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Type 1 cytokines polarize thymocytes during T cell development in adult thymus organ cultures

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Abstract

Peripheral T cells can be polarized towards type 1 or type 2 cytokine immune responses during TCR engagement. Because T cell selection by peptide plus self-MHC in the thymus requires TCR engagement, we hypothesized that type 1 cytokines may polarize developing T cells. We cultured thymi from BBDR rats in adult thymus organ cultures (ATOC) under type 1 cytokine conditions in the absence of exogenous antigen. Type 1 cytokine-conditioned ATOC generated cells that spontaneously secreted high levels of IFNγ, but not IL-4. A second exposure to type 1 cytokines further increased IFNγ secretion by these cells, most of which were blasts that expressed the activation markers CD25, CD71, CD86, and CD134. Studies using blocking antibodies and pharmacological inhibitors suggested that both IL-18 and cognate TCR–MHC/ligand interactions were important for activation. Blocking anti-MHC class I plus anti-MHC class II antibodies, neutralizing anti-IL-18 antibody, and the p38 MAP-kinase inhibitor SB203580 each reduced IFNγ production by $\sim$75–80%. Cyclosporin A, which prevents TCR signaling, inhibited IFNγ production by $\sim$50%. These data demonstrate that exposure to type 1 cytokines during intrathymic development can polarize differentiating T cells, and suggest a mechanism by which intrathymic exposure to type 1 cytokines may modulate T cell development.
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1. Introduction

A key determinant of the response of CD4^{+} and CD8^{+} effector T cells to antigen is the cytokine environment they encounter during cognate engagement of the TCR [1–3]. IL-12 polarizes T cells towards a type 1 cytokine profile characterized by high IFNγ and low IL-4 secretion, whereas IL-4 polarizes T cells towards a type 2 cytokine profile characterized by high IL-4 and low IFNγ secretion [4]. Type 1 polarized T cells are effective at controlling intracellular pathogens [5] and have been implicated in some T cell-mediated autoimmune disorders [6,7]. Type 2 polarized T cells are

effective at controlling extracellular pathogens, such as helminths [8,9] and also promote humoral immunity, including IgE-mediated allergic responses [10]. Ex vivo exposure of T cells to type 1 or type 2 cytokine environments during cognate TCR interaction with MHC plus antigen rapidly polarizes their cytokine secretion profile.

It is not known which cells produce the IL-4 needed to generate type 2 responses in vivo. NK T cells, basophils, mast cells, and T cells themselves are all candidate sources [11,12]. The IL-12 that promotes type 1 cytokine responses in vivo is produced primarily by dendritic cells and macrophages; IL-12 is secreted rapidly in response to infection by many intracellular pathogens [5,13–15]. Microbial LPS, bacterial DNA containing CpG motifs, and Leishmania antigen are also potent inducers of IL-12 in phagocytic cells [5]. The response of the innate immune system to some infectious agents and microbial products is vigorous enough to

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increase levels of circulating IL-12. For example, infection with murine cytomegalovirus [13,16] or *Toxoplasma gondii* [17], or exposure to LPS [18] or the dsRNA viral mimetic polyinosinic:polycytidylic acid (poly I:C) [19] markedly increases circulating IL-12 levels in vivo in mice. Activation of APCs by pathogens, causing them to secrete IL-12 could, therefore, have systemic effects on T cells. IL-12 is also produced, in the absence of infection, by dendritic cells after cognate interaction with T cells [20,21] and in the thymus [22,23].

The role of polarizing cytokine environments in regulating peripheral T cell responses has been studied extensively, but it is not known if T cells differentiating in the thymus are also susceptible to polarization. Because positive and negative selection by self-peptide–MHC complexes during thymocyte development requires engagement of the TCR [24–26], we hypothesized that thymic T cells can be polarized. To test this hypothesis, we used rat adult thymus organ cultures (ATOC) to examine the effect of exposure to a type 1 cytokine environment on T cell development. We chose to study thymi from BBDR rats because we have shown previously that BBDR rat ATOC recapitulate normal thymocyte development, and generate normal ratios of functional CD4$^+$ and CD8$^+$ single positive TCR^{hi} cells [27,28]. The BBDR rat is phenotypically normal and disease-free, but can be induced to develop autoimmune diabetes by environmental perturbants, including viral infection [29–31]. Diabetes in these animals is associated with a type 1 immune response [32].

We report that exposure of BBDR rat ATOC to a type 1 cytokine environment generated activated T cells that spontaneously secreted high levels of IFNγ. Using blocking antibodies and pharmacological inhibitors, we further documented that both TCR–MHC/ligand interactions and endogenous IL-18 were important for activation. Both pre-selection CD4$^+$ and CD8$^+$ thymocytes and CD4 and CD8 single positive thymocytes were polarized to increased IFNγ production. These data document that developing rat thymocytes, like peripheral T cells, are susceptible to the polarizing effects of type 1 cytokines.

2. Materials and methods

2.1. Animals

Viral antibody-free BBDR rats were purchased from BRM, Inc. (Worcester, MA) and used between 8 and 10 weeks of age. All sentinel animals screened during these studies were certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham's rat virus, H-1, mouse poliovirus (GD7), Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus,

Hantaan virus, and *Encephalitozoon cuniculi*. All animals were housed in a viral-antibody-free facility and provided with sterile food and water ad libitum. Animals were maintained in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

2.2. Antibodies and cytokines

For flow microfluorometry of surface antigens, fluorochrome- or biotin-conjugated mAbs directed against the $\alpha\beta$ TCR (clone R73), CD25 (IL-2Rα, clone OX-39), CD71 (transferrin receptor, clone OX-26), CD4 (clone OX-35), CD8α chain (clone OX-8), CD8β chain (clone 341), CD134 (clone OX-40), CD45RC (clone OX22), CD80 (clone 3H5), CD86 (clone 24F), CD3 (clone G4.18), CD62L (clone HRL1), IL-4 (clone OX81, and IFNγ (clone DB-1) were purchased from BD Biosciences (San Diego, CA). Isotype control mAbs mouse IgG1, mouse IgG2a, rat IgG2b, hamster Ig, and PE- or CyChrome®-conjugated streptavidin were also purchased from BD Biosciences. Antibodies were used either directly conjugated with FITC, PE, or CyChrome®, or were used as biotin conjugates and developed for visualization by PE- or CyChrome®-streptavidin.

Mouse anti-rat IL-4 mAb (OX81), anti-rat MHC class II (OX6), anti-rat MHC class I (OX18), and isotype control anti-dengue virus mIgG1 (3H5-1) antibodies were produced in our laboratory and purified using Protein G-Plus affinity chromatography columns (Calbiochem, San Diego, CA). A purified neutralizing rabbit antibody against rat IL-18 has been described in Ref. [33] and was generously provided by Dr Nathan Karin (Technion, Haifa, Israel). Purified polyclonal rabbit antibody to irrelevant peptide was used as an isotype control. Purified polyclonal rabbit anti-rat IFNγ (Torrey Pines, La Jolla, CA) and anti-ART2.1 antibodies were used as biotin-conjugated antibodies [34].

2.3. Culture of ATOC and lymph node cells

ATOC were established from BBDR rat thymi as described in Ref. [27]. Thymic fragments were cultured for 5 days in a humidified incubator in an atmosphere of 7% CO$_2$ and air at 37 °C. In some experiments, syngeneic BBDR rat serum was substituted for fetal bovine serum. To expose developing BBDR thymocytes to type 1 cytokines, ATOC were cultured in 10 ng/ml mouse IL-12, 10 ng/ml human IL-2, and 25 μg/ml of anti-rat IL-4 mAb. Cells were harvested after 5 days of culture,

and in some experiments were incubated in type 1 cytokines in secondary cultures at 1×10^6 cells/ml with 10 ng/ml IL-12, 10 ng/ml IL-2, and 10 µg/ml of anti-rat IL-4 mAb for an additional 5 days. Throughout this report, the term 'secondary suspension culture' is used to describe secondary cultures of thymocytes in type 1 cytokines following a primary ATOC in the presence of type 1 cytokines.

Primary cultures of BBDR cervical and mesenteric lymph node cell suspensions were cultured in type 1 cytokines as previously described for secondary thymus cultures. Cells recovered from cultures were washed three times in HEPES buffered-RPMI (Gibco, Rockville, MD). Viable cells were counted by the method of Trypan blue exclusion using a hemocytometer.

Randomly selected supernatants from cultures were tested and confirmed to contain <0.5 EU of endotoxin per milliliter (Endosafe, Charles River Laboratories, Charleston, SC).

2.4. Flow microfluorometry

Two and three-color flow microfluorometry analyses were performed as described in Ref. [27]. Briefly, 1×10^6 viable cells were first incubated with biotin-conjugated mAbs for 30 min on ice. Cells were then washed and incubated for an additional 30 min on ice with FITC-conjugated, PE-conjugated, and CyChrome®-conjugated antibodies, or fluorochrome-conjugated streptavidin to visualize biotinylated mAbs. FITC-, biotin-, PE-, and CyChrome®-conjugated isotype control immunoglobulins were used for all analyses. Staining for detection of intracellular IFNγ was performed on BBDR ATOC cultured at 37 °C under various conditions for 5 days, or cultured in ATOC for 5 days followed by re-stimulation under the same conditions in secondary cultures for an additional 5 days. Cells from both sets of cultures were harvested, counted, and re-cultured in the day 5 supernatant at 1×10^6 cells/ml in the presence of GolgiPlug™ for 5–6 h. Cells were then reacted with antibodies to surface proteins, washed, and fixed and permeabilized overnight at 4 °C using Cytofix/Cytoperm™ reagent (BD Biosciences). Intracellular cytokine staining was performed using a 2 h incubation on ice with FITC-anti-rat IFNγ or FITC-mouse IgG1 isotype control mAb.

Cells were washed, fixed with 1% paraformaldehyde, and analyzed using a FACScan® instrument (Becton Dickinson, Sunnyvale, CA). Lymphoid cells were identified electronically by their forward and side light-scatter characteristics. For surface staining alone, 10,000 events were analyzed. For combined staining for intracellular cytokines and surface markers, 50,000 events were analyzed in most samples.

2.5. ELISA and ELISPOT assays

ELISA assays for rat IFNγ, TNFα, and IL-10 were initially performed using commercial kits according to manufacturer's directions (BioSource International, Camarillo, CA). Later studies used commercially available antibodies (BD Biosciences, San Diego, CA; Torrey Pines, La Jolla, CA) and recombinant rat cytokines as standards. Recombinant mouse IL-12, human IL-2, rat IFNγ, rat TNFα, and rat IL-10 were purchased from R&D Systems (Minneapolis, MN). ELISAs were developed using a detection system of avidin-peroxidase and OPD substrate. Absorbance was read at 490 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). ELISPOT assays for IFNγ and IL-4 were performed as previously described [35].

2.6. Western blot analysis and pharmacological inhibitors

Western blots for rat IL-18 used recombinant rat IL-18 as a standard, purified goat anti-rat IL-18 antibody, and secondary biotin-conjugated rabbit anti-goat IgG antibody (R&D Systems). Frozen and thawed cell pellets (2.5×10^5) from the indicated cultures were applied to each lane. Gels were developed using streptavidin-horseradish peroxidase (Vector Labs) and ECL™ Western blotting chemiluminescent detection reagent (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

In some experiments, antibodies were added to type 1 cytokine polarized cultures as follows. Neutralizing purified rabbit anti-rat IL-18 antibody (10 µg/ml) [33] or irrelevant purified rabbit Ig (10 µg/ml) was added to both the ATOC and secondary cultures. Purified anti-MHC class I and anti-MHC class II mAb (50 µg/ml each), or an irrelevant isotype control mAb (at an equivalent amount of protein, either 50 or 100 µg/ml), was added on day 0 of secondary culture. Antibodies were not added to the ATOC cultures because anti-MHC antibodies would have interfered with T cell selection [36]. IFNγ production was measured in the supernatants from day 5 secondary suspension cultures by ELISA assays.

For analyses of pharmacological inhibitors, adult BBDR rat thymi were first cultured for 5 days in the presence of type 1 cytokines as previously described. The ATOC were harvested and the cells were then incubated at a concentration of 1×10^6 cells/ml either on anti-CD3 mAb coated plates (5 µg/ml), or in the presence of IL-12, IL-2, and anti-IL-4 mAb as previously described. Pharmacological inhibitors were added on day 0 of secondary cultures, and were dissolved in sterile DMSO to $1000 \times$ stocks just prior to use. The specific compounds tested were the calcineurin inhibitor Cyclosporin A (100 ng/ml) [37], the p38 map kinase inhibitor
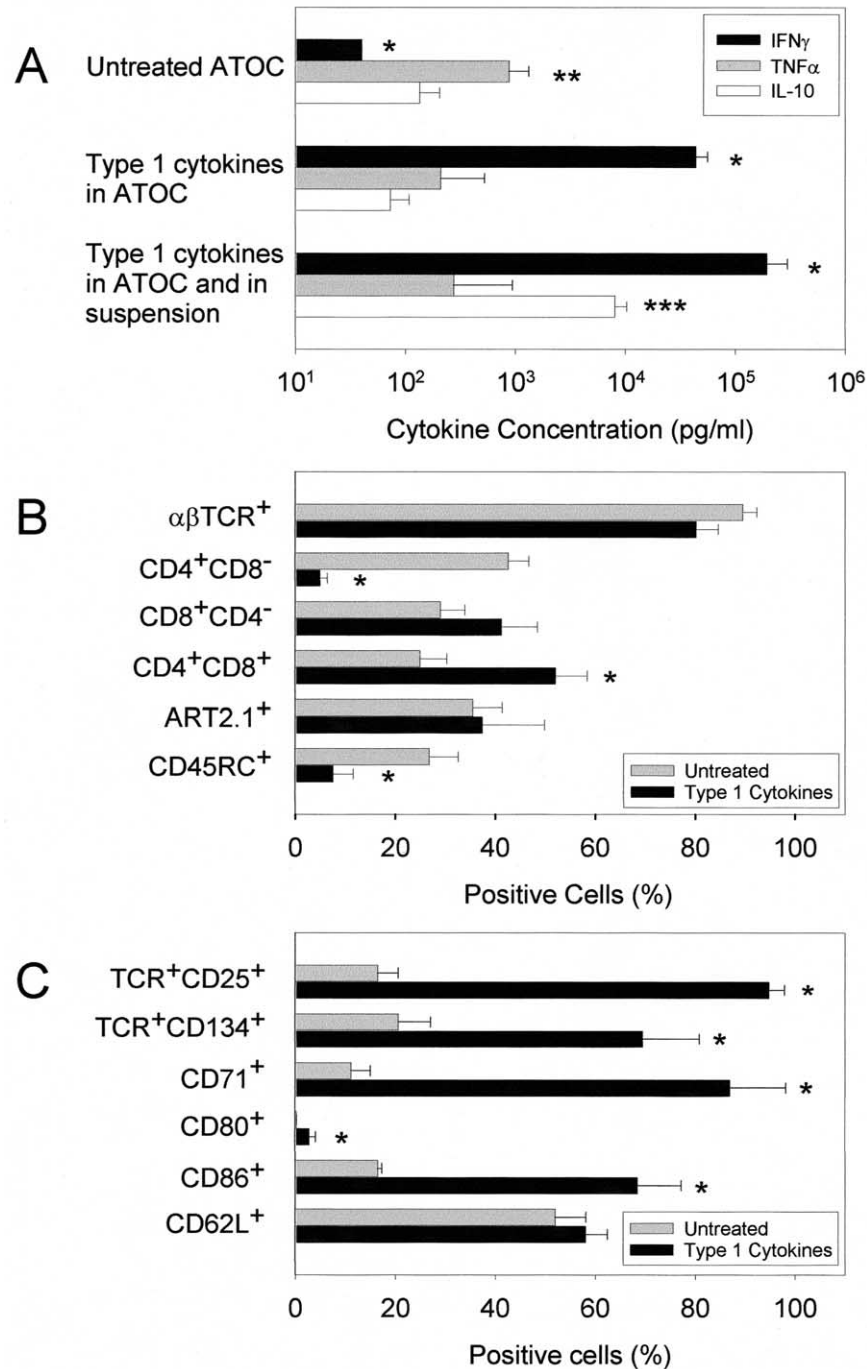
Fig. 1. Cytokine production and phenotype of cells generated in ATOC. Panel A: Adult BBDR thymi were cultured in the absence of cytokines (uppermost three bars), in the presence of type 1 cytokines (middle three bars), or in ATOC followed by suspension culture, both in the presence of type 1 cytokines (lowest three bars), as described in Section 2. Supernatants were harvested and the concentrations of IFNγ, TNFα, and IL-10 were measured by ELISA. Each data point represents the mean ± 1 SD of 3–12 independent trials. Cytokine concentration is shown on a logarithmic scale. *Concentration of IFNγ in each of the three culture conditions is statistically different from that in the other culture conditions ($p < 0.01$). **The concentration of TNFα in the control culture is significantly higher than the other two culture conditions ($p < 0.025$). ***IL-10 concentration in the combined ATOC and suspension culture is significantly higher than in both other culture conditions ($p < 0.001$). No other paired comparisons were statistically significant. Panels B and C: BBDR thymi were cultured in ATOC in the absence of cytokines (gray bars) or in ATOC in the presence of type 1 cytokines followed by suspension culture with type 1 cytokines (black bars). Cells were analyzed by flow microfluorometry for expression of differentiation (Panel B) and activation (Panel C) markers. Each data point represents the mean percentage of cells with the indicated phenotype (± 1 SD) from three to five independent replicates. Percentages obtained using isotype control antibodies were uniformly <1% and were not subtracted from the values shown. *$p < 0.01$ vs. control, untreated cultures.

SB203580 (5 μM) [38], tyrphostin A1 (5 μM), which is used as a control for inhibitors of protein tyrosine kinases [39] and DMSO, which was the vehicle for each reagent. Inhibitors were purchased from Calbiochem (San Diego, CA). IFNγ production was measured in the supernatants on the days indicated by ELISA assays.

2.7. Statistics

Parametric data are presented as arithmetic means ± 1 standard deviation (SD). Groups of three or more means were compared by one-way analysis of variance and the least significant differences procedure for a posteriori contrasts [40]. Where required, data were log transformed before analysis to account for broad variances. Pairs of means were compared using two-tailed t-tests with separate variance estimates and the Bonferroni adjustment for multiple comparisons was applied as necessary [41].

3. Results

3.1. Cells generated in BBDR rat ATOC exposed to type 1 cytokines secrete increased amounts of IFNγ

BBDR rat ATOC were used to determine whether exposure of differentiating T cells to type 1 cytokines during intrathymic development would alter their cytokine production or activation state. We quantified cytokine production by cells generated in untreated ATOC, in ATOC exposed to type 1 cytokines, and in ATOC exposed to type 1 cytokines and then re-exposed to these same conditions in secondary suspension cultures. (Throughout the results, 'secondary suspension culture' refers to cultures performed with the addition of type 1 cytokines.)

As shown in Fig. 1 (Panel A), cells cultured in the presence of type 1 cytokines in ATOC produced large amounts of IFNγ. Addition of a secondary suspension culture containing type 1 cytokines further increased the amount of IFNγ produced. In both cases, the concentration of IFNγ in the culture supernatant was $>10^3$-fold greater than in control cultures. The increase in the amount of cytokine secreted into the culture supernatant in the experimental groups was associated with a progressive increase in the number of cells that stained positively for IFNγ in the ELISPOT assay (Table 1).

Because all cultures were performed in the presence of bovine serum, it could be argued in theory that the increase in IFNγ concentration observed in the experimental cultures was due to the activation of xenoreactive T cells. To address this possibility, we performed an additional set of experiments consisting of ATOC plus type 1 cytokines followed by secondary suspension

Table 1
Frequency of IFNγ and IL-4 secreting cells in ATOC

Culture Condition	IFNγ (spots/10^5 cells)	IL-4 (spots/10^5 cells)
1 Control ATOC	3, 21, 30	6, 12, 18
2 Type 1 cytokines in ATOC only	30, 80, 85	5, 8, 8
3 Type 1 cytokines in ATOC and in secondary cultures	>500, >750, 1333	0, 2, 3

Adult BBDR rat thymocytes were cultured in the absence of cytokines (condition 1) or in the presence of type 1 cytokines either with (condition 3) or without (condition 2) a secondary suspension culture as described in Section 2. The number of IFNγ and IL-4 secreting cells was quantified by ELISPOT assay. Individual results are shown from three independent trials.

culture using rat serum ($N=4$) or fetal bovine serum ($N=4$). We observed that the concentration of IFNγ generated by both cultures was statistically similar ($42.7 \pm 17.9 \times 10^5$ pg/ml using rat serum and $82.1 \pm 30.0 \times 10^5$ pg/ml using bovine serum). The latter value is similar to that shown in Fig. 1 for the same conditions. The result suggests that elevated IFNγ secretion was not due solely to xenogeneic T cell reactivity.

The secretion of TNFα was not increased by exposure to type 1 cytokines. It was, in fact, higher in control ATOC than in either of the culture conditions performed in the presence of type 1 cytokines.

The secretion of IL-10 was similar in ATOC performed with and without the addition of type 1 cytokines, but was significantly increased by re-exposure to type 1 cytokines in secondary suspension culture. The observation that IL-10 was increased in secondary suspension cultures is consistent with published observations that IL-12, which was added to these cultures, can induce T cells to produce IL-10 [42].

IL-4 could not be measured by ELISA, since anti-IL-4 antibody was added to the cultures. ELISPOT data shown in Table 1 indicate, however, that the number of cells that were producing IL-4 was small in all of the culture conditions.

3.2. Cells generated in BBDR rat ATOC exposed to type 1 cytokines acquire an activated phenotype

We next defined the phenotype of thymocytes cultured under the same three conditions. Consistent with our previous report [27], the majority of cells recovered from day 5 untreated BBDR rat ATOC were CD4$^+$ or CD8$^+$ single-positive T cells (Fig. 1, Panel B). Many of the cells expressed ART2.1, which is not expressed on thymocytes [43], and CD45RC and CD62L, which are expressed only at low levels in the thymus [44,45] (Fig. 1, Panels B and C). On day 5 of untreated ATOC, 11–21% of cells expressed the activation antigens CD25,
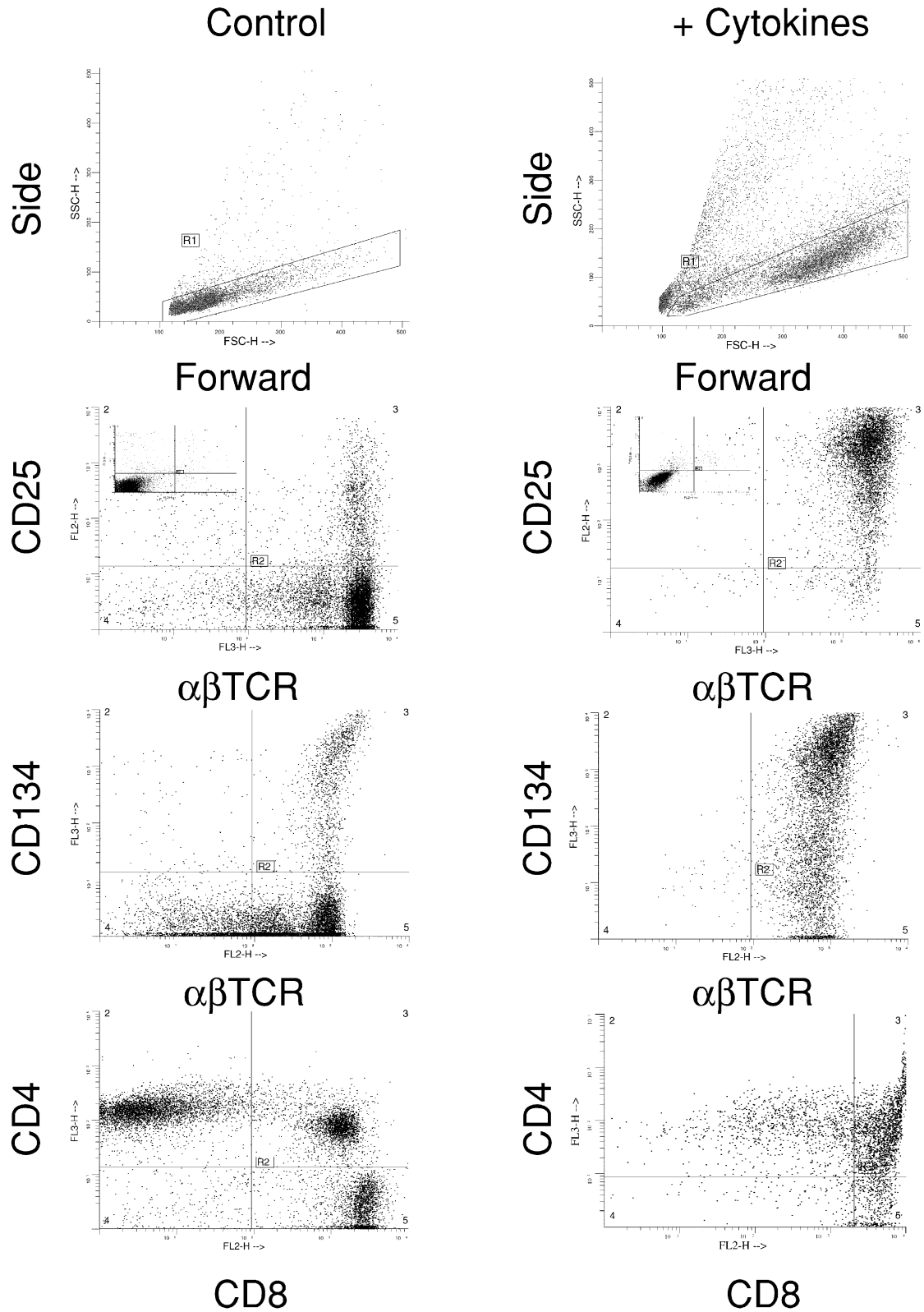
Fig. 2. Flow cytometry profiles of cells generated in ATOC. Adult BBDR thymi were cultured in the presence of type 1 cytokines followed by secondary culture, again in the presence of type 1 cytokines (right panels), or in primary cultures in absence of cytokines and with no secondary suspension culture (left panels). Cells were harvested and reacted with antibodies directed against the indicated surface antigens. Representative flow cytometry profiles are shown; the complete dataset is presented in Fig. 1. Isotype controls are shown as insets and were less than 1% in all cases.
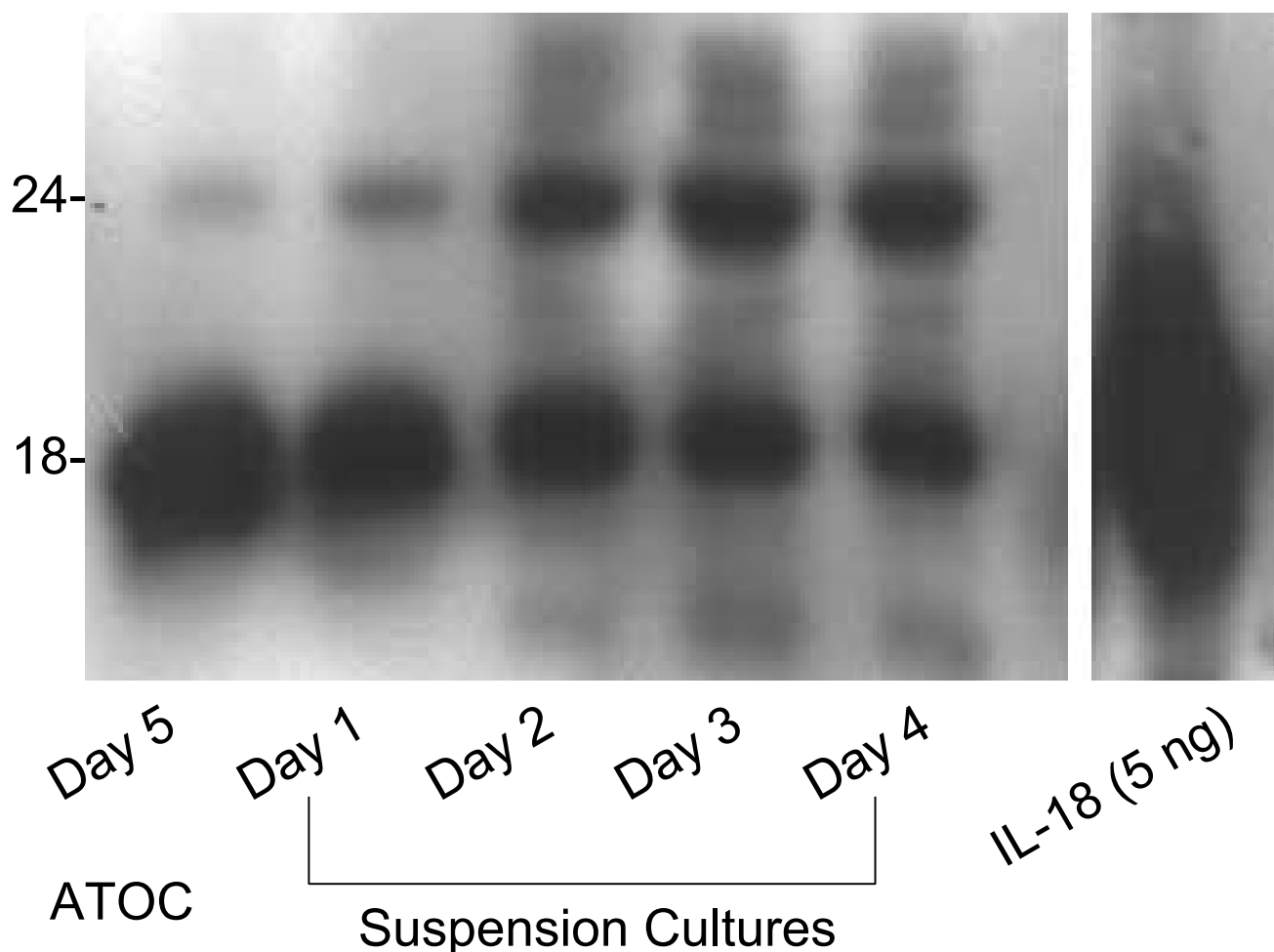
Fig. 3. IL-18 generated in ATOC and secondary cultures. Adult BBDR thymi were cultured in the presence of type 1 cytokines for 5 days followed by 1–4 days of secondary culture, again in the presence of type 1 cytokines. On the indicated days, cells were recovered and the analyzed for the presence of IL-18 by Western blotting as described in Section 2. Both cleaved biologically active (18 kD) and uncleaved inactive precursor (24 kD) forms of IL-18 were detected. The right-most lane was prepared using 5 ng of recombinant rat IL-18. Molecular masses were established with a panel of markers (not shown).

CD134 (OX40), and CD71 (transferrin receptor) (Fig. 1, Panel C).

The addition of type 1 cytokines to BBDR rat ATOC had no effect on the percentage of day 5 cells that expressed CD4 or CD8 or the activation markers CD25, CD134 and CD71 (data not shown).

Cells harvested from secondary suspension cultures, however, showed clear evidence of activation. The majority of these cells expressed CD25 (95%), CD134 (70%), CD71 (87%), and CD86 (68%) (Fig. 1, Panels B and C). Expression of CD45RC was reduced (Fig. 1, Panel B), consistent with their being activated [46]. Most of the cells were large, again suggesting that they were activated (Fig. 2). Compared to control ATOC, cells from the secondary suspension cultures had a smaller percentage of CD4$^+$ single positive cells and an increased percentage of CD4$^+$CD8$^+$ cells (Fig. 1, Panel B). We observed, however, that the CD4$^+$CD8$^+$ cells were in fact CD4$^+$CD8α^+ cells with a TCR^{hi}

phenotype (data not shown). Because activated CD4$^+$ T cells in the rat express the CD8α chain [47], the data suggest that the double positive cells were actually activated CD4$^+$ 'single' positive cells. Secondary suspension culture was also associated with an increase in the cell surface densities of CD25 and CD8α chain (Fig. 2).

3.3. Both IL-18 and TCR/MHC-ligand interactions are important for cell activation in ATOC incubated in the presence of type 1 cytokines

3.3.1. Pharmacologic inhibitors

IL-18 can synergize with IL-12 to induce antigen-independent activation of T cells [37,39,48,49]. It also amplifies TCR-driven type 1 polarized responses [20,21]. To analyze the role of IL-18 in the activation of ATOC-derived cells, Western blot analysis was performed on
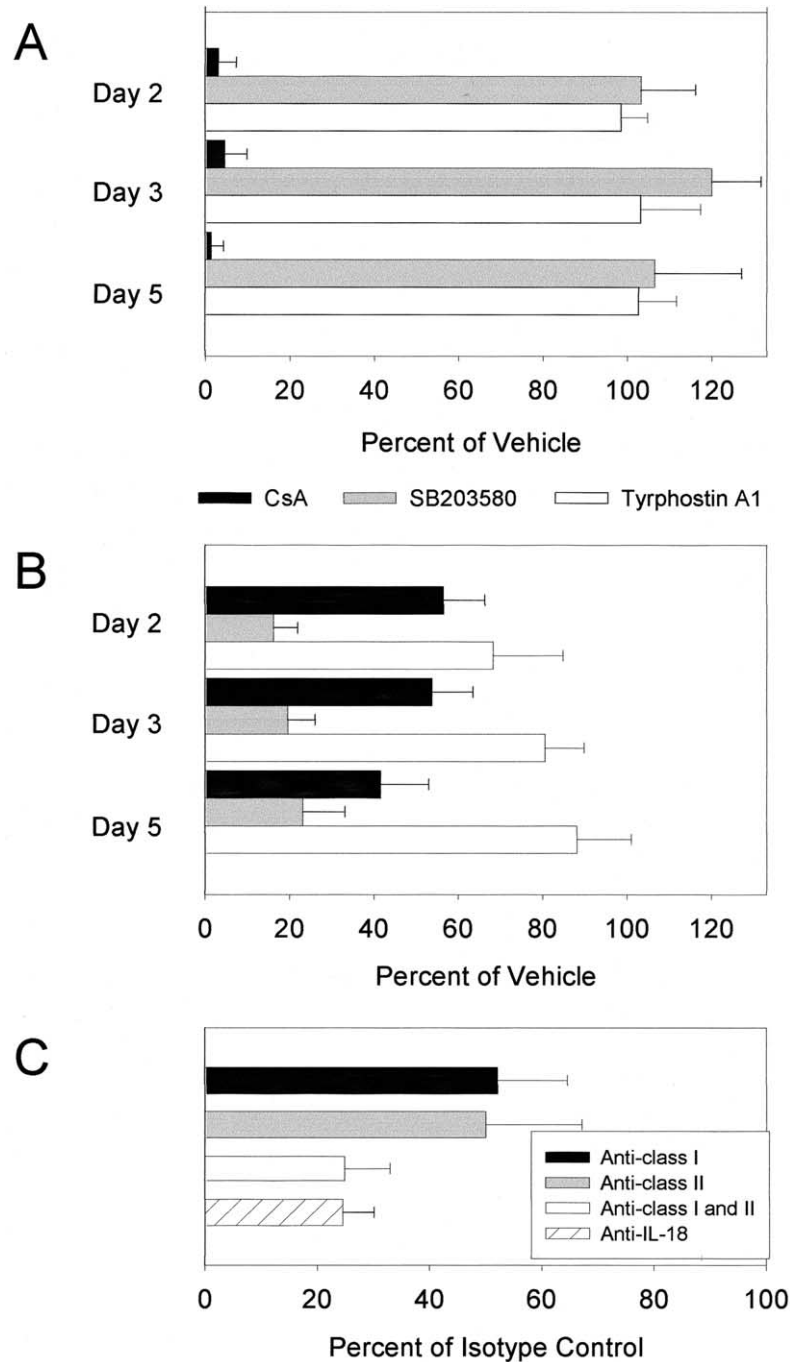
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Fig. 4. IFNγ production by thymocytes in the presence or absence of pharmacological inhibitors and antibodies. Panels A and B: Adult BBDR thymi were cultured in the presence of type 1 cytokines for 5 days followed by secondary suspension culture performed either in the presence of plate-bound anti-CD3 mAb (Panel A) or type 1 cytokines (Panel B). To individual secondary cultures was also added either cyclosporin A (black bars), the p38 map kinase inhibitor SB203580 (gray bars), tyrphostin A1 (white bars), or DMSO. Aliquots of supernatant were harvested from the same cultures on days 2, 3, and 5 of the secondary culture and the concentration of IFNγ was measured by ELISA. Results are expressed as percent of the IFNγ concentration present in the control cultures performed in the presence of the vehicle DMSO. Each data point represents the mean ± 1 SD of seven to eight individual cultures from two independent trials. Panel C: Adult BBDR thymi were cultured in the presence of type 1 cytokines for 5 days followed by secondary suspension culture with type 1 cytokines. Cultures were performed in the presence of antibodies directed against MHC class I and/or MHC class II (secondary cultures only) or antibody against IL-18 (primary and secondary cultures), or isotype control antibody as described in Section 2. The concentration of IFNγ in the culture supernatants was measured by ELISA on day 5 of suspension culture. Results are expressed as the mean percent of the IFNγ concentration (± 1 SD) present in the control cultures performed in the presence of the isotype control antibody. Data for IL-18 blocking experiments are from six cultures performed in the course of three independent trials. Data for MHC class I and class II blocking experiments are from seven cultures performed during two independent trials.

lysates of cells obtained from ATOC. We detected both the cleaved active form and the inactive precursor form of rat IL-18 in cells recovered from both ATOC cultured in type 1 cytokines and from secondary suspension cultures (Fig. 3). The amount of the active form decreased over time, and the amount of precursor increased. This observation is probably due to the fact that the active form is secreted into the medium, which was not tested in this analysis.

The presence of IL-18 suggests that it may have been involved in the antigen-independent activation of T cells and the induction of IFNγ secretion in this system [39]. Alternatively, it is possible that cell activation was mediated by signaling through the TCR following interaction with MHC/self-peptides. We used pharmacological inhibitors to discriminate these possibilities. Cells from ATOC incubated with type 1 cytokines were recovered and then cultured in suspension in the presence of either type 1 cytokines or immobilized anti-CD3 mAb (a positive control for TCR-mediated signaling). Both types of secondary culture were then treated with either: (1) CsA, which inhibits TCR-mediated signaling, but does not affect T cell activation mediated by IL-12 plus IL-18 [37]; (2) the p38 MAP-kinase inhibitor SB203580, which inhibits IL-12 and IL-18-mediated, but not TCR-mediated T cell activation [39]; (3) tyrphostin A1, which reportedly does not inhibit either pathway [39]; or (4) DMSO, the vehicle for each of the other compounds. Concentrations of IFNγ in supernatants on days 2, 3, and 5 of secondary culture were quantified by ELISA.

In comparison with DMSO control cultures, cyclosporin A inhibited IFNγ production induced by anti-CD3 by >95% at all time points (Fig. 4, Panel A), confirming published data [39]. As expected [39], neither SB203580 nor tyrphostin A1 had any effect on anti-CD3 mAb induced IFNγ production. In contrast (Fig. 4, Panel B), T cell activation induced by type 1 cytokines was inhibited ∼50% by CsA and ∼80% by SB203580 at all time points. It was also inhibited by tyrphostin A1, but to a much lesser extent, 15–30%. This effect of tyrphostin A1 may represent its recently described ability to inhibit tyrosine kinase activity that is associated with IL-12 signaling [50]. The inhibitor data suggest that both TCR-mediated activation and cytokine signaling pathways are involved in the activation of cells in ATOC exposed to type 1 cytokines.

3.3.2. Studies with antibodies

To investigate TCR-mediated activation further, we added antibodies against rat MHC class I, MHC class II, or both to secondary suspension cultures performed with type 1 cytokines. The anti-MHC mAbs were added only at the time of secondary suspension culture in order to permit intrathymic selection to occur during the primary ATOC. We also tested antibody against rat

IL-18, in this case adding the antibody both to the ATOC and to the suspension cultures.

In comparison with control cultures performed using isotype control antibodies, IFNγ production was reduced ∼50% by anti-MHC class I or anti-MHC class II antibody alone, ∼75% by the combination of these two antibodies, and ∼75% by neutralizing anti-IL-18 antibody (Fig. 4, Panel C). The results are consistent with the inhibitor data and suggest that both TCR-mediated and IL-18-mediated signaling are involved in the activation of ATOC-derived cells incubated in type 1 cytokines.

3.4. Requirements for type 1 cytokine polarization and the cellular origin of IFNγ

We next determined the relative contributions of IL-12, IL-2, and anti-IL-4 in the production of IFNγ by rat ATOC. The standard conditions that we have used to polarize rat cells (10 ng/ml IL-12, 10 ng/ml IL-2 and 25 μg/ml anti-IL-4 in ATOC) were assigned a value of 100% (123,844 ± 30,540 pg/ml of IFNγ on day 5 ATOC in three to four independent trials). Fig. 5 (Panel A) shows the levels of IFNγ produced under different treatment conditions as a percentage of the IFNγ production under the standard conditions. The combination of IL-12 and IL-2 was the most effective. IFNγ levels in cultures that received 10 ng/ml IL-12 plus 10 ng/ml IL-2 averaged 58% of the levels observed in cultures where anti-IL-4 was also included (standard conditions). However, anti-IL-4 alone was not sufficient to polarize the cells.

We evaluated the doses of IL-12 that could effectively polarize thymocytes to a type 1 cytokine profile in IL-12 dose titration studies. Five-fold and 25-fold lower concentrations of IL-12 were used together with IL-2 and anti-IL-4. Fig. 5 (Panel A) shows that even at these very low concentrations of IL-12, IFNγ production still averaged 64% of the levels observed with the standard conditions of 10 ng/ml of IL-12.

The cellular origin of the IFNγ in the ATOC was determined by surface labeling for TCR, CD4, and CD8, followed by intracellular staining for IFNγ. Table 2 shows the distribution of IFNγ$^+$ cells within the TCR$^+$ and TCR$^-$ compartments, and within the pre-selection CD4$^+$CD8$^+$ population and the positively selected CD4 and CD8 single positive populations. On day 5 of the ATOC, approximately one-third of total cells are CD4 CD8 double positive cells and approximately two-thirds are single positive cells in all of the treatment groups (data not shown). For the standard conditions of polarization (ATOC treatment B), all of the IFNγ$^+$ cells were TCR$^+$, with a similar percentage of IFNγ$^+$ cells in the immature CD4$^+$CD8$^+$, and the selected CD4 and CD8 single positive cells. IFNγ$^+$ cells were observed in both CD4 and CD8 single positive populations at a ratio of
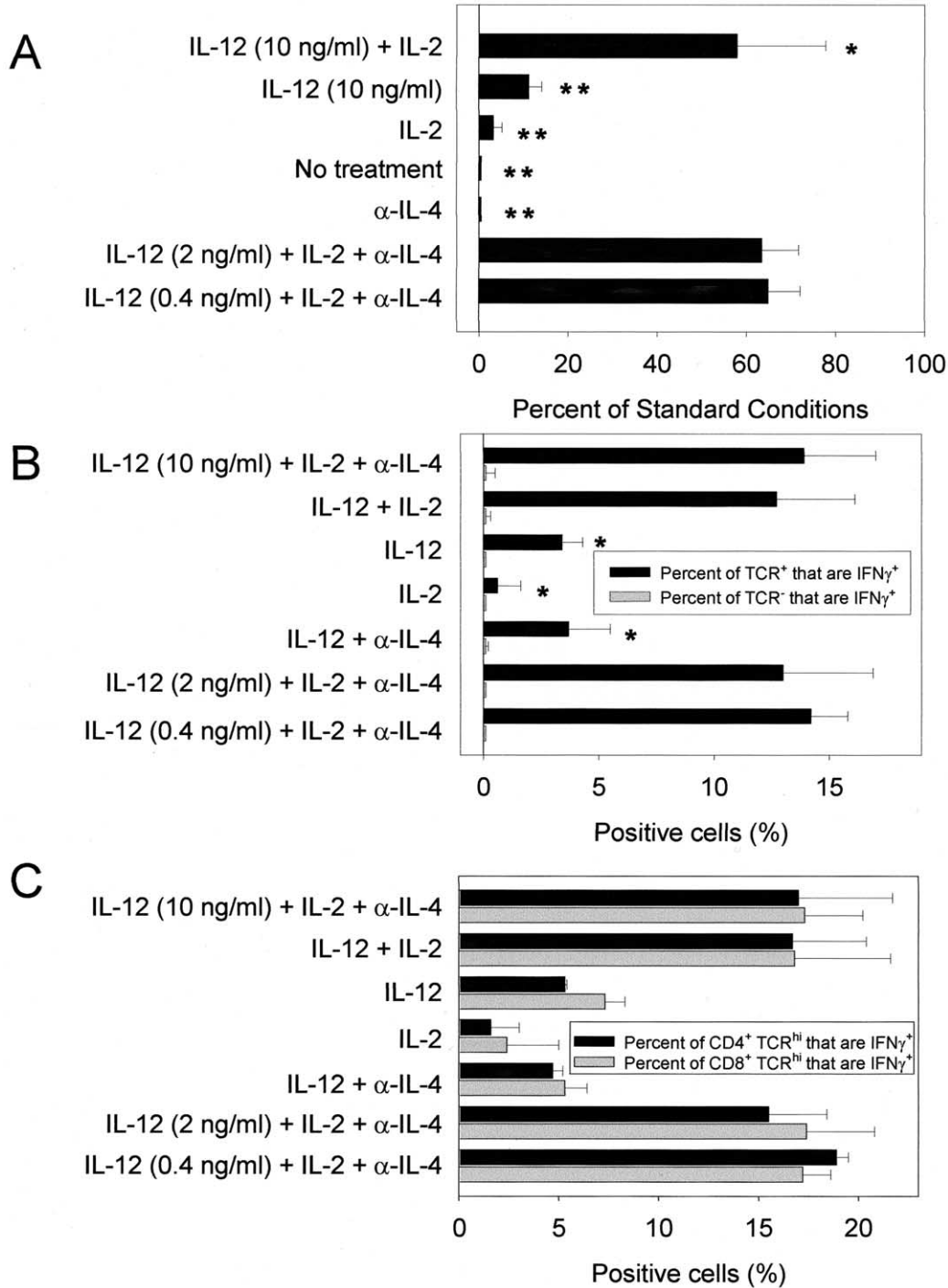
Fig. 5. Requirements for type 1 cytokine polarization and the cellular origin of IFNγ. Panel A: Requirements for type 1 cytokine polarization in day 5 ATOC. Type 1 cytokine polarization was measured by IFNγ concentrations in the supernatants by ELISA assay. Results are expressed as the mean percent of IFNγ concentration (± 1 SD) present in the cultures performed in the presence of 10 ng/ml IL-12, 10 ng/ml IL-2 and 25 μg/ml anti-IL-4 ('standard conditions'). Data are from three to four independent trials. *IFNγ levels are significantly different from standard conditions ($p<0.05$). **IFNγ levels are significantly different from standard conditions ($p<0.005$). Panels B and C: Requirements for type 1 cytokine polarization and cellular origin of IFNγ in day 5 secondary cultures. Cells were cultured under the indicated conditions for 5 days in ATOC. The IFNγ production by these ATOC are the data shown in Table 2 and in Fig. 5 (Panel A). ATOC were then harvested and re-cultured under the same treatment conditions for an additional 5 days in secondary culture. Type 1 cytokine polarization was measured by the frequency of IFNγ⁺ cells as determined by intracellular staining. A mouse IgG1 isotypic control antibody was used to determine background staining, which is subtracted from the percent values shown. Results shown are the mean frequency of IFNγ⁺ cells (± 1 SD) for three to four independent determinations. Panel B: Frequencies of IFNγ⁺ cells in TCR⁺ and TCR⁻ populations on day 5 of secondary cultures. *IFNγ levels are significantly different from standard conditions shown in the uppermost bars ($p<0.05$). Panel C: Frequencies of IFNγ⁺ cells in CD4⁺ and CD8⁺ populations on day 5 of secondary cultures. No statistically significant differences were observed between the frequencies of IFNγ⁺ cells in the CD4 or CD8 T cell compartments.

Table 2
Cellular origin of IFNγ producing cells in BBDR rat ATOC

ATOC treatment	% IFNγ$^+$ cells in the TCR$^+$ population	% IFNγ$^+$ cells in the TCR$^-$ population	% IFNγ$^+$ cells in the CD4$^+$CD8$^+$ population	% IFNγ$^+$ cells in CD4 and CD8 single positive populations
A. No treatment	0.4 ± 0.3	0 ± 0	0.6 ± 0.5	0.6 ± 0.6
B. IL-12 (10 ng/ml) + IL-2 + anti- IL-4	2.0 ± 1.2	0 ± 0	3.6 ± 2.3	2.4 ± 1.5
C. IL-12 (10 ng/ml) + IL-2	2.0 ± 1.7	0 ± 0	2.3 ± 0.7	1.3 ± 1.1
D. IL-12	0.6 ± 0.4	0 ± 0	1.2 ± 0.8	0.6 ± 0.1
E. Anti-IL-4	0.5 ± 0.2	0 ± 0	0.5 ± 0.3	0.7 ± 0.1
F. IL-2	0.8 ± 0.2	0 ± 0	0.8 ± 0.3	0.9 ± 0.2
G. IL-12 (2 ng/ml) + IL-2 + anti-IL-4	2.5 ± 2.0	0 ± 0	3.4 ± 1.3	2.4 ± 1.3
H. IL-12 (0.4 ng/ml) + IL-2 + anti-IL-4	1.4 ± 0.8	0 ± 0	1.4 ± 0.2	2.0 ± 0.9

ATOC were established on day 0 with the indicated treatments. On day 5, cultures were harvested and stained for surface markers and intracellular IFNγ as described in Section 2. Results shown are the mean ± 1 SD for three to four independent trials. The ratio of CD4$^+$CD8$^+$ cells to CD4 and CD8 single positive cells in these experiments was approximately 1:3 for all treatment conditions.

1:4 (data not shown). Omission of the anti-IL-4 did not significantly decrease the frequency of IFNγ$^+$ cells (treatment C) relative to the standard conditions. Both IL-12 and IL-2 appear to be necessary for optimal induction of IFNγ in day 5 ATOC (treatments D and F vs. B and C). The frequency of IFNγ$^+$ cells was similar between groups that received 10, 2, or 0.4 ng/ml of IL-12 (treatments B, G, and H).

The cellular origin of IFNγ was also determined after secondary culture under these same treatment conditions. At this time, 10 days after the initiation of the ATOC cultures, virtually all of the cells have a positively selected phenotype with up-regulated αβTCR. Only the αβTCR$^+$ cells T cells produced IFNγ (Fig. 5, Panel B), and they were equally divided between the CD4$^+$ and CD8$^+$ TCR subsets for all of the treatment conditions (Fig. 5, Panel C). As we observed for ATOC cultures, IL-12 and IL-2 synergized in the induction of IFNγ$^+$ cells, and all doses of IL-12 induced comparable frequencies of IFNγ$^+$ cells in secondary cultures. There was no difference in the frequency of IFNγ$^+$ cells between cultures that received anti-IL-4 and those that did not. The effect of anti-IL-4 alone without cytokines could not be determined because cells in this treatment group did not survive in the secondary cultures.

3.5. Comparative analysis of BBDR lymph node T cells cultured in type 1 cytokines

Because the polarization of adult rat thymocytes in culture has not, to our knowledge, previously been reported, we sought to compare the responses of these cultures to more conventional cultures of peripheral lymph node cells. BBDR rat lymph node cell suspensions were cultured either in type 1 cytokines at the same concentrations used for the culture of ATOC-derived cells, or in the presence of the mitogen Con A. On day 5

(corresponding to the end of the ATOC), lymph node cells cultured in type 1 cytokines secreted ∼10-fold more IFNγ than did cells from Con A stimulated cultures (Fig. 6, Panel A, $p < 0.02$). The concentration of IFNγ in supernatants of lymph node cultures performed with type 1 cytokines was ∼65% of that found in ATOC supernatants (Fig. 1, Panel A), but the data do not permit any determination of the production of IFNγ on a per cell basis. Lymph node cells cultured in type 1 cytokines secreted ∼two-fold more IL-10 and TNFα than did cells in Con A-activated cultures, but the concentrations were low under both the conditions.

We also compared the cell surface phenotype of lymph node cells cultured in the presence of type 1 cytokines with that of freshly isolated lymph node cells (Fig. 6, Panels B and C). There was little evidence of activation in freshly isolated lymph node cells. After culture of lymph node cells in type 1 cytokines, small populations (<30%) of cells expressing both CD4 and CD8α chain, and the activation markers CD25 and CD86 were detected.

We attempted to extend the comparison between polarization of lymph node cell and thymocytes by harvesting the lymph node cell cultures and performing a secondary culture, as was done for thymocytes. We found, however, that a secondary culture of lymph node cells in type 1 cytokines led to the death of >90% of the cells by day 2 of culture, presumably due to activation induced cell death [51,52].

4. Discussion

Cytokine polarization of peripheral T cells during TCR engagement is an important mechanism regulating the type of immune response that is generated to foreign antigens and to autoantigens [53,54]. In the present
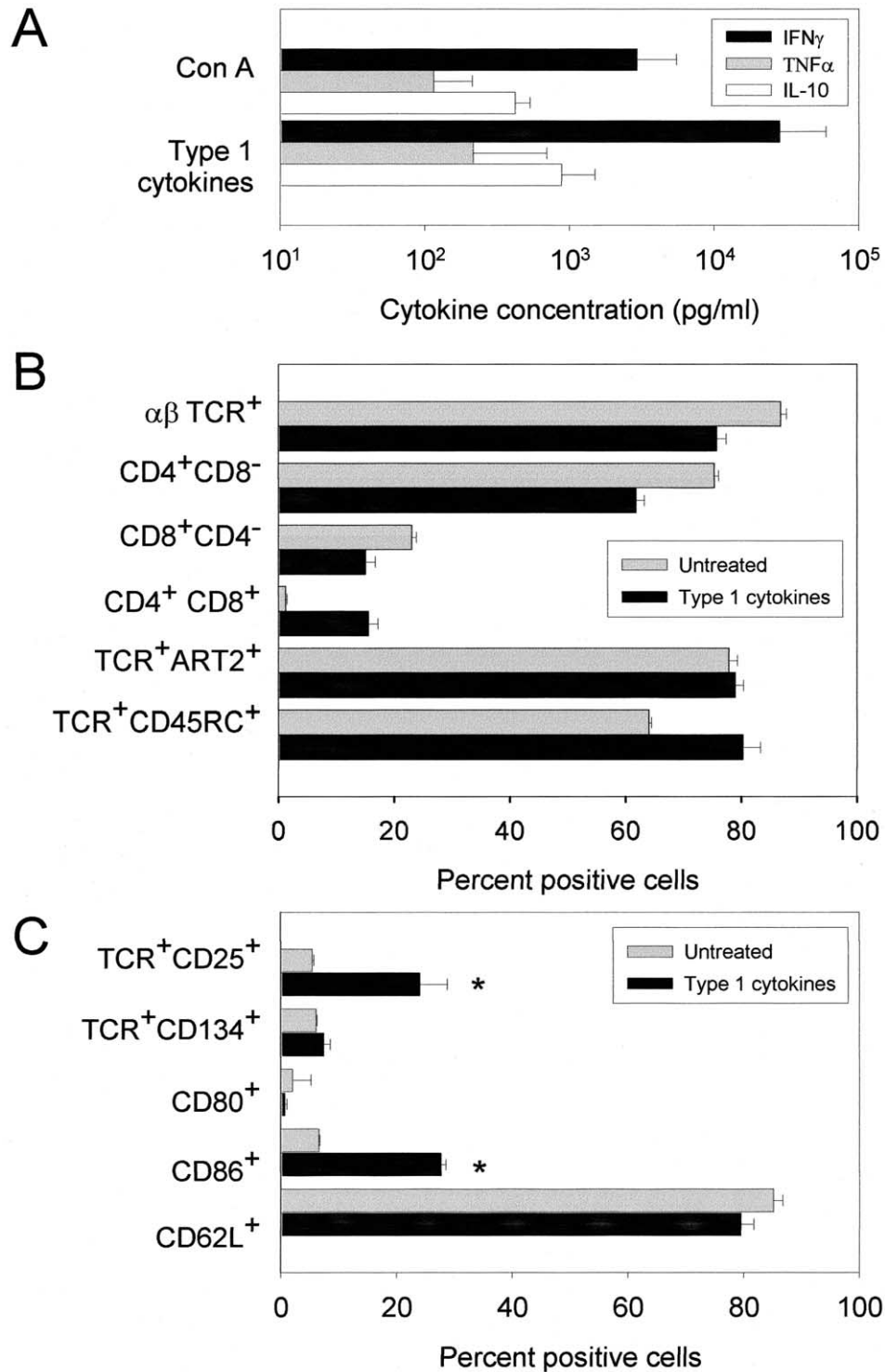
Fig. 6. Cytokine production and cellular phenotype of BBDR lymph node cells. Panel A: BBDR lymph node cells were cultured in the presence of Con A (5 μg/ml) or type 1 cytokines for 5 days. The concentrations of IFNγ, TNFα, and IL-10 in the culture supernatant were determined by ELISA. Each data point represents the mean ± 1 SD of four independent trials. There were no statistically significant differences between the two groups. Panel B: BBDR lymph node cells cultured in type 1 cytokines for 5 days (black bars) and a sample of freshly isolated BBDR rat lymph node cells (gray bars) were harvested and analyzed by flow cytometry for the expression of differentiation (Panel B), and activation (Panel C) antigens. Each data point represents the mean percentage of cells (± 1 SD) in the lymphocyte fraction expressing the indicated antigen in three to five independent experiments. Percentages obtained using isotype control antibodies were uniformly <1% and were not subtracted from the values shown. *$p<0.01$ vs. freshly isolated cells.

study, we have demonstrated that exposure of thymocytes to type 1 cytokines during intrathymic selection also modulates T cell activation and cytokine production. T cells derived from type 1 cytokine-conditioned rat ATOC spontaneously produced large amounts of IFNγ. Re-exposure of these cells to type 1 cytokines induced even more production of IFNγ and was associated with T cell activation. These events occurred in the absence of exogenous foreign antigen. The IFNγ datasets suggest that the susceptibility of adult thymocytes to polarization is qualitatively similar to that of peripheral lymph node cells exposed to type 1 cytokines in the absence of antigen.

The polarization of developing thymocytes by type 1 cytokines has not, to our knowledge, been reported previously. Our data clearly document the secretion of copious amounts of IFNγ and of T cell activation following exposure of ATOC to type 1 cytokines. Most importantly, the data suggest that intrathymic polarization could be an important process in vivo as well as in vitro. We note that IL-12, the principal agent of polarization used in these studies, is synthesized in the thymus [22,23] and circulates at high levels after exposure to certain pathogens [5,13,16–18] [55,56] or following administration of CpG DNA [57]. Nonlethal doses of *Toxoplasma gondii*, *Toxoplasma cruzi*, MCMV or CpG DNA given to mice result in circulating levels of IL-12 that range from 0.45 to 15 ng/ml, with persistence in the serum from 36 h to 8 days. Ex vivo production of IL-12 by cells from mice given CpG DNA, *T. gondii*, or *Mycoplasma bovis* range from 1–5 ng/ml in 24–48 h cultures of spleen or lymph node cells, or cultures of APCs [18,58,59]. Culture of human PBL or monocytes with seven different species of gram positive bacteria or *Heliobacter pylori* induces the production of ∼1–4 ng/ml of IL-12 after 18–24 h in culture [59,60]. In addition, virus infections in humans and mice can further increase sensitivity to concurrent or subsequent bacterial challenges by a mechanism that involves elevated serum levels of proinflammatory cytokines, including IL-12 [61]. Circulating IL-12 levels following exposure to certain pathogens are therefore comparable to those able to polarize thymocytes in ATOC.

The detection of intrathymic polarization in vivo would be a difficult undertaking due to the complex kinetics associated with continuous influx of bone marrow immigrants, intrathymic death of most of those immigrants, and efflux of maturing T cells. The key to our discovery was the use of ATOC [27]. Thymocytes survive poorly in cell suspension cultures, irrespective of whether the cells are cultured in the presence or absence of type 1 cytokines (B.J.W., unpublished observations). In contrast, rat ATOC allows normal thymocyte development and selection to occur in vitro. It overcomes the problem of in vivo studies by allowing for analysis of T cell development in the absence of seeding of the thymus

with thymocyte progenitors and in the absence of emigration from the thymus [27]. Rat ATOC recapitulates faithfully normal rat intrathymic T cell developmental kinetics and phenotypes. It generates CD4 and CD8 single positive cells that up-regulate the TCR, as is characteristic of peripheral T cells [27].

Our observation that rat ATOC generates single-positive T cells was the basis of our hypothesis that thymocytes might respond to the polarizing effects of type 1 cytokines as a result of TCR engagement during selection [24–26]. We also hypothesized that exposure of ATOC to type 1 cytokines might lead to T cell activation by an antigen-independent mechanism, as has been described for mouse and human peripheral T cells cultured in IL-12 and IL-18 [37,39,48,49]. The present data confirm both of these hypotheses and suggest that both antigen-dependent and antigen-independent mechanisms contribute to intrathymic T cell polarization.

With respect to the antigen-dependent mechanism, we documented that anti-MHC mAbs, which block TCR/MHC-ligand cognate interactions [62], reduced T cell polarization. The participation of TCR engagement was further confirmed by the use of cyclosporin A, which blocks T cell activation mediated by TCR signaling. We interpret these data to suggest that TCR–MHC-ligand interactions play an important role in the activation of the T cells that are cultured in type 1 cytokines. We recognized that xenogeneic reactivity against bovine serum could have been an alternative explanation for these results, but our control cultures performed with rat serum largely excluded this possibility.

With respect to the antigen-independent mechanism of intrathymic polarization, we documented that both neutralizing anti-IL-18 antibody and the p38 MAP-kinase inhibitor SB203580 also reduced T cell polarization. These observations are consistent with the view that a cytokine-driven antigen-independent pathway participates in intrathymic polarization. We do recognize, however, that IL-18 can synergize with IL-12 to activate T cells in the presence or absence of antigen, implying that IL-18 could participate in both antigen-dependent and antigen-independent polarizing processes.

We demonstrated that the IFNγ produced in ATOC and in secondary cultures originates from T cells. In ATOC, polarization occurs in both pre-selection CD4+CD8+ and post-selection CD4 and CD8 single positive thymocytes (Table 2). Polarization of double positive thymocytes might be predicted from the observations that these cells show heightened sensitivity to stimulation with low affinity peptide–MHC complexes compared to mature T cells, even though their surface TCR levels are lower [63,64]. After a secondary exposure to type 1 cytokines, there is a substantial increase in the percent of IFNγ+ cells. Exposure of thymocytes to a

type 1 cytokine environment can clearly 'prime' cells for heightened responses on secondary exposure to these conditions.

The combination of IL-12 and IL-2 is optimal for type 1 cytokine polarization of both ATOC and secondary cultures (Table 2; Fig. 5). Anti-IL-4 by itself is not sufficient to bias cells to a type 1 cytokine response, but it was shown to facilitate IFNγ production in the ATOC but not in secondary cultures. We were unable to detect IL-4 in day 5 ATOC by ELISA in cultures that received IL-12 plus IL-2 (<8 pg/ml in four determinations), but it is possible that IL-4 could have been produced earlier in the ATOC and/or utilized by day 5. Based on our observation that exposure of developing thymocytes to polarizing conditions generates activated T cells, we speculate that the exposure of thymocytes in vivo to analogous conditions may represent a mechanism underlying the generation and survival of self-reactive T cells in genetically pre-disposed individuals. If polarizing conditions were to be present in the thymus, self-reactivity might result from interference with the negative selection of high affinity self-reactive T cells or from the promotion of positive selection. Alternatively, self-antigens might be cross-presented to T cells as a result of cytokine induced maturation of APCs, a mechanism that has been described for peripheral T cells [65,66]. Many self-antigens are expressed by thymic stromal cells, and correlative studies suggest that intrathymic self-antigen expression may play an important role in T cell tolerance [67–71]. Self-antigens that are normally expressed at levels too low to induce positive selection might do so in the context of a pro-inflammatory environment, up-regulated APC function [5,72], and a lowered threshold for T cell activation [73,74]. The role of type 1 cytokines in altering selection during intrathymic development is unknown, but perturbation of intrathymic selection in experimental systems has been shown to lead to the generation of autoreactive T cells [75–77]. Environmental perturbation in general, and infection in particular, have been implicated in the pathogenesis of several autoimmune diseases [78,79]. Polarizing conditions within the thymus resulting from high circulating levels of IL-12 generated by infection could represent a mechanism linking genetic susceptibility to autoreactivity to the environmental 'triggers' of autoimmune disease.

The hypothesis that intrathymic exposure to type 1 polarizing conditions could lead to a loss of self-tolerance implies that the activated T cells derived from BBDR rat ATOC exposed to type 1 cytokines should be self-reactive. Preliminary studies using adoptive transfer methods suggest that this is, in fact, the case (B.J.W., unpublished observations).

In summary, we have documented that developing thymocytes exposed to type 1 cytokines in vitro become polarized towards a type 1 cytokine profile through a combination of antigen-dependent and cytokine-driven mechanisms. We speculate that thymocytes in vivo could be exposed to a type 1 cytokine environment as a result of IL-12 produced endogenously or in response to infection. The relative importance of intrathymic polarization as a normal physiological response mechanism and a pathophysiological process leading to autoimmunity remains under study in our laboratory.

Acknowledgements

References

[1] Mosmann T, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145–73.

[2] Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787–93.

[3] O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 1998;8:275–83.

[4] Seder RA. Acquisition of lymphokine-producing phenotype by CD4+ T cells. J Allergy Clin Immunol 1994;94:1195–202.

[5] Romani L, Puccetti P, Bistoni F. Interleukin-12 in infectious diseases. Clin Microbiol Rev 1997;10:611–36.

[6] Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55:1139–49.

[7] Miossec P. Cytokines in rheumatoid arthritis: is it all TNF-alpha? Cell Mol Biol 2001;47:675–8.

[8] Finkelman FD, Urban JF Jr. The other side of the coin: the protective role of the TH2 cytokines. J Allergy Clin Immunol 2001;107:772–80.

[9] Grencis RK. Th2-mediated host protective immunity to intestinal nematode infections. Philos Trans R Soc Lond B Biol Sci 1997; 352:1377–84.

[10] Mazzarella G, Bianco A, Catena E, De Palma R, Abbate GF. Th1/Th2 lymphocyte polarization in asthma. Allergy 2000;55(Suppl 61):6–9.

[11] Seder RA, Paul WE, Ben Sasson SZ, LeGros GS, Kagey-Sobotka A, Finkelman FD et al. Production of interleukin-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. Int Arch Allergy Appl Immunol 1991;94:137–40.

[12] Croft M, Swain SL. Recently activated naive CD4 T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. J Immunol 1995;154:4269–82.

[13] Dalod M, Salazar-Mather TP, Malmgaard L, Lewis C, Asselin-Paturel C, Briere F et al. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. J Exp Med 2002; 195:517–28.

[14] de Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell- promoting dendritic cells in vitro with diverse Th cell-polarizing signals. J Immunol 2002;168:1704–9.

[15] Wang J, Wakeham J, Harkness R, Xing Z. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. J Clin Investig 1999;103:1023–9.

[16] Ruzek MC, Miller AH, Opal SM, Pearce BD, Biron CA. Characterization of early cytokine responses and an interleukin (IL)-6-dependent pathway of endogenous glucocorticoid induction during murine cytomegalovirus infection. J Exp Med 1997; 185:1185–92.

[17] Mordue DG, Monroy F, La Regina M, Dinarello CA, Sibley LD. Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. J Immunol 2001;167:4574–84.

[18] Sousa CR, Hieny S, Scharton-Kersten T, Jankovic D, Charest H, Germain RN et al. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J Exp Med 1997;186:1819–29.

[19] Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732–8.

[20] Robinson D, Shibuya K, Mui A, Zonin F, Murphy E, Sana T et al. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. Immunity 1997;7:571–81.

[21] Chang JT, Segal BM, Nakanishi K, Okamura H, Shevach EM. The costimulatory effect of IL-18 on the induction of antigen-specific IFN-gamma production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor beta2 subunit. Eur J Immunol 2000;30:1113–9.

[22] Ludviksson BR, Ehrhardt RO, Strober W. Role of IL-12 in intrathymic negative selection. J Immunol 1999;163:4349–59.

[23] Godfrey DI, Kennedy J, Gately MK, Hakimi J, Hubbard BR, Zlotnik A. IL-12 influences intrathymic T cell development. J Immunol 1994;152:2729–35.

[24] Jameson SC, Bevan MJ. T-cell selection. Curr Opin Immunol 1998;10:214–9.

[25] Amsen D, Kruisbeek AM. Thymocyte selection: not by TCR alone. Immunol Rev 1998;165:209–29.

[26] Chidgey AP, Boyd RL. Thymic stromal cells and positive selection. Acta Pathol Microbiol Immunol Scand 2001; 109:481–92.

[27] Whalen BJ, Weiser P, Marounek J, Rossini AA, Mordes JP, Greiner DL. Recapitulation of normal and abnormal BioBreeding rat T cell development in adult thymus organ culture. J Immunol 1999;162:4003–12.

[28] Whalen BJ, Marounek J, Weiser P, Appel MC, Greiner DL, Mordes JP et al. BB rat thymocytes cultured in the presence of islets lose their ability to transfer autoimmune diabetes. Diabetes 2001;50:972–9.

[29] Mordes J, Bortell R, Doukas J, Rigby MR, Whalen BJ, Zipris D et al. The BB/Wor rat and the balance hypothesis of autoimmunity. Diabetes Metab Rev 1996;2:103–9.

[30] Mordes JP, Bortell R, Groen H, Guberski DL, Rossini AA, Greiner DL. Autoimmune diabetes mellitus in the BB rat. In: Sima AAF, Shafrir E, editors. Animal models of diabetes: a primer. Amsterdam: Harwood Academic Publishers; 2001;1–41.

[31] Ellerman KE, Richards CA, Guberski DL, Shek WR, Like AA. Kilham rat triggers T-cell-dependent autoimmune diabetes in multiple strains of rat. Diabetes 1996;45:557–62.

[32] Zipris D. Evidence that Th1 lymphocytes predominate in islet inflammation and thyroiditis in the BioBreeding (BB) rat. J Autoimmun 1996;9:315–9.

[33] Wildbaum G, Youssef S, Grabie N, Karin N. Neutralizing antibodies to IFN-gamma-inducing factor prevent experimental autoimmune encephalomyelitis. J Immunol 1998;161:6368–74.

[34] Waite DJ, Appel MC, Handler ES, Mordes JP, Rossini AA, Greiner DL. Ontogeny and immunohistochemical localization of thymus-dependent and thymus-independent RT6+ cells in the rat. Am J Pathol 1996;148:2043–56.

[35] Hylkema MN, van der DM, Pater JM, Kampinga J, Nieuwenhuis P, Groen H. Single expression of CD45RC and RT6 in correlation with T-helper 1 and T-helper 2 cytokine patterns in the rat. Cell Immunol 2000;199:89–96.

[36] Jenkinson EJ, Owen JJ. T-cell differentiation in thymus organ cultures. Semin Immunol 1990;2:51–8.

[37] Yang J, Murphy TL, Ouyang W, Murphy KM. Induction of interferon-gamma production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. Eur J Immunol 1999;29:548–55.

[38] Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF et al. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 1995;364:229–33.

[39] Yang J, Zhu H, Murphy TL, Ouyang W, Murphy KM. IL-18-stimulated GADD45 beta required in cytokine-induced, but not TCR- induced, IFN-gamma production. Nat Immunol 2001; 2:157–64.

[40] Nie NH, Hull CH, Jenkins JG, Steinbrenner K, Bent DH. Statistical package for the social sciences. New York: McGraw-Hill, 1975.

[41] Glantz SA. Primer of biostatistics. New York: McGraw-Hill, 1981.

[42] Gerosa F, Paganin C, Peritt D, Paiola F, Scupoli MT, Aste-Amezaga M et al. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. J Exp Med 1996;183:2559–69.

[43] Bortell R, Kanaitsuka T, Stevens LA, Moss J, Mordes JP, Rossini AA et al. The RT6 (Art2) family of ADP-ribosyltransferases in rat and mouse. Mol Cell Biochem 1999;193:61–8.

[44] Tamatani T, Kitamura F, Kuida K, Shirao M, Mochizuki M, Suematsu M et al. Characterization of rat LECAM-1 (L-selectin) by the use of monoclonal antibodies and evidence for the presence of soluble LECAM-1 in rat sera. Eur J Immunol 1993;23:2181–8.

[45] Johnson P, Maiti A, Ng DHW. CD45: a family of leukocyte-specific cell surface glycoproteins. In: Herzenberg LA, Weir DM, Herzenberg LA, editors. Weir's handbook of experimental immunology. Cambridge: Blackwell Science; 2001;62.1–6.

[46] Sarawar SR, Sparshott SM, Sutton P, Yang CP, Hutchinson IV, Bell EB. Rapid re-expression of CD45RC on rat CD4 T cells in vitro correlates with a change in function. Eur J Immunol 1993; 23:103–9.

[47] Bevan DJ, Chisholm PM. Co-expression of CD4 and CD8 molecules and de novo expression of MHC class II antigens on activated rat T cells. Immunology 1986;59:621–5.

[48] Yoshimoto T, Takeda K, Tanaka T, Ohkusu K, Kashiwamura S, Okamura H et al. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. J Immunol 1998;161:3400–7.

[49] Tominaga K, Yoshimoto T, Torigoe K, Kurimoto M, Matsui K, Hada T et al. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. Int Immunol 2000; 12:151–60.

[50] Du C, Bright JJ, Sriram S. Inhibition of CD40 signaling pathway by tyrphostin A1 reduces secretion of IL-12 in macrophage, Th1 cell development and experimental allergic encephalomyelitis in SJL/J mice. J Neuroimmunol 2001;114:69–79.

[51] Budd RC. Activation-induced cell death. Curr Opin Immunol 2001;13:356–62.

[52] Janssen O, Sanzenbacher R, Kabelitz D. Regulation of activation-induced cell death of mature T-lymphocyte populations. Cell Tissue Res 2000;301:85–99.

[53] Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. Immunol Res 1999;20:147–61.

[54] Romagnani S. Th1 and Th2 in human diseases. Clin Immunol Immunopathol 1996;80:225–35.

[55] Antunez MI, Cardoni RL. IL-12 and IFN-gamma production, and NK cell activity, in acute and chronic experimental *Trypanosoma cruzi* infections. Immunol Lett 2000;71:103–9.

[56] Bliss SK, Butcher BA, Denkers EY. Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. J Immunol 2000;165:4515–21.

[57] Krieg AM, Love-Homan L, Yi AK, Harty JT. CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. J Immunol 1998;161:2428–34.

[58] Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. J Immunol 2000;165:1228–35.

[59] Jiao X, Lo-Man R, Guermonprez P, Fiette L, Deriaud E, Burgaud S et al. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. J Immunol 2002; 168:1294–301.

[60] Hessle C, Andersson B, Wold AE. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect Immun 2000;68:3581–6.

[61] Nguyen KB, Biron CA. Synergism for cytokine-mediated disease during concurrent endotoxin and viral challenges: roles for NK and T cell IFN-gamma production. J Immunol 1999;162:5238–46.

[62] Jenkinson EJ, Owen JJ. T-cell differentiation in thymus organ cultures. Semin Immunol 1990;2:51–8.

[63] Lucas B, Stefanova I, Yasutomo K, Dautigny N, Germain RN. Divergent changes in the sensitivity of maturing T cells to structurally related ligands underlies formation of a useful T cell repertoire. Immunity 1999;10:367–76.

[64] Davey GM, Schober SL, Endrizzi BT, Dutcher AK, Jameson SC, Hogquist KA. Preselection thymocytes are more sensitive to T cell receptor stimulation than mature T cells. J Exp Med 1998; 188:1867–74.

[65] Den Haan JMM, Lehar SM, Bevan MJ. CD8+ but not CD8− dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med 2000;192:1685–95.

[66] Hoffmann TK, Meidenbauer N, Müller-Berghaus J, Storkus WJ, Whiteside TL. Proinflammatory cytokines and CD40 ligand enhance cross-presentation and cross-priming capability of human dendritic cells internalizing apoptotic cancer cells. J Immunother 2001;24:162–71.

[67] Kyewski B, Rottinger B, Klein L. Making central T-cell tolerance efficient: thymic stromal cells sample distinct self-antigen pools. Curr Top Microbiol Immunol 2000;251:139–45.

[68] Hanahan D. Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. Curr Opin Immunol 1998;10:656–62.

[69] Farr AG, Rudensky A. Medullary thymic epithelium: a mosaic of epithelial 'self'? J Exp Med 1998;188:1–4.

[70] Egwuagu CE, Charukamnoetkanok P, Gery I. Thymic expression of autoantigens correlates with resistance to autoimmune disease. J Immunol 1997;159:3109–12.

[71] Sospedra M, Ferrer-Francesch X, Dominguez O, Juan M, Foz-Sala M, Pujol-Borrell R. Transcription of a broad range of self-antigens in human thymus suggests a role for central mechanisms in tolerance toward peripheral antigens. J Immunol 1998; 161:5918–29.

[72] Kissler S, Anderton SM, Wraith DC. Antigen-presenting cell activation: a link between infection and autoimmunity? J Autoimmun 2001;16:303–8.

[73] Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. Science 1996;273:104–6

[74] Ludewig B, Junt T, Hengartner H, Zinkernagel RM. Dendritic cells in autoimmune diseases. Curr Opin Immunol 2001; 13:657–62.

[75] Damoiseaux JG, Beijleveld LJ, Breda Vriesman PJ. Multiple effects of cyclosporin A on the thymus in relation to T-cell development and autoimmunity. Clin Immunol Immunopathol 1997;82:197–202.

[76] Page DM. Cutting edge: thymic selection and autoreactivity are regulated by multiple coreceptors involved in T cell activation. J Immunol 1999;163:3577–81.

[77] Laufer TM, Fan L, Glimcher LH. Self-reactive T cells selected on thymic cortical epithelium are polyclonal and are pathogenic in vivo. J Immunol 1999;162:5078–84.

[78] Powell JJ, Van de Water J, Gershwin ME. Evidence for the role of environmental agents in the initiation or progression of autoimmune conditions. Environ Health Perspect 1999;107:667–72.

[79] Todd JA, Steinman L. Autoimmunity. The environment strikes back. Curr Opin Immunol 1993;5:863–5.