

## **The Role of Transforming Growth Factor $\beta$ in the Generation of Suppression: An Interaction between CD8<sup>+</sup> T and NK Cells**

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### **Summary**

CD8<sup>+</sup> T cells have suppressor effector functions, but the mechanisms involved in the generation of this activity are poorly understood. We report that natural killer (NK) cells have an important role in the acquisition of this function. CD8<sup>+</sup> cells induce NK cells to produce transforming growth factor- $\beta$  (TGF- $\beta$ ) which, in turn, stimulates CD8<sup>+</sup> T cells to become suppressors of antibody production. Using a monocyte-dependent and -independent method to induce antibody production, we first observed that the addition of NK cells to CD8<sup>+</sup> cells was required for optimal suppression. Next, we determined that the interaction of CD8<sup>+</sup> T cells with NK cells resulted in a striking increase NK cell TGF- $\beta$  mRNA and its production. This cytokine appeared to be involved in the induction of T suppressor cell activity since: (a) anti-TGF- $\beta$ 1 completely abrogated the suppression of immunoglobulin G synthesis; (b) TGF- $\beta$ 1 could substitute for NK cells in inducing CD8<sup>+</sup> T cells to develop suppressor activity; and (c) a short exposure of T cells to TGF- $\beta$ 1 in the absence of B cells was sufficient for the generation of suppressor activity by CD8<sup>+</sup> T cells. Interferon  $\gamma$  did not have this property. These studies provide strong evidence that in addition to its suppressive properties, TGF- $\beta$  is involved in the generation of CD8<sup>+</sup> T suppressor effector cells. Because NK cell function is decreased in many autoimmune diseases, these cells may fail to interact properly with these individuals' CD8<sup>+</sup> cells in generating suppressors of aggressive anti-self responses.

Autoimmune diseases occur in genetically susceptible individuals after the breakdown of self-tolerance. Suppressor lymphocytes represent one mechanism that normally protects against autoimmunity (1, 2). CD8<sup>+</sup> T cells have well-recognized suppressor functions, but in SLE, for example, CD8<sup>+</sup> T cells enhance rather than suppress B cell function for reasons that are not fully understood (3). In addition to their cytolytic functions, NK cells also have suppressive properties (4–6). NK cell activity is decreased in autoimmune diseases such as SLE (7), Sjogren's syndrome (8), and multiple sclerosis (9). The regulatory role of NK cells is unclear since these cells have also been shown to support Ig production (10–12). Mechanisms reported to be involved in NK cell regulatory effects have included perturbation of Fc $\gamma$  receptors (CD16) (6), or NK cell-derived cytokines such as TNF- $\alpha$  and IFN- $\gamma$  (4, 12).

Another cytokine that has prominent effects on host defense is TGF- $\beta$ . Mice with an inherited deficiency of this cytokine develop a multifocal inflammatory disease that results in organ failure and ultimately, in death (13). TGF $\beta$  is a multifunctional cytokine with predominantly suppressive effects on the immune system (14). Recently, TGF $\beta$  has been reported to selectively activate CD8<sup>+</sup> T cells to proliferate (15).

Herein, we report a novel interaction between NK and CD8<sup>+</sup> T cells resulting in the generation of TGF $\beta$ , which, in turn, becomes involved in the activation of CD8<sup>+</sup> lymphocytes to become suppressor effectors of antibody production.

### **Materials and Methods**

**Cell Isolation.** PBMC were prepared from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. To obtain NK cells, PBMC was added to a nylon wool column and the eluted, nonadherent cells were immediately rosetted with AET-treated SRBC (16). The nonrosetting fraction was then stained with anti-CD3 and anti-CD74 (anti-HLA-DR) antibodies and depleted of reacting cells using immunomagnetic beads (Dynal, Great Neck, NY). This resultant population usually contained >98% CD11b<sup>+</sup>, <0.5% CD3<sup>+</sup>, and <0.5% CD20<sup>+</sup> cells.

B cells were prepared by recovering the nylon adherent cells and immediately rosetting the recovered cells. The nonrosetting fraction was depleted of monocytes and functional NK cells by incubation with 40 mM L-leucine methyl ester (17). The resultant population was usually >92% CD20<sup>+</sup> and <0.5% CD3<sup>+</sup>.

CD4<sup>+</sup> and CD8<sup>+</sup> cells were prepared from the nylon nonad-

herent cells were stained with antibodies to CD8, CD16, CD11b, and CD74. The same antibodies were used to obtain CD8<sup>+</sup> cells except that CD4 was substituted for CD8. Monocytes were prepared by adhering PBMC to plastic. After vigorous washing, the adherent cells were recovered by scraping using a cell scraper (GIBCO BRL, Gaithersburg, MD). Monocytes were irradiated with 3,000 rad from a 137 cesium source.

**Reagents.** Antibodies used were anti-CD3 (454, a gift from W. Stohl, University of Southern California; anti-CD3, purchased from Dako Corp., Carpinteria, CA), anti-CD4 (408, W. Stohl; anti-CD3, Dako), anti-CD8 (284.1.3, W. Stohl; anti-CD8, Dako), anti-CD11b (OKM1, American Type Culture Collection [ATCC], Rockville, MD), anti-CD20 (Leu 16, Becton Dickinson), anti-CD74 (L243, ATCC), anti-CD56 (Coulter Immunology, Hialeah, FL), and anti-CD2 (GT2, D. Cantrell, Imperial Cancer Research Fund, London, UK and OKT11, ATCC). Chicken anti-human TGF $\beta$  and chicken Ig were purchased from R&D Systems, Inc. (Minneapolis, MN).

Recombinant human IL-2 was kindly provided by Cetus, Corp. (Emeryville, CA) and TGF- $\beta$  was purchased from R&D Systems. Recombinant IFN- $\gamma$  was kindly provided by Genentech (South San Francisco, CA).

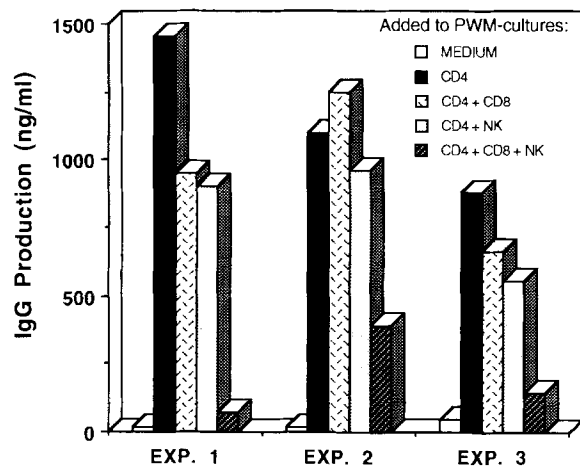
**T Cell-dependent Antibody Production.** Cultures were carried out in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FCS (Gemini Bioproducts Inc., Calabasas, CA), 10 g/ml gentamicin (Irvine Scientific), 2 mM L-glutamine (Flow Laboratories, McLean, VA), and 10 mM HEPES (United States Biochemical Corp., Cleveland, OH).

Where PWM was used to induce antibody synthesis, NK cells were cultured overnight at 37°C in the presence or absence of 100 U/ml rIL-2. These cells were then extensively washed before being added to culture wells.  $0.5 \times 10^5$  CD4<sup>+</sup> T and B cells were added to the wells of a 96-well flat bottom microtiter plate (Flow Laboratories) along with  $3 \times 10^4$  monocytes (plastic adherent cells irradiated with 3,000 rad). CD8<sup>+</sup> or NK cells were added generally at  $0.5 \times 10^5$  cells per well although in some experiments graded concentrations of each were added to determine maximum regulatory effects. PWM (Sigma Chemical Co., St. Louis, MO) was added to the wells at 0.1  $\mu$ g/ml. After 7 d at 37°C in 5% CO<sub>2</sub>, cell-free supernatants from these cultures were harvested and assayed for IgG content by an ELISA, performed as previously described (5). Where anti-CD2 mAbs were used to induce antibody production, the NK cells were not treated with IL-2.

**Cytokine mRNA and TGF $\beta$  Measurement.** NK cells, CD8<sup>+</sup> cells, or both were added to CD4<sup>+</sup> cells, B cells, and monocytes which were then cultured in the presence of PWM. After 6 or 18 h, the cultures were stained with CD56-PE and CD8-FITC and then sorted by flow cytometry by positive selection. These cells were pelleted and processed for PCR analysis. Cytoplasmic RNA was extracted and cDNA was synthesized and amplified using appropriate primers whose sequences have been reported (18, 19). In two experiments, the NK cells were isolated after 18 h of incubation and cultured for a further 72 h. The supernatants were collected and assayed for TGF $\beta$  activity using an ELISA purchased from Genzyme Corp. (Cambridge, MA) and performed according to the manufacturer's instructions.

## Results

Using two different methods to assess T cell-dependent B cell differentiation, we observed that the addition of CD8<sup>+</sup> cells to cultures containing CD4<sup>+</sup> and B cells gener-



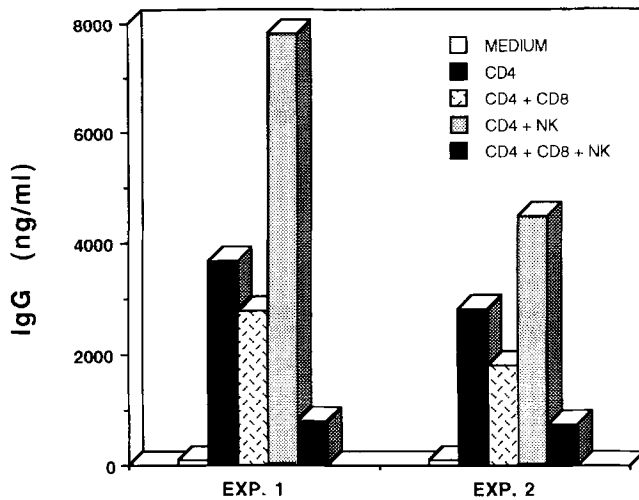
**Figure 1.** Marked suppression of antibody production requires both CD8<sup>+</sup> and NK cells. To B cells and irradiated monocytes ( $10^4$ ) cultured with PWM were added CD4<sup>+</sup> cells (solid bars), CD4<sup>+</sup> and CD8<sup>+</sup> cells (light hatched bars), CD4<sup>+</sup> and NK cells (stippled bars), and CD4<sup>+</sup>, CD8<sup>+</sup>, and NK cells (dark hatched bars). The NK cells had been pretreated with rIL-2 (100 U/ml). All lymphocyte subsets were added at  $5 \times 10^4$  cells/well. After 7 d of culture with PWM, supernatants were harvested from the wells and assayed for IgG content (ng/ml) by an ELISA. (Open bars) Background IgG in cultures.

ally resulted in only modest inhibition of antibody synthesis. These studies suggested that an additional mononuclear cell population was needed for the generation of potent suppressor activity. We considered that NK cells might have this property. We first tested this hypothesis using PWM to induce antibody synthesis. Neither the addition of CD8<sup>+</sup> cells, NK cells, nor both together markedly suppressed antibody synthesis. Whereas the addition of IL-2-activated NK cells did not have this effect, the combination of activated NK and CD8<sup>+</sup> cells markedly suppressed Ig production in eight separate experiments. Three experiments illustrating the range of effects observed are shown in Fig. 1. The suppressive effect of adding both NK and CD8<sup>+</sup> cells was not due to alterations in the number of cells in the wells since increased numbers

**Table 1.** Suppression of IgM and IgG Requires Both CD8<sup>+</sup> and NK Cells

Added to PWM cultures	Exp. 1		Exp. 2	
	IgM	IgG	IgM	IgG
Medium	4,100	875	2,800	440
CD8	3,000	650	2,400	460
NK <sub>IL-2</sub>	3,900	550	5,200	460
CD8 + NK <sub>IL-2</sub>	260	145	840	280

PWM cultures consisted of CD4<sup>+</sup> cells, B cells, irradiated (3,000 rad) monocytes, and PWM at the concentrations indicated in Fig. 1. To the cultures were added medium, CD8<sup>+</sup> cells, and/or NK cells at  $5 \times 10^4$ /well each. After 7 d of culture, supernatants were harvested and assayed for IgM and IgG content by an ELISA.



**Figure 2.** Marked suppression of antibody production in a monocyte-independent system requires both CD8<sup>+</sup> and NK cells. CD4<sup>+</sup> ( $5 \times 10^4$ ) and B cells ( $5 \times 10^4$ ) were cultured in the presence of a mitogenic combination of anti-CD2 antibodies (OKT11 and GT2). Added to the wells were CD8<sup>+</sup> cells ( $5 \times 10^4$ ) and/or untreated NK cells ( $5 \times 10^4$ ). After 7 d of culture, supernatants were assayed for IgG content by an ELISA.

of CD8<sup>+</sup> or NK cells alone were ineffective. In addition to suppression of IgG, IgM production was also markedly suppressed as shown in Table 1.

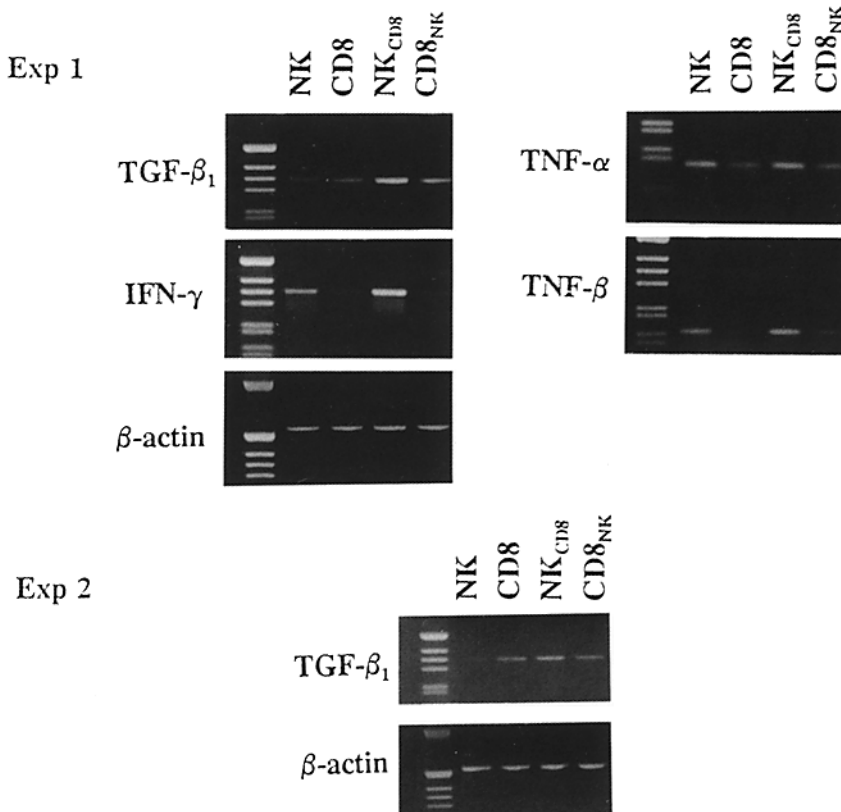
We have recently reported that one can assess the direct

effects of T cells on B cells in the absence of exogenous accessory cells with a mitogenic combination of anti-CD2 mAbs (20). As shown in Fig. 2, with this second method we also observed that the addition of CD8<sup>+</sup> T cells to CD4<sup>+</sup> and B cells had modest suppressive effects. The addition of NK cells generally enhanced antibody production. Since NK cells display CD2 receptors, these cells were not pretreated with IL-2. Nonetheless, the presence of both CD8<sup>+</sup> and NK cells resulted in optimal suppression of IgG synthesis.

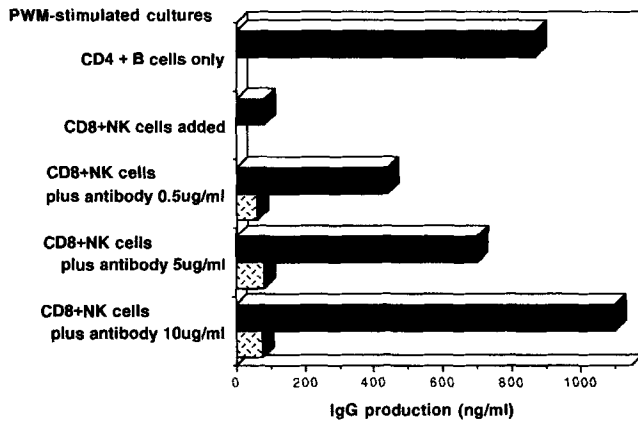
As many lymphocyte functions can be mediated by cytokines, the expression of cytokine mRNA was measured to identify possible candidates for the suppressive effect. Cytokine mRNA expression by CD8<sup>+</sup> and NK cells was determined after these cells were cultured separately or together in PWM-stimulated cultures. After 6 h of incubation, CD8<sup>+</sup> T and CD56<sup>+</sup> NK cells were sorted by positive selection. The isolated cell populations were then analyzed for the expression of cytokine mRNA by PCR. Cytokines produced by NK and CD8<sup>+</sup> cells include TNF- $\alpha$ , TNF- $\beta$  and IFN- $\gamma$  (21). Because of the inhibitory effects of TGF $\beta$  (22, 23), this cytokine was also examined.

There was only one consistent result of culturing both NK and CD8<sup>+</sup> cells together. This was a marked increase in NK cell TGF $\beta$  mRNA. In some experiments, there was also a modest increase in CD8<sup>+</sup> cell TGF $\beta$  mRNA and an increase in NK cell IFN- $\gamma$  transcripts. Two experiments are shown in Fig. 3.

Using a similar protocol, two additional experiments were performed where NK cells were isolated after an overnight



**Figure 3.** Cytokine mRNA expression in NK and/or CD8<sup>+</sup> cells after these cells were cultured separately or together. PWM cultures containing CD4<sup>+</sup> cells ( $10^6$ ), B cells ( $10^6$ ), and irradiated (3,000 rad) monocytes were cultured with: lane 1, IL-2-pretreated NK cells ( $10^6$ ); lane 2, CD8<sup>+</sup> cells ( $10^6$ ); lane 3, NK cells after culture with CD8 cells; and lane 4, CD8<sup>+</sup> cells after culture with NK cells. After 6 h at 37°C in 3-ml culture tubes, the cells were stained with anti-CD56-PE and anti-CD8-FITC and sorted by flow cytometry for NK and CD8 cells. Cells were then processed for PCR analysis. A similar pattern of cytokine expression was observed after 18 h of culture.

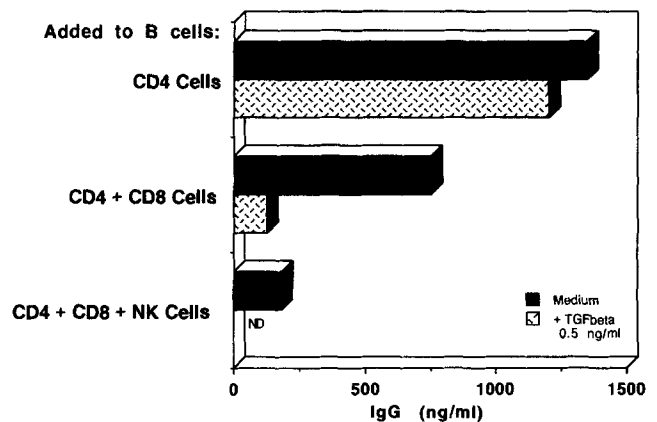


**Figure 4.** Anti-TGF $\beta$  abolished NK-CD8 cell-induced suppression. Cultures were initiated as described in the legend to Fig. 1. Added to these cultures were different concentrations of anti-TGF $\beta$  antibody (0.5–10  $\mu$ g/ml) or similar concentrations of control chicken antibody.

incubation and cultured for an additional 72 h. When culture supernatants were assayed for TGF $\beta$  by an ELISA, NK cells isolated from cultures containing CD8 $^+$  cells produced 660 pg/ml, whereas in the absence of CD8 $^+$  cells TGF $\beta$  was <50 pg/ml.

These results raised the possibility that production of TGF $\beta$  might be important in the generation of suppressor activity. To test this hypothesis, the effect of anti-TGF $\beta$  on IgG production was determined. Graded doses of anti-TGF $\beta$  antibody were added to PWM-stimulated cultures containing both IL-2-treated NK and CD8 $^+$  cells. As shown in Fig. 4, anti-TGF $\beta$ , but not the control antibody reversed the suppressive effects. These results demonstrate that TGF $\beta$  contributes to the suppression of IgG secretion.

Since NK cells can act as a source of TGF $\beta$ , we determined whether TGF $\beta$  could take the place of NK cells in the generation of suppressive activity by CD8 $^+$  cells. Because of the known suppressive effects of this cytokine on antibody production, we first added graded doses of TGF $\beta$  to CD4 $^+$  and B cells to determine the minimum concentration that would not be directly inhibitory. This concentration of TGF $\beta$  (0.5 ng/ml) was then added to cultures where CD8 $^+$  cells were present or absent. Cultures containing both CD8 $^+$  and NK cells served as the positive control. In each of three separate

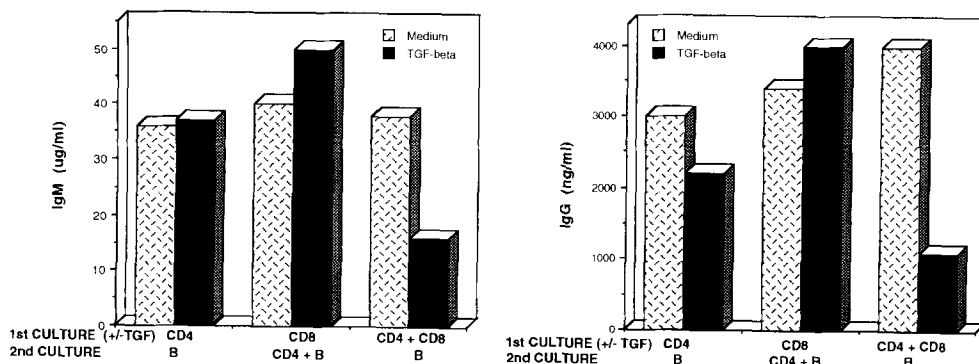


**Figure 5.** TGF $\beta$  can substitute for NK cells in the generation of T cell suppressor activity. TGF $\beta$  (0.5 ng/ml) or medium was added to PWM cultures containing CD4 $^+$  cells, B cells, and irradiated monocytes with or without CD8 $^+$  cells as described in the legend to Fig. 1. Some PWM cultures received both CD8 $^+$  and NK cells that had been incubated with 100 U/ml rIL-2. After 7 d of culture, supernatants were assayed for Ig content by an ELISA.

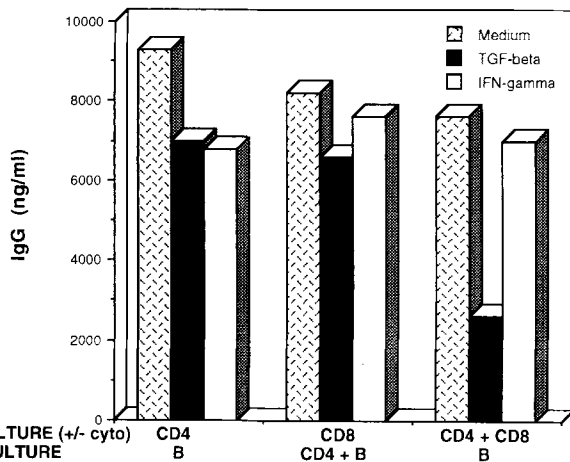
experiments, we have observed that the effects of adding either TGF $\beta$  or NK cells to CD8 $^+$  cells are similar. In each case, there was marked suppression of IgG production. Fig. 5 shows one representative experiment.

These observations suggested that TGF $\beta$  was involved in the activation of CD8 $^+$  T cells to become suppressors of antibody production. However, other explanations included the priming of B cells by TGF $\beta$  to become susceptible to the action of suppressor cells. To address this, two-stage experiments were performed where T cells were stimulated with PWM overnight in the presence or absence of TGF $\beta$ . The cells were then extensively washed before being added to B cells. Exposure of either CD4 $^+$  or CD8 $^+$  cells alone to TGF $\beta$  did not induce any suppressive activity. However, this activity did develop when cultures containing both CD4 $^+$  and CD8 $^+$  cells were preincubated with TGF $\beta$ . Both IgG and IgM production were significantly inhibited (Fig. 6).

IFN- $\gamma$  is another cytokine reported to be associated with the development of suppressor activity (24). Therefore, a direct comparison was made between TGF $\beta$  and IFN- $\gamma$  for their ability to induce suppressor activity using the two-step culture system. As before, preincubation of cultures containing



**Figure 6.** T lymphocytes preincubated with TGF $\beta$  mediate suppression. CD4 $^+$  cells, CD8 $^+$  cells, or both were cultured overnight with irradiated monocytes and PWM in the presence or absence of TGF $\beta$  (0.1 ng/ml). Next day, the wells were extensively washed. B cells and fresh PWM were then added to all the wells. In the case of the CD8 $^+$  only wells, CD4 $^+$  cells were also added in the second culture. All cell concentrations were as described the legend to Fig. 1.



**Figure 7.** Comparison of TGF $\beta$  and IFN- $\gamma$  on the development of suppressive activity as was described in Fig. 6, except that IFN- $\gamma$  (100 U/ml) was also tested.

both CD4<sup>+</sup> and CD8<sup>+</sup> cells with TGF $\beta$  stimulates suppressor activity. However, IFN- $\gamma$ , over a wide range of concentrations, did not have any suppressive effect (Fig. 7).

### Discussion

In this study, CD4<sup>+</sup> cells were unable to induce CD8<sup>+</sup> cells to become potent suppressors of antibody production. It has been well established that CD4<sup>+</sup> CD45RA<sup>+</sup> cells have important roles in the development of CD8<sup>+</sup> T cell suppressor activity (25). Here we show that NK cells were also required for the development of optimal suppressor activity. This finding was observed using both a monocyte-dependent and -independent culture system to induce polyclonal B cell activation.

Previously, mechanisms responsible for the suppression of antibody production by NK cells were poorly understood. The addition of NK cells to PBMC could either decrease CD4<sup>+</sup> cell helper activity or increase CD8<sup>+</sup> cell suppressor activity. On the basis of the present experiments, the former possibility is unlikely since the addition of NK cells to CD4<sup>+</sup> and B cells did not notably decrease antibody production. In fact, when antibody production was stimulated with anti-CD2, NK cells enhanced the amount of antibody produced. What we have demonstrated is that the generation of suppression requires both NK and CD8<sup>+</sup> cells. When PWM was used to induce antibody production, the NK cells had to be preactivated with IL-2 in order for suppression to occur. When anti-CD2 was used, however, this was not the case because NK cells express CD2 on their cell surface and have been reported to be activated by these mAbs.

To identify potentially critical cytokines that might be in-

involved in the activation of suppression, we examined NK and CD8<sup>+</sup> cells for cytokine mRNA after they were cultured together or separately in PWM-stimulated cultures. The striking observation was the upregulation of TGF $\beta$  mRNA in NK cells that had been cultured together with CD8<sup>+</sup> cells. NK cells isolated from cultures containing CD8<sup>+</sup> cells produced TGF $\beta$ , whereas the absence of CD8<sup>+</sup> cells, little or none was produced. Although TGF $\beta$  message has been described previously in activated NK cells (26), this, to our knowledge, is the first report of this cytokine produced by these cells. Moreover, the TGF $\beta$  detected in the supernatant was in the active form, indicating that conversion from the latent form had occurred.

Evidence that TGF $\beta$  was actually involved in the generation of suppressor cell activity was provided by (a) the observation that anti-TGF $\beta$  abolished the suppressive effects of the NK-CD8<sup>+</sup> cell interaction; and (b) the ability of TGF $\beta$  to replace NK cells in generating optimal CD8<sup>+</sup> cell suppressor cell activity. It is important to emphasize that with the low concentration of TGF $\beta$  used, there was no inhibitory effect of this cytokine on IgG production in the absence of CD8<sup>+</sup> cells.

TGF $\beta$  is an immunosuppressive cytokine with marked inhibitory effects on T, B, and NK cell effector functions (14). It is interesting to note that although TGF $\beta$  had a marked effect on antibody production, proliferative activity in PWM cultures was unaffected by TGF $\beta$  (data not shown). This suggests that some cellular functions are more susceptible to the effects of TGF $\beta$  than others. In addition to downregulating effector functions, the present study suggests that this cytokine can also activate lymphocytes to develop suppressive effects. This suggestion is consistent with the recent demonstration that TGF $\beta$  selectively stimulates CD45RB<sub>hi</sub> CD8<sup>+</sup> cells to proliferate (15). Moreover, in chickens, there is one report that TGF $\beta$  is important in the generation of CD8<sup>+</sup> cell suppressor activity (27). Other positive effects on the growth and development of lymphocyte subsets have been reported (28, 29). Our results demonstrate that TGF $\beta$  alone is insufficient to stimulate CD8<sup>+</sup> cells to become suppressor effector cells but that CD4<sup>+</sup> cells are also required. This is wholly consistent with previous reports describing the role of CD4<sup>+</sup> cell suppressor inducer cells. Our data indicate that for optimal suppression of Ig production, CD4<sup>+</sup> cells and a source of TGF $\beta$  are needed.

As stated above, NK cell function is decreased in many autoimmune diseases including SLE, Sjogren's syndrome, and multiple sclerosis (7-9). If NK cells interact with CD8<sup>+</sup> cells in a manner similar in vivo as they do in this in vitro model, then impaired NK cell function may reflect a defect that contributes to the inability of CD8<sup>+</sup> cells to effectively suppress the aggressive anti-self responses in these diseases.

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