Either IL-2 or IL-12 Is Sufficient to Direct Th1 Differentiation by Nonobese Diabetic T Cells

Weisong Zhou, Feng Zhang and Thomas M. Aune

*J Immunol* 2003; 170:735-740; doi: 10.4049/jimmunol.170.2.735
http://www.jimmunol.org/content/170/2/735

**References**
This article cites 31 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/170/2/735.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Either IL-2 or IL-12 Is Sufficient to Direct Th1 Differentiation by Nonobese Diabetic T Cells

Weisong Zhou,* Feng Zhang* and Thomas M. Aune

Th cell differentiation from naive precursors is a tightly controlled process; the most critical differentiation factor is the action of the driving cytokine: IL-12 for Th1 development, IL-4 for Th2 development. We found that CD4+ T cells from nonobese diabetic mice spontaneously differentiate into IFN-γ-producing Th1 cells in response to polyclonal TCR stimulation in the absence of IL-12 and IFN-γ. Instead, IL-2 was necessary and sufficient to direct T cell differentiation to the Th1 lineage by nonobese diabetic CD4+ T cells. Its ability to direct Th1 differentiation of both naive and memory CD4+ T cells was clearly uncoupled from its ability to stimulate cell division. Autocrine IL-2-driven Th1 differentiation of nonobese diabetic T cells may represent a genetic liability that favors development of IFN-γ-producing autoreactive T cells. The Journal of Immunology, 2003, 170: 735–740.

The nonobese diabetic (NOD) mouse strain spontaneously develops autoimmune diabetes. The disease is characterized by insulitis of the pancreas, followed by selective destruction of β cells in pancreatic islets (17). Evidence suggests that β cell destruction is mediated, at least in part, by effector CD4+ T cells that preferentially secrete IFN-γ and TNF-α (18, 19). NOD CD4+ T cells have been shown to have an increased propensity to become effector Th1 cells in tissue culture assays (20). We wanted to determine whether Th1 differentiation by NOD T cells had the same cytokine requirements as found in other strains. We found that IL-2 was necessary and sufficient to direct naive NOD T cells into the Th1 lineage.

Materials and Methods

Mice

Female NOD, NOD.B10 Idd9, nonobese diabetes-resistant (NOR), C57BL/6 (B6), C57BL10 (B10), and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at 6–8 wk of age.

Reagents

Complete RPMI 1640 medium supplemented with 10% FBS (Lot ALK 14837; HyClone Laboratories, Logan, UT), 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM l-glutamine, and 0.05 mM 2-ME (J. T. Baker, Phillipsburg, NJ) was used for cell culture. Human rIL-2 was a gift from Hoffmann-LaRoche (Nutley, NJ). Recombinant murine cytokines (IL-2, IL-4, and IL-12), purified mAbs (neutralizing anti-IL-12, C17.8; anti-CD8a, 53-6.7), labeled mAbs (FITC anti-CD4, APC anti-IFN-γ, APC anti-IL-2 α-chain, PE anti-IL-2R β-chain, PE anti-IL-2R γ-chain), and GolgiPlug reagent were purchased from BD PharMingen (San Diego, CA). The following mAbs were purified from tissue culture supernatant of hybridoma cells purchased from American Type Culture Collection (Manassas, VA): anti-I-A (specific for I-A g7, k, r f), or s haplotypes, 10-3.6.2; specific for d haplotype, 34-5-3S; or specific for b, d, g haplotypes, M5/114.15.2, anti-CD3 (145-2C11), anti-CD8 (243), and neutralizing anti-cytokine mAbs, including anti-IL-2 (54B6-1), anti-IL-4 (11B11), and anti-IFN-γ (R4-6A2). CFSE was obtained from Molecular Probes (Eugene, OR).

Cell preparation and culture

T cells were purified by negative selection from pooled splenocytes of three to four mice, as previously described (21). Briefly, single cell suspensions were prepared from mouse spleen. RBCs were removed by hypotonic lysis. I-A-expressing cells and CD8+ T cells were removed by incubation with anti-I-A (10–3-6.2 for NOD, NOD.B10 Idd9, and NOR; M5/114.15.2 for B6 and B10; 34-5-3S for BALB/c) and anti-CD8 mAbs.
(53-6.7 for NOD, NOD.B10 Idd9, and NOR; 2.43 for B6, B10, and BALB/c) at 4°C for 30 min, washed, and incubated with goat anti-mouse and goat anti-rat IgG bound to magnetic beads (Polysciences, Warrington, PA) at room temperature for 30 min with rocking. Cells bound to the beads were removed with a magnet. The purity of CD4+ T cells was >90%, as determined by flow cytometry. RBC-depleted splenocytes were irradiated at 2000 rad from a 137Ce source and used as APCs. Purified T cells (1 x 10^6/ml) combined with irradiated splenocytes (1 x 10^6/ml) were cultured in complete RPMI 1640 medium in 24- or 96-well tissue culture plates (1 or 0.2 ml/well, respectively) at 37°C in 5% CO2 in air. CD4+CD44low and CD4+CD44high T cells (2 x 10^5/ml) were purified by cell sorting and cultured with irradiated splenocytes (1 x 10^6/ml) in 24-well tissue culture plates. In some experiments, CD4+ T cells were prepared by positive selection using magnetic cell sorting (MACS) CD4 (L3T4) microbeads and MS separation columns (Miltenyi Biotec, Auburn, CA), according to manufacturer’s instructions. Briefly, spleen cells of three to four mice were pooled, and RBCs and tissue debris were removed. After labeling with MACS CD4 microbeads, the cells were applied to a separation column. The magnetically labeled CD4+ cells were retained in the column and then eluted as the positively selected cell fraction. The purity of the CD4+ T cells was >92%, as determined by flow cytometry.

A 7-day protocol was used to generate effector T cells. Purified T cells were cultured and stimulated with immobilized anti-CD3 plus irradiated splenocytes in the absence (neutral condition) or presence of IL-12 at 5 ng/ml plus anti-IL-4 at 10 μg/ml (Th1 condition) or IL-4 at 5 ng/ml plus anti-IFN-γ at 10 μg/ml (Th2 condition). For 5 days, washed twice, and restimulated with immobilized anti-CD3 for 2 days. To immobilize anti-CD3 on culture plates, anti-CD3 diluted to 10 μg/ml in 0.1 M sodium bicarbonate (pH 9.6) was added to the plates (0.5 and 0.1 ml/well for 24- and 96-well plates, respectively) and incubated at 37°C for 4–6 h at 4°C overnight. The plates were washed thoroughly before use. Where indicated, neutralizing mAbs including anti-IL-2 (0.1–10 μg/ml), anti-IL-4 (10 μg/ml), anti-IL-12 (10 μg/ml), anti-IFN-γ (10 μg/ml), or anti-IL-2 plus either recombinant human IL-2 (50 U/ml) or mouse IL-12 (5 ng/ml) were included in the cultures during the 5 days of primary stimulation. Rat IgG2a (isotype matched with S4B-6) was used as a negative control in neutralization experiments with anti-IL-2 Ab. All priming cytokines and Abs were added on day 0 of the culture period.

**Proliferation assay**

Purified CD4+ T cells were cultured under neutral condition in the absence or presence of increasing levels of anti-IL-2 Ab for 3 days. [3H]Thymidine (5 μCi) was added during the last 8–12 h. [3H]Thymidine incorporation was determined with a beta scintillation counter.

**Detection of cytokines**

IFN-γ, IL-2, IL-4, IL-12, IL-18, and TNF-α ELISAs were performed with mAbs from BD Pharmingen, according to the manufacturer’s procedures. The concentration of cytokine was calculated from a curve constructed with a recombinant cytokine standard.

**Flow cytometry**

CFSE-labeled or unlabeled CD4+ T cells were stained for surface markers and intracellular cytokines. For CFSE labeling, 1–2 x 10^5 cells/ml in PBS were incubated with 10 μM CFSE for 8 min at room temperature. After stopping the labeling reaction by addition of 1 vol FBS, the cells were washed three times and cultured under conditions, as indicated in the text, for 3 days. GolgiPlug containing 1 μg/ml of brefeldin A was added to cultures 4 h before harvest for intracellular IFN-γ staining. FITC-labeled anti-CD4 and APC-labeled anti-IFN-γ were used to stain cells according to BD Pharmingen’s recommended method. Cells were analyzed by flow cytometry using a FACS Calibur (BD Biosciences, San Jose, CA). In some experiments, cells cultured 2 days after restimulation were stained with FITC-labeled anti-CD4 and APC-labeled anti-IFN-γ for flow cytometric analysis. For separation of CD4+CD44low and CD4+CD44high populations, purified CD4+ T cells were stained with PE-labeled anti-CD44 Ab and sorted using a FACStarPlus.

**Results**

**NOD CD4+ T cells differentiate into strong producers of IFN-γ in the absence of IL-12**

Purified NOD T cells respond poorly to polyclonal TCR stimuli, as measured by the extent of proliferation and production of IL-2 and IL-4 (2). We wanted to determine whether NOD CD4+ T cells also...
produced low levels of IFN-γ in response to a polyclonal TCR stimulation. Purified CD4+ T cells from NOD, NOD.B10 Idd9, NOR, B6, B10, and BALB/c mice were stimulated with immobilized anti-CD3 plus irradiated splenocytes without (neutral conditions) or with either IL-12 plus anti-IL-4 (Th1 condition) or IL-4 plus anti-IFN-γ (Th2 condition) for 5 days and restimulated with anti-CD3 alone for 2 days. Culture fluids were harvested daily after the primary and secondary stimulation and analyzed for levels of IFN-γ. Surprisingly, NOD CD4+ T cells produced levels of IFN-γ under neutral conditions that were comparable to those of cells cultured under Th1 conditions (Fig. 1A). This was the case after both the primary and secondary stimulation. CD4+ T cells from NOD.B10 Idd9 and NOR mice also produced elevated levels of IFN-γ during culture under neutral conditions. The NOR strain is highly related to NOD, but is relatively free of autoimmune disease. The NOD.B10 Idd9 strain exhibits a reduced incidence of diabetes. These data indicate that the high level of IFN-γ production under neutral condition is a NOD genetic trait rather than a diabetes-related phenomena. As expected, IFN-γ production by B6, B10, and BALB/c T cells was highly dependent upon culture with IL-12.

Elevated IFN-γ expression under neutral conditions could result from generation of more cells that produce IFN-γ or elevation of IFN-γ expression per cell. To address this question, we measured intracellular IFN-γ production by flow cytometry. T cells were cultured under neutral, Th1, or Th2 conditions. After 5 days, cultures were harvested and restimulated with immobilized anti-CD3. The same percentage of CD4+ T cells produced IFN-γ after secondary stimulation following primary culture under either neutral or Th1 conditions (Fig. 1B). The overall number of IFN-γ producers and the distribution of the cells that expressed various amounts of intracellular IFN-γ were similar under the two conditions. This indicated that the elevated IFN-γ production in NOD T cells observed under neutral conditions did not result from increased IFN-γ expression by a small number of cells, but rather a uniform increase in the number of NOD CD4+ T cells that had differentiated into IFN-γ-producing cells. Culture under Th2 conditions suppressed the number of IFN-γ cells (Fig. 1B). The number of NOD CD4+ T cells that produced IFN-γ after culture under neutral or Th1 conditions was comparable to the number of B6 CD4+ T cells that produced IFN-γ after culture under Th1 conditions. However, the number of NOD T cells that expressed high levels of IFN-γ was greater than that of B6 cells (Fig. 1B).

**Elevated IFN-γ production and Th1 differentiation by NOD T cells require IL-2**

A possible explanation for elevated IFN-γ expression after culture under neutral conditions may be the presence of Th1-promoting cytokines in the primary T cell cultures. To test this possibility, we examined levels of IL-2, IL-12, IL-18, TNF-α, and IL-4 in primary cultures stimulated under neutral conditions. NOD and B6 CD4+ T cells produced comparable levels of IL-2 with a peak at day 2, whereas NOD cells produced less amounts of IL-4 than B6 cells (Fig. 2A). Cultures from both strains did not contain detectable levels of IL-12, IL-18, or TNF-α. When neutralizing mAb against mouse IL-2, IL-12, IFN-γ, or IL-4 were added to the primary

---

**FIGURE 2.** IL-2 is required for IFN-γ production by NOD T cells cultured under neutral conditions. *A,* Cytokine production by NOD and B6 CD4+ T cells during the primary stimulation. Purified CD4+ T cells were stimulated with anti-CD3 and irradiated splenocytes for 5 days. ELISA results of IL-2, IL-4, IL-12, and IFN-γ production in the culture supernatant from one of two representative experiments conducted in triplicate are shown (mean ± SD). *B,* Inhibition of IFN-γ production by anti-IL-2 mAb. Purified CD4+ T cells were stimulated with anti-CD3 plus irradiated splenocytes in the presence of 10 μg/ml of neutralizing anti-cytokine mAb. After 5 days, cultures were restimulated with anti-CD3 for 2 days. ELISA results of IFN-γ production in the culture supernatant after secondary stimulation from one of three representative experiments conducted in triplicate are shown (mean ± SD).
culture, IFN-γ production by NOD and B6 T cells in secondary culture was markedly inhibited by anti-IL-2, but not by the other Abs (Fig. 2B) or a control rat IgG2a (data not shown).

Segregation of IL-2-dependent IFN-γ expression from IL-2-dependent cell proliferation

Because IL-2 is a growth factor that stimulates T cells to undergo cell division, the suppression of IFN-γ production by NOD CD4\(^+\) T cells may result from inhibition of cell proliferation or differentiation. Surprisingly, inhibition of IFN-γ production by anti-IL-2 was readily dissociated from inhibition of cell proliferation based upon mAb concentration. Low concentrations of anti-IL-2 markedly inhibited IFN-γ production in cultures after primary stimulation (Fig. 3A) without a detectable effect on cell proliferation (Fig. 3B). Analysis of cell division and intracellular IFN-γ expression in primary cultures by flow cytometry showed comparable numbers of cell divisions in the presence or absence of anti-murine IL-2 mAb (1 μg/ml) (Fig. 3C), but marked inhibition of IFN-γ production in the presence of this amount of anti-IL-2 mAb (Fig. 3D). Similarly, we observed marked inhibition of IFN-γ production by NOR T cells by anti-IL-2 mAb (9.6 vs 0.9%). For the B6 strain, the percentage of IFN-γ-producing cells without or with anti-IL-2 treatment was less than 1%. Because we wanted to directly compare the number of cell divisions with IFN-γ production on a per cell basis, these cultures were not restimulated with PMA and ionomycin or anti-CD3 before addition of inhibitors of protein export and flow cytometric analysis. These data indicate that IL-2-dependent IFN-γ expression and Th1 differentiation can be dissociated from IL-2-dependent T cell proliferation.

IL-2 is a potent Th1 differentiation factor for NOD T cells

Because IL-12 is a known Th1 differentiation factor, we compared IL-2 and IL-12 for their ability to direct CD4\(^+\) T cells from NOD, NOR, and B6 mice into Th1 lineage. MACS-purified CD4\(^+\) T cells were cultured under neutral conditions with anti-murine IL-2 mAb in the absence or presence of either human IL-2 or murine IL-12 during primary culture and IFN-γ examined at 2 days after restimulation. Supplementation with human IL-2 resulted in a marked increase in IFN-γ production by NOD and NOR T cells, but not by B6 T cells (Fig. 4). As expected, addition of IL-12 caused T cells from all the three strains to produce high levels of IFN-γ (Fig. 4). Therefore, IL-2 functioned as a potent Th1-driving cytokine as IL-12 did for NOD and NOR T cells, but not B6 T cells.

Strain-dependent differences in the ability of IL-2 to direct Th1 differentiation may reflect differences in IL-2R expression, differences in downstream signaling pathways, or differences in responsiveness of the IFN-γ gene. We analyzed expression levels of the

**FIGURE 3.** IL-2-induced cell proliferation is uncoupled from IFN-γ production in NOD CD4\(^+\) T cells. A, Effect of anti-IL-2 on IFN-γ production. Cells were stimulated with anti-CD3 mAb for 5 days. ELISA results of IFN-γ production in the culture supernatant at day 5 from one of two representative experiments conducted in quadruplicate are shown (mean ± SD). B, Effect of anti-IL-2 mAb on cell proliferation. Purified CD4\(^+\) T cells were stimulated with anti-CD3 plus irradiated splenocytes under neutral condition in the presence of various amounts (0.3, 1, 3, or 10 μg/ml) of anti-IL-2. [\(^{3}H\)]Thymidine was added to the cultures during the last 12 h before the cells were harvested at day 3. Results are from one of two representative experiments conducted in quadruplicate (mean ± SD). C and D, Segregation of IL-2-associated cell division and IFN-γ production. Purified CD4\(^+\) T cells were CFSE labeled and stimulated with anti-CD3 plus irradiated splenocytes in the absence or presence of anti-murine IL-2 (1 μg/ml) for 3 days. After blocking of cytokine secretion with brefeldin A for 4 h, the cells were stained and analyzed for cell division (C) and intracellular IFN-γ expression (D) by flow cytometry. Results are representative of three independent experiments.
FIGURE 4. IL-2 and IL-12 are potent inducers of IFN-γ production by NOD T cells. Purified CD4⁺ T cells of NOD, NOR, and B6 mice were stimulated with anti-CD3 plus irradiated splenocytes under neutral conditions with anti-IL-2 mAb (10 μg/ml) and without (mock) or with cytokines (recombinant human IL-2 at 50 U/ml or mouse IL-12 at 5 ng/ml) for 5 days. ELISA results of IFN-γ levels in the culture supernatant at day 5 from one of three representative experiments conducted in triplicate are shown (mean ± SD).

three (α, β, γ) subunits of the IL-2R on CD4⁺ T cells by flow cytometry. The percentage of NOD T cells that expressed IL-2R for NOD, NOR, and B6 strains was 6, 8, and 7% for α subunit; 13, 11, and 13% for β subunit; and 62, 60, and 59% for γ subunit, respectively. After 24-h stimulation, the percentage of cells expressing α, β, and γ subunits went up to 90, 97, and 99%, respectively, for all three strains. These data indicated that the expression levels of all three IL-2R subunits were equivalent in the three strains.

IL-2-dependent IFN-γ production of NOD T cells is independent of previous activation status

To examine the responses of naive and memory CD4⁺ T cells to IL-2 and IL-12, CD4⁺ CD44low (naive) and CD4⁺ CD44high (memory) cells were purified by flow cytometry and stimulated with anti-CD3 and irradiated splenocytes plus either IL-2 or IL-12 for 5 days. IFN-γ production was determined 2 days after restimulation. Both naive and memory T cells of NOD mice differentiated into effector T1 cells with comparable levels of IFN-γ in the presence of IL-2 or IL-12 (Fig. 5A). In contrast, only memory T cells of B6 mice responded with high levels of IFN-γ production to IL-2 or IL-12 stimulation (Fig. 5B). The optimal IFN-γ production by B6 naive T cells was IL-12 dependent (Fig. 5B).

Discussion

In this study, we have examined the cytokine requirements for the elevated IFN-γ production by NOD CD4⁺ T cells. We have shown that IL-2 effectively directs NOD CD4⁺ T cell into the Th1 differentiation pathways, and that IL-2 is equivalent to IL-12 as a Th1 differentiation factor for NOD T cells. The equivalence between IL-2 and IL-12 as a Th1 differentiation factor is strain specific. IL-2 also directs T cells from the highly related, but autoimmune free, NOR strain to the Th1 lineage, suggesting that this is a genetic trait rather than a reflection of the disease process. In contrast to NOD, T cells from B6, B10, and BALB/c strains do not differentiate into IFN-γ producers to an equivalent extent when cultured with either IL-2 or IL-12. This is true even when these T cells are cultured with supraphysiologic amounts of IL-2. Therefore, this limitation in IL-2-dependent differentiation of T cells into IFN-γ producers by these strains appears to reflect an intrinsic limitation in Th1 differentiation, rather than altered responsiveness to IL-2. This difference was most pronounced in the naive CD4⁺ T cell population, suggesting that differences in prior Ag stimulation do not account for these strain-dependent differences in Th1 differentiation.

Generally, IL-2 is thought to represent a Th1 cytokine. Its ability to drive Th1/Th2 differentiation has never been clearly delineated because of its critical role in promoting T cell division and survival. The requirement of IL-2 for development of Th2 effectors has been shown in vitro for T cells from BALB/c (22), B10.A (23), or B6 strains (22, 24). Neutralization of IL-2 (22, 23) or blockade of the IL-2R (24) during primary stimulation of CD4⁺ T cells results in diminished IL-4 expression. Our study extends the investigation of the function of IL-2 by showing that this cytokine can direct Th1 differentiation in the absence of IL-12 or IFN-γ. However, this ability is clearly strain dependent.

Our results support the hypothesized two-phase model for naive CD4⁺ T cell activation and differentiation. In this model, CD4⁺ T cells undergo an early phase of Ag-dependent cell activation and proliferation, followed by a late phase of cytokine-dependent effector cell differentiation (24, 25). In NOD T cells, these two events, cell proliferation and differentiation into effector cells, are both IL-2 dependent, but apparently require different levels of IL-2. This is indicated by suppression of cell proliferation and therefore IFN-γ production in the presence of high levels of anti-IL-2 mAb, but only inhibition of IFN-γ expression with low levels of anti-IL-2 mAb (Fig. 3, A and B). Uncoupling of IFN-γ production and cell proliferation in NOD T cells strongly argues that the elevated IFN-γ production by T cells cultured under neutral conditions (in the presence of autocrine IL-2) actually reflects Th1 differentiation.

In contrast to other strains, engagement of IL-2R or IL-12R is sufficient to direct Th1 differentiation by NOD T cells. IL-2R and IL-12R trigger distinct intracellular signaling pathways, e.g., STAT5 and STAT4 for IL-2 and IL-12, respectively (9, 26, 27). The important role of IL-12/STAT4 in Th1 development is underscored by studies of gene knockout mice (10, 11, 14). In contrast,
STAT4-independent pathways of Th1 differentiation have also been demonstrated (28). T cells that exhibit both STAT4 and STAT6 gene deficiencies differentiate into IFN-γ producers or effector Th1 cells under neutral culture conditions. The IL-2-induced IFN-γ production and Th1 differentiation by NOD T cells, in vitro, support the existence of such a pathway. Consistent with this view, it has been shown that injection of human rIL-2 into DBA/1 mice, an animal model of rheumatoid arthritis, results in markedly increased IFN-γ production, in vivo (29). The comparable levels of IL-2 production and IL-2R expression by NOD and B6 CD4+ T cells suggest that signaling downstream of IL-2/IL-2R engagement plays a role in the IL-2-induced Th1 differentiation.

Normally, a rate-limiting factor in IFN-γ expression by T cells and Th1 differentiation, in vivo, is the requirement for both an antigenic stimulus and an inflammatory stimulus (6–8). The antigenic stimulus is required to stimulate expansion of T cells (30), and the inflammatory stimulus is required to stimulate IL-12 production by macrophages or dendritic cells (31). Presumably, these checks limit the ability of T cells to differentiate into Th1 cells in an inappropriate context, such as in response to self Ag. In principle, this restriction may serve as a safeguard against development of autoimmunity. In contrast, NOD T cells are able to bypass this potential safeguard and differentiate, in vitro, into IFN-γ producers using a simple autocrine pathway because T cells can produce their own differentiation factor, IL-2. These differences may contribute to the ability of NOD T cells to easily differentiate into IFN-γ producers that invade islets in the pancreas in the apparent absence of an inflammatory stimulus.

References