The Role of Transforming Growth Factor β in the Generation of Suppression: An Interaction between CD8+ T and NK Cells

By J. Dixon Gray, Makoto Hirokawa, and David A. Horwitz

From the Division of Rheumatology and Immunology, Department of Medicine, University of Southern California School of Medicine, Los Angeles, California 90033

Summary
CD8+ 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+ cells induce NK cells to produce transforming growth factor-β (TGF-β) which, in turn, stimulates CD8+ 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+ cells was required for optimal suppression. Next, we determined that the interaction of CD8+ T cells with NK cells resulted in a striking increase NK cell TGF-β mRNA and its production. This cytokine appeared to be involved in the induction of T suppressor cell activity since: (a) anti-TGF-β1 completely abrogated the suppression of immunoglobulin G synthesis; (b) TGF-β1 could substitute for NK cells in inducing CD8+ T cells to develop suppressor activity; and (c) a short exposure of T cells to TGF-β1 in the absence of B cells was sufficient for the generation of suppressor activity by CD8+ T cells. Interferon γ did not have this property. These studies provide strong evidence that in addition to its suppressive properties, TGF-β is involved in the generation of CD8+ 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+ cells in generating suppressors of aggressive anti-self responses.

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-DP) antibodies and depleted of reacting cells using immunomagnetic beads (Dynal, Great Neck, NY). This resultant population usually contained >98% CD11b+, <0.5% CD3+, and <0.5% CD20+ cells.

B cells were prepared by recovering the nylon adherent cells and immediately rosetting the recovered cells. 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+, <0.5% CD3+, and <0.5% CD20+ cells.

Herein, we report a novel interaction between NK and CD8+ T cells resulting in the generation of TGFβ, which, in turn, becomes involved in the activation of CD8+ lymphocytes to become suppressor effectors of antibody production.
herent cells were stained with antibodies to CD8, CD16, CD11b, and CD74. The same antibodies were used to obtain CD8+ cells except that CD4 was substituted for CD8. Monocytess 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). Monocytess 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 TGFβ 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-β was purchased from R&D Systems. Recombinant IFN-γ was kindly provided by Genentech (South San Francisco, CA).

T Cell–dependent Antibody Production. Cultures were carried out in RPMI 1640 medium (Irvin Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FCS (Gemini Bioproducts Inc., Calabasas, CA), 10 g/ml gentamicin (Irvin 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 × 10⁶ CD4+ T and B cells were added to the wells of a 96-well flat bottom microtiter plate (Flow Laboratories) along with 3 × 10⁴ monocytes (plastic adherent cells irradiated with 3,000 rad). CD8+ or NK cells were added generally at 0.5 × 10⁶ cells/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 µg/ml. After 7 d at 37°C in 5% CO₂, 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 TGFβ Measurement. NK cells, CD8+ cells, or both were added to CD4+ 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 TGFβ activity using an ELISA purchased from Genzyme Corp. (Cambridge, MA) and performed according to the manufacturer’s instructions.

Results

Figure 1. Marked suppression of antibody production requires both CD8+ and NK cells. To B cells and irradiated monocyte (10⁶) cultured with PWM were added CD4+ cells, (solid bars), CD4+ and CD8+ cells (light hatched bars), CD4+ and NK cells (stippled bars), and CD4+, CD8+, 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 × 10⁴ 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.

Table 1. Suppression of IgM and IgG Requires Both CD8+ and NK Cells

| Added to PWM cultures | Exp. 1 | Exp. 2 |
|-----------------------|-------|-------|
| Medium                | IgM   | IgG   | IgM   | IgG   |
| CD8                   | 4,100 | 875   | 2,800 | 440   |
| NKil-2                | 3,900 | 550   | 5,200 | 460   |
| CD8 + NKil-2          | 260   | 145   | 840   | 280   |

PWM cultures consisted of CD4+ cells, B cells, irradiated (3,000 rad) monocytes, and PWM at the concentrations indicated in Fig. 1. To the cultures were added medium, CD8+ cells, and/or NK cells at 5 × 10⁴/well each. After 7 d of culture, supernatants were harvested and assayed for IgM and IgG content by an ELISA.

Using two different methods to assess T cell–dependent B cell differentiation, we observed that the addition of CD8+ cells to cultures containing CD4+ and B cells generally 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+ 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+ 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+ cells was not due to alterations in the number of cells in the wells since increased numbers...
of CD8+ 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+ T cells to CD4+ 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+ 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+ and NK cells was determined after these cells were cultured separately or together in PWM-stimulated cultures. After 6 h of incubation, CD8+ T and CD56+ 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+ cells include TNF-α, TNF-β and IFN-γ (21). Because of the inhibitory effects of TGFβ (22, 23), this cytokine was also examined.

There was only one consistent result of culturing both NK and CD8+ cells together. This was a marked increase in NK cell TGFβ mRNA. In some experiments, there was also a modest increase in CD8+ cell TGFβ mRNA and an increase in NK cell IFN-γ 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 2.** Marked suppression of antibody production in a monocyte-independent system requires both CD8+ and NK cells. CD4+ (5 x 10⁴) and B cells (5 x 10⁴) were cultured in the presence of a mitogenic combination of anti-CD2 antibodies (OKT11 and GT2). Added to the wells were CD8+ cells (5 x 10⁴) and/or untreated NK cells (5 x 10⁴). After 7 d of culture, supernatants were assayed for IgG content by an ELISA.

**Figure 3.** Cytokine mRNA expression in NK and/or CD8+ cells after these cells were cultured separately or together. PWM cultures containing CD4+ cells (10⁶), B cells (10⁶), and irradiated (3,000 rad) monocytes were cultured with: lane 1, IL-2-pretreated NK cells (10⁶); lane 2, CD8+ cells (10⁶); lane 3, NK cells after culture with CD8 cells; and lane 4, CD8+ 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.
PWM-stimulated cultures

CD4 + B cells only

CD8+NK cells added

CD8+NK cells plus antibody 0.5ug/ml

CD8+NK cells plus antibody 5ug/ml

CD8+NK cells plus antibody 10ug/ml

1st CULTURE (+/-TGF) CD4 CD8

2nd CULTURE B

Added to B cells:

CD4 Cells

CD4 + CD8 Cells

CD4 + CD8 + NK Cells

IgG production (ng/ml)

Figure 4. Anti-TGFβ 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-TGFβ antibody (0.5–10 µg/ml) or similar concentrations of control chicken antibody.

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

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

Since NK cells can act as a source of TGFβ, we determined whether TGFβ 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 TGFβ to CD4+ and B cells to determine the minimum concentration that would not be directly inhibitory. This concentration of TGFβ (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 experiments, we have observed that the effects of adding either TGFβ 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 TGFβ 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 TGFβ 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 TGFβ. The cells were then extensively washed before being added to B cells. Exposure of either CD4+ or CD8+ cells alone to TGFβ did not induce any suppressive activity. However, this activity did develop when cultures containing both CD4+ and CD8+ cells were preincubated with TGFβ. Both IgG and IgM production were significantly inhibited (Fig. 6).

IFN-γ is another cytokine reported to be associated with the development of suppressor activity (24). Therefore, a direct comparison was made between TGFβ and IFN-γ for their ability to induce suppressor activity using the two-step culture system. As before, preincubation of cultures containing
both CD4+ and CD8+ cells with TGFβ stimulates suppressor activity. However, IFN-γ, over a wide range of concentrations, did not have any suppressive effect (Fig. 7).

**Discussion**

In this study, CD4+ cells were unable to induce CD8+ cells to become potent suppressors of antibody production. It has been well established that CD4+ CD45RA+ cells have important roles in the development of CD8+ 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+ cell helper activity or increase CD8+ cell suppressor activity. On the basis of the present experiments, the former possibility is unlikely since the addition of NK cells to CD4+ 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+ 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 involved in the activation of suppression, we examined NK and CD8+ cells for cytokine mRNA after they were cultured together or separately in PWM-stimulated cultures. The striking observation was the upregulation of TGFβ mRNA in NK cells that had been cultured together with CD8+ cells. NK cells isolated from cultures containing CD8+ cells produced TGFβ, whereas the absence of CD8+ cells, little or none was produced. Although TGFβ 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 TGFβ detected in the supernatant was in the active form, indicating that conversion from the latent form had occurred.

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

TGFβ is an immunosuppressive cytokine with marked inhibitory effects on T, B, and NK cell effector functions (14). It is interesting to note that although TGFβ had a marked effect on antibody production, proliferative activity in PWM cultures was unaffected by TGFβ (data not shown). This suggests that some cellular functions are more susceptible to the effects of TGFβ 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 TGFβ selectively stimulates CD45RBhi CD8+ cells to proliferate (15). Moreover, in chickens, there is one report that TGFβ is important in the generation of CD8+ cell suppressor activity (27). Other positive effects on the growth and development of lymphocyte subsets have been reported (28, 29). Our results demonstrate that TGFβ alone is insufficient to stimulate CD8+ cells to become suppressor effector cells but that CD4+ cells are also required. This is wholly consistent with previous reports describing the role of CD4+ cell suppressor inducer cells. Our data indicate that for optimal suppression of Ig production, CD4+ cells and a source of TGFβ 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+ 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+ cells to effectively suppress the aggressive anti-self responses in these diseases.

---

The authors gratefully appreciate the technical expertise of Ms. Lillie Hsu and Hua Wang. We also thank Drs. William Stohl, Chaim Jacob and Gunther Dennert for their helpful comments.

This work was supported by grants from the National Institutes of Health (AR-29846), the Nora Eccles Treadwell Foundation, and the Southern California Chapter of the Arthritis Foundation.

1941 Gray et al. Brief Definitive Report
References

1. Kumar, V., and E.E. Sercarz. 1991. Regulation of autoimmunity. *Cur. Opin. Immunol.* 3:888.

2. Bloom, B.R., P. Salgame, and B. Diamond. 1992. Revisiting and revising suppressor T cells. *Immunol. Today* 13:131.

3. Linker-Israeli, M., F.P. Quismorio, Jr., and D.A. Horwitz. 1990. CD8+ lymphocytes from patients with systemic lupus erythematosus sustain rather than suppress spontaneous polyclonal IgG production and synergize with CD4+ cells to support autoantibody synthesis. *Arthritis Rheum.* 33:1216.

4. Kuwano, K., S. Arai, T. Munakata, Y. Tomita, Y. Yoshitake, and K. Kumagai. 1986. Suppressive effect of human natural killer cells on Epstein-Barr virus-induced immunoglobulin synthesis. *J. Immunol.* 137:1462.

5. Abo, W., J.D. Gray, A.C. Bakke, and D.A. Horwitz. 1987. Studies on human blood lymphocytes with IC3b (type 3) complement receptors. II. Characterization of subsets which regulate pokeweed mitogen–induced lymphocyte proliferation and immunoglobulin synthesis. *Clin. Exp. Immunol.* 67:544.

6. Tilden, A.B., T. Abo, and C.M. Balch. 1983. Suppressor cell function of human granular lymphocytes identified by the HNK-1 (Leu 7) monoclonal antibody. *J. Immunol.* 130:1171.

7. Katz, P., A. Zaytoun, and J.H. Lee. 1982. Abnormal natural killer cell activity in SLE: an intrinsic defect in the lytic event. *J. Immunol.* 129:1966.

8. Goto, J., K. Tanimoto, T. Chihara, and Y. Horiuchi. 1981. Natural cell–mediated cytotoxicity in Sjögren’s syndrome and rheumatoid arthritis. *Arthritis Rheum.* 24:1377.

9. Benczur, M., G.G. Petranyi, G. Palfy, M. Varga, M. Talas, B. Kotsy, I. Foldes, and S.R. Hollan. 1980. Dysfunction of natural killer cells in multiple sclerosis: a possible pathogenic factor. *Clin. Exp. Immunol.* 39:657.

10. Vyakamam, A., M.K. Brenner, J.E. Reitite, C.H. Houlker, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, and T. Doetschman. 1992. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature (Lond.)* 359:693.

11. Massague, J. 1990. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* 6:597.

12. Lee, H.-M., and S. Rich. 1993. Differential activation of CD8+ T cells by transforming growth factor-β1. *J. Immunol.* 151:668.

13. Salgame, P., J.S. Abrams, C. Clayberger, H. Goldberg, J. Convit, R.L. Modlin, and B.R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science (Wash. DC)* 254:279.

14. Shau, H., and S.H. Golub. 1985. Depletion of NK cells with the lysosomotropic agent L-leucine methyl ester and the in vitro generation of NK cell activity from NK precursor cells. *J. Immunol.* 134:1136.

15. Lee, H.-M., and S. Rich. 1988. Mechanism of K562-induced human natural killer cell inactivation using highly enriched effector cells isolated via a new single-step sheep erythrocyte rosette assay. *Ann. Inst. Pasteur Immunol.* 139:361.

16. Abrams, S.I., and Z. Brahmi. 1988. Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.* 170:1039.

Received for publication 25 October 1993 and in revised form 13 June 1994.