reveal additional mechanisms leading to the total resistance to EAE in T cell-specific Id2-deficient mice.

We have shown in this study that mice with T cell-specific Id2 deficiency do not develop EAE. However, whether removal of Id2 after the disease already develops, such as by use of chemical inhibitors of Id2 or inducible Id2 deletion models, can alter the disease course is another interesting question. We predict that removal of Id2 after T cell activation should also be effective in inhibiting the T cell response, possibly by shrinking the effector T cell population through increased cell death. If confirmed, modulation of E protein–Id protein activity may become a possible direction of the development of future immune suppressive treatments.

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Disclosures

The authors have no financial conflicts of interest.

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