Peripheral T Cells

Production in

γ

Proliferation and IFN-γ Mediated Notch Signaling Regulates

Osborne

Tanapat Palaga, Lucio Miele, Todd E. Golde and Barbara A.

J Immunol 2003; 171:3019-3024; doi: 10.4049/jimmunol.171.6.3019

http://www.jimmunol.org/content/171/6/3019

Why The JJ?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References

This article cites 29 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/171/6/3019.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

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2003 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
TCR-Mediated Notch Signaling Regulates Proliferation and IFN-γ Production in Peripheral T Cells

Tanapat Palaga,* Lucio Miele,† Todd E. Golde,‡ and Barbara A. Osborne2*

Notch genes encode membrane receptors that regulate cell fate decisions in metazoan. Notch receptors and ligands are expressed in developing lymphoid tissue and mature lymphocytes and the role of Notch signaling in early T and B cell development has been studied extensively. However, its contribution to mature T cell function is unknown. TCR-mediated T cell activation is a fundamental process of the adaptive immune system that has been studied for decades; however, the details of this process are incompletely understood. In this study, we present evidence that Notch is required for TCR-mediated activation of peripheral T cells. Inhibition of Notch activation dramatically decreases T cell proliferation in both CD4 and CD8 cells and blocks both NF-κB activity and IFN-γ production in peripheral T cells. Our data reveal a new, nondevelopmental function of Notch as a previously unknown key link in peripheral T cell activation and cytokine secretion. The Journal of Immunology, 2003, 171: 3019–3024.

The activation of a mature T cell requires the engagement of the TCR by Ag presented by MHCs on the surface of an APC. A second costimulatory signal through CD28-B7 interaction is required for sustained activation and function of T cells. TCR- and CD28-mediated signals together induce vigorous proliferation of T lymphocytes and drive them to produce a variety of effector cytokines that mediate immune responses. Proximal signals resulting from TCR/CD28 engagement are well documented; however, a detailed understanding of the steps leading from these proximal signals to the many distal effects of Ag presentation to T cells is lacking. In this report, we demonstrate that TCR/CD28 signaling induces the expression and activation of Notch proteins.

In mammals, four Notch proteins and several ligands have been identified. Notch receptors and their ligands are expressed on lymphoid progenitors and their expression persists throughout lymphoid development (1, 2). The spatial and temporal expression of Notch receptors and ligands suggest a role for Notch in lymphoid ontogeny. Notch is an ~300-kDa, heterodimeric transmembrane protein which signals in response to ligand binding. Binding of Notch to a ligand on a neighboring cell initiates the proteolytic cleavage of the Notch receptor. This occurs in two stages, an extracellular cut catalyzed by an ADAM family protease and a second cut at the inner surface of the plasma membrane catalyzed by γ-secretase (3–5). Cleavage by γ-secretase releases a 97-kDa intracellular portion of Notch (NIC), which translocates to the nucleus where, through the formation of a heterodimeric complex with CBFI and various coactivator molecules, it functions as a transcriptional activator. Activation of the Notch signaling pathway results in the transcription of a broad range of target genes including the Hes basic helix-loop-helix family of genes. Recent data demonstrate that Notch signaling plays a crucial role at multiple steps of T lineage development (6–9).

The spatial and temporal expression of Notch receptors and ligands suggest a role for Notch in hemopoietic and lymphoid development (1, 2). Although Notch expression in pluripotent hematopoietic stem cells promotes self-renewal of this population (10), its expression in lymphoid cells can regulate lineage specification (2). In particular, accumulating evidence demonstrates that Notch activation drives a common lymphoid precursor toward T cell development at the expense of B cell differentiation, implying that Notch is active in cells destined to become T cells, while inactivated in those cells that assume a B cell fate. Notch expression has also been implicated in the CD4/CD8 lineage decision (11–13). Although the function of Notch in regulating lineage decisions in hemopoiesis and in the developing thymus has been studied, the role of Notch in peripheral T function is largely unexplored. In this report, we show that Notch expression and activation is induced by TCR signaling and that Notch activity is associated with the proliferation of T cells as well as the activation of NF-κB and the production of the effector cytokine IFN-γ.

Materials and Methods

Cell culture, activation, drug treatment, and cytokine assays

Lymphocytes were isolated from either spleen or lymph nodes and stimulated to proliferate with plate-bound anti-TCR (H57) plus anti-CD28 or anti-CD3 (2C11) plus anti-CD28 for 48 h unless otherwise indicated. All T cell Abs were purchased from BD PharMingen (San Diego, CA) and used at 10 μg/ml. Anti-Notch-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-Notch-2 was obtained from the Developmental Studies Hybridomas Bank (University of Iowa, Ames, IA). In some experiments, lymphocytes were activated with Con A (Sigma-Aldrich, St. Louis, MO) at 2.5 μg/ml. Proliferation of cells was assayed by thymidine uptake. CFSE staining was conducted as previously described (14). For experiments using the γ-secretase inhibitor, IL-CHO cells were pretreated for 30 min with the inhibitor or DMSO vehicle and inhibitor was present during the time course of the experiment. IL-CHO was synthesized as previously described (15) and used at either 10, 25, or 50 μM as indicated. Secreected cytokines were assayed with an ELISA-based assay using anti-IL-2 and anti-IFN-γ purchased from BD PharMingen.

Intracellular cytokine staining and flow cytometry

Lymphocytes were activated as described above for 72 or 96 h and restimulated with PMA (80 nM) and A23187 (2.5 μM). After a 1-h restimulation, monesin (3 μM) was added and cells were further incubated for 3 h before

Received for publication April 14, 2003. Accepted for publication July 17, 2003.

*Address correspondence and reprint requests to Dr. Barbara A. Osborne, Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003-6410. E-mail address: osborne@vasci.umass.edu

1 Abbreviations used in this paper: NIC, intracellular Notch; AS, antisense.
harvesting for analysis by flow cytometry. In experiments using IL-CHO, cells were treated with the indicated concentrations of IL-CHO or DMSO vehicle for 12 h before restimulation. After harvesting, cells were fixed with 2% paraformaldehyde, washed, and permeabilized with 0.5% saponin as previously described (16) and analyzed by flow using a FACS Calibur (BD Biosciences, Mountainview, CA).

**EMSA**

Gel shift assays were conducted using nuclear extracts isolated from cells activated as described above. In some experiments, cells were treated with IL-CHO as indicated above. Nuclear extracts were prepared and 5 μg of extract was incubated with radioactive oligonucleotide. These assays were conducted using the Gel Shift Assay system (Promega, Madison, WI) and conditions used were those suggested by the manufacturer.

**Results**

**Notch expression is induced by TCR signaling**

To confirm that Notch family members are expressed in mature T cells, lymphocytes isolated from adult lymph nodes or spleen were stimulated with anti-CD3ε and anti-CD28 Abs. Both stimulated and unstimulated cells were assayed for Notch-1 expression using an Ab that specifically recognizes the Notch-1 protein. Unstimulated cells expressed little or no Notch-1 but in contrast, stimulation of T cells dramatically induced Notch-1 protein levels (Fig. 1A). Although Notch protein has been observed in thymic and peripheral T cell populations, this is the first evidence that signals through the TCR induce Notch-1 expression. Notch-2 expression has been reported in thymocytes so we also asked whether TCR signaling induced Notch-2 in mature T cells. Indeed, when lymph node T cells were stimulated with anti-CD3ε and anti-CD28 Abs, Notch-2 protein levels also were induced at a low level (Fig. 1B).

The data shown in Fig. 1, A and B, used whole cell populations from either spleen or lymph nodes but we also have obtained identical data using purified T cells from both spleen and lymph nodes, indicating that TCR signals induce Notch-1 and Notch-2 in T cells (data not shown).

Hes-1 is a known target of activated Notch in lymphoid cells as well as other cell lineages (17, 18). Therefore, to determine whether Notch-1 and Notch-2 expression has functional consequences, we asked whether TCR or TCR plus costimulatory signals induced Hes-1 expression. We found that both anti-CD3ε and anti-CD28 plus anti-CD28 induced expression of Hes-1 protein, indicating that signals through the TCR activate the Notch signaling pathway (Fig. 1C). In addition to the observation of Hes-1 expression, using an Ab specific for activated Notch-1, we detected the cleaved free intracellular form of Notch-1 when T cells were stimulated as described above (data not shown).

**Inhibition of Notch signaling blocks T cell activation**

To determine the functional consequences of Notch activation, it is necessary to inhibit Notch activity. Therefore, we used both genetic and pharmacological approaches to inhibit Notch activity. First, we used a pharmacological inhibitor specific for γ-secretase activity (15). Upon ligand binding, Notch is proteolytically processed by γ-secretase at a site within or in close proximity to the transmembrane domain (4). Notch processing by γ-secretase activity is required by all Notch receptors (Notch-1–4) to activate downstream signaling events (19, 20). Since there are multiple Notch receptors in mammals and at least Notch-1 and 2 are activated in stimulated T cells, functional redundancy among the family members is a consideration when studying the role of the Notch receptors. Although the targeted deletion of a single family member will not completely abrogate Notch signaling, the functional redundancy among the Notch receptors can be circumvented by the use of γ-secretase; an enzyme is required for the processing of all Notch receptors. In the presence of the γ-secretase inhibitor IL-CHO, we found that TCR-induced Notch-1 expression is strongly inhibited (Fig. 1D). Because activation of Notch drives new transcription of Notch in several systems, including T cells and Drosophila, complete inhibition of Notch activation should decrease Notch protein expression as well (21, 22). Indeed, we found that blockade of Notch activation by IL-CHO resulted in reduced expression of Notch-1 (Fig. 1D) and Notch-2 (data not shown).

We used another approach to examine the biological significance of Notch signaling during T cell activation. We used a transgenic line of mice that contain a Notch-1 antisense (AS) transgene driven by the mouse mammary tumor virus-long terminal repeat (Notch AS). These mice have specifically reduced levels of Notch-1 in several cell types, including splenic lymphocytes, and show phenotypic changes in hemopoietic precursors and B cells (23). In a previous study, we found that Notch levels are reduced from 1.5- to 4-fold in spleens from Notch AS animals and there is some variation from animal to animal (23). Furthermore, the expression of other Notch family members is not altered in Notch AS.
mice (23). In this study, we show that when splenic lymphocytes from these animals were activated with either Con A or anti-CD3 plus anti-CD28, the level of Notch-1 induction was reduced (Fig. 1E). When these data were quantitated, we found that Notch-1 levels were reduced ~4-fold in Con A-stimulated cells from the Notch AS mice while Notch-1 levels in AS mice treated with anti-CD3 plus anti-CD28 were reduced ~3-fold as compared with controls. The reduction of Notch activity in these mice is partial but highly reproducible and provides a specific, nonpharmacological approach to assessing the effects of Notch activity on T cell function. Notch effects are well known to be exquisitely dose dependent (24), thus even an incomplete suppression of Notch-1 expression can have striking biological consequences (23).

A well-characterized effect of TCR signaling is a rapid proliferative response. To determine whether Notch plays a role in this proliferative response, splenic T cells were incubated with either anti-CD3ε or anti-CD3ε plus anti-CD28 (Fig. 2A, left and right panel, respectively) in the presence or absence of IL-CHO. IL-CHO completely blocked the ability of splenic T cells to proliferate in response to anti-CD3ε and significantly blocked anti-CD3ε plus anti-CD28-induced proliferation. Furthermore, while exogenous IL-2 could sustain the proliferative response in anti-CD3ε-treated cells, in the presence of IL-CHO, exogenous IL-2 had little to no effect in rescuing either anti-CD3ε or anti-CD3ε plus anti-CD28-induced proliferation. We also found that IL-CHO inhibited the expression of the activation marker CD69 in a dose-dependent fashion (Fig. 2B). The surface expression of CD25 also was moderately reduced in a dose-dependent manner (data not shown). The fact that IL-CHO only partially blocks CD69 expression may indicate that both TCR signals as well as Notch activation are required for sustained maximal expression of this early T cell activation Ag. Thus reduction of Notch activation by IL-CHO only partially blocks CD69 up-regulation. These data demonstrate that inhibition of Notch activation results in a profound blockade of TCR-induced proliferation. It is important to note that IL-CHO blocks the activation of all Notch family members, suggesting the possibility that multiple Notch family members regulate the proliferation of peripheral T cells.

We then investigated whether the effects of IL-CHO were due to specific inhibition of proliferation rather than cytotoxicity and whether both CD4 and CD8 cells were sensitive to Notch inhibition. To verify that the effects on proliferation were not merely a cytotoxicity effect, we first asked whether IL-CHO-induced death in stimulated T cells. We found that IL-CHO did not induce significant levels of death, as compared with controls, in stimulated T cells at either 10 or 25 μM IL-CHO, the concentrations of inhibitor used in these studies (Fig. 2C). To further confirm that IL-CHO is not toxic to cells, we assayed proliferation using CFSE-loaded cells. CFSE is passively taken up by cells and, as they divide, the CFSE fluorescence decreases by half at each division, thus providing an accurate measure of the number of cell divisions. CFSE-loaded CD4 or CD8 cells were incubated with anti-CD3ε plus anti-CD28 in the presence of either 10 or 25 μM IL-CHO or vehicle control and analyzed for proliferation at 72 and 96 h after stimulation (Fig. 2D). CFSE profiles were obtained by gating on live cells, thus demonstrating that while IL-CHO inhibits cell division, it does not kill the cells. Indeed, at 96 h, a time when IL-CHO is no longer effective due to the half-life of the compound in cell culture (15), both CD8 and, to a lesser extent, CD4 cells begin to proliferate, confirming once again that these cells are viable but unable to proliferate vigorously in the presence of IL-CHO. These data clearly suggest a role for Notch signaling in both CD4 and CD8 TCR-induced proliferation.

We also asked whether Notch AS displayed defects in proliferation. However when we repeated the experiments presented in Fig. 2A using splenocytes from Notch AS mice, we detected no obvious proliferation defects (data not shown). Notch AS mice have reduced levels of Notch-1 but Notch-2, -3, and -4 levels are normal in these animals. Therefore, it is possible that several Notch family members regulate T cell proliferation.

**Notch signaling regulates IL-2 and IFN-γ secretion in activated T cells**

To investigate the role Notch signaling plays in regulating effector functions of activated T cells, we examined the effect of IL-CHO treatment on cytokine production. IL-2 and IFN-γ are cytokines produced by Th1 cells, CD8 CTLs, and NK cells upon stimulation (16). Both cytokines are produced and secreted at a high level by splenocytes after stimulation for 48 h (Fig. 3A). The amount of both IL-2 and IFN-γ decreased in a dose-dependent manner when cells were pretreated with IL-CHO inhibitor. To confirm these data, IL-2 and IFN-γ production was assayed in TCR-stimulated splenocytes from transgenic mice expressing AS Notch-1. As mentioned previously, expression of Notch-1 in splenocytes from these mice showed an incomplete reduction in Notch-1 induction upon stimulation, suggesting that Notch-1 expression is only partially disrupted in this transgenic mouse model (23). When splenocytes from these mice were stimulated through soluble anti-CD3ε and anti-CD28, both IL-2 and IFN-γ levels were significantly decreased in the Notch-1 AS splenocytes as compared with control splenocytes (Fig. 3B). We also have examined the expression of intracellular cytokine levels of both IL-2 and IFN-γ in Notch AS lymph node cells and found a 25% reduction in IL-2-positive cells and approximately a 40% reduction of IFN-γ-positive cells (data not shown and Fig. 4A). These data are consistent with results obtained using the γ-secretase inhibitor and indicate that Notch signaling is necessary for maximal expression and production of IL-2 and IFN-γ during T cell activation. Additionally, because the data obtained with Notch AS mice agree completely with the data obtained with IL-CHO, these data suggest that the effects of Notch on IL-2 and IFN-γ synthesis may be entirely due to Notch-1. Lastly, we recognize that reduced levels of cytokines in IL-CHO-treated cells may well be due to reduced numbers of cells since proliferation is blocked in cells cultured with IL-CHO. But we observed the same level of reduction in cytokine levels in Notch AS mice, where, as mentioned above, proliferation is not affected. These data reinforce the interpretation that the defect in cytokine secretion observed in these experiments is due to a defect in Notch signaling and not merely a matter of cell number.

Multiple transcriptional regulators including NF-κB, NFAT, and AP-1 have been shown to play a crucial role in regulating early transcriptional events following stimulation of T lymphocytes. The NF-κB signaling pathway has been shown by several investigators to play an important role in cell survival, proliferation, and cytokine production in peripheral T cells (25–27). Using transgenic mice expressing the NF-κB superrepressor IκBzα, it was shown that defects in activation of the NF-κB pathway have a strong negative impact on activation of T lymphocytes (25, 26). In particular, defects in proliferation and decreased IFN-γ production were observed. These phenotypes are similar to what we observed in our system. These observations and evidence suggesting that Notch regulates NF-κB activity (22, 28–29) prompted us to hypothesize that the defects we observed in IL-CHO-treated or Notch AS splenocytes may be due in part to decreased NF-κB activation. Thus, we examined NF-κB activity in stimulated splenocytes either treated with IL-CHO or from Notch-1 AS mice and respective
controls. As shown in Fig. 3C, decreased NF-κB activity was consistently observed under conditions that decreased Notch-1 activity. However, the activity of NFAT, another transcription factor that plays a critical role in activated T cells, remained unaltered. Therefore, the defects we observed in IL-CHO-treated splenocytes or those from AS Notch-1-transgenic mice may be, at least in part, due to defects in NF-κB activation.

Notch signaling regulates IFN-γ production in activated T cells
To determine whether the observed defects in IFN-γ production are specific to either CD4⁺ or CD8⁺ SP T, we stimulated splenocytes from Notch AS-transgenic mice with Con A, restimulated with PMA and calcium ionophore (A23187), and analyzed either CD4 or CD8 subsets for intracellular production of IFN-γ. The number of IFN-γ-producing cells was significantly reduced in the CD8 subset of Notch AS-transgenic (Fig. 4A) mice. There were very few IFN-γ-producing CD4 cells irrespective of Notch status. When we repeated this experiment treating splenocytes with the γ-secretase inhibitor, we found that IL-CHO treatment caused a
significant dose-dependent reduction of the number of IFN-γ-producing CD8 cells (Fig. 4B). However IL-CHO treatment did not alter the fraction of IFN-γ-producing CD4 cells. These results are parallel to those obtained with the Notch AS-transgenic mice. Taken together, these data suggest that Notch plays a crucial role in regulating IFN-γ in CD8 T cells.

**Discussion**

The data reported above reveal multiple, previously unrecognized roles for Notch signaling in peripheral T cell function. Our data support a model in which TCR engagement causes Notch activation and subsequent up-regulation of Notch protein. In turn, Notch
activation is likely important for NF-κB activation and activation-induced T cell proliferation. This appears to apply equally to both CD4 and CD8 T cells. Additionally, we have uncovered a new role for Notch-1 in regulating IFN-γ production in CD8 cells. This suggests that Notch-1 may regulate effector functions of CD8 cells.

Notch-1 has been shown to affect intrathymic maturation of CD8 cells (12). Thus, it is likely that Notch-1 may control several steps in the life cycle of CD8 cells from the thymus to the periphery.

The role of Notch in T cell proliferation is suggested by experiments using the use of the γ-secretase inhibitor IL-CHO. As discussed above, IL-CHO inhibits the activation of all four Notch proteins, thus it is possible that multiple Notch proteins regulate T cell proliferation. This is supported by the fact that we did not observe proliferation defects in Notch AS mice, mice that have reduced levels of Notch-1 but normal levels of Notch-2, -3, and -4. Alternatively, it is also possible that the effects of the γ-secretase inhibitor IL-CHO are due to inhibition of an unknown γ-secretase substrate. Although we favor the first interpretation, either explanation is important. The effects of IL-CHO on T cell proliferation are profound and whatever the mechanism of inhibition may be, this observation identifies substrate(s) of γ-secretase as important regulators of T cell proliferation.

The observations presented in this report are significant for several reasons. They reveal a new, important link in the chain of events that trigger T cell proliferation in response to TCR engagement. This offers new potential opportunities for modulating T cell activation in vivo. Furthermore, our data suggest that manipulation of Notch signaling may specifically affect the cellular arm of the immune response. This knowledge may have wide implications in the development of tumor and virus vaccines, as well as the treatment of organ rejection and autoimmune disorders. Finally, our data expand the importance of Notch signaling beyond embryogenesis and cell maturation events. Our observations suggest that Notch signaling may participate in intercellular communications that modulate cellular function throughout the life cycle of a cell from lineage choice during development to ultimate effector functions.

Acknowledgements

We thank members of the Osborne laboratory for stimulating discussions, Alfred Singer, Richard Goldsby, and Susanne Szabo for thoughtful comments and suggestions, and Becky Lawlor and Kathie Curnick for excellent technical assistance.

References