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Study of the target cell of the dengue virus-induced suppressor signal

Manohar Indra Shukla and U.C. Chaturvedi*

Virus Laboratory, Postgraduate Department of Pathology and Bacteriology, KG Medical College, Lucknow 226 003, India

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Summary. Dengue type 2 virus (DV) induces two generations of T suppressor cells (Ts_1 and Ts_2) in murine spleen. The Ts_2 cells produce a soluble factor (SF_2) which suppresses DV-specific IgM antibody plaque-forming cells (PFC). In this study the target cell of SF_2 was examined. The results show that SF_2 requires a third generation of T cells (T_3) in order to mediate suppression. The surface phenotype of this T_3 cell is Ly 1^+ and the suppression mediated is antigen specific.

Keywords: dengue virus, suppressor signal, target cell

We have observed that there is a suppressor pathway in the spleens of dengue virus type 2 (DV)-infected mice. This pathway is one of antigen-specific suppression of IgM antibody plaque-forming cells (PFC). The pathway includes induction of the first generation of suppressor T lymphocytes (Ts_1) by DV which produce a soluble factor, the suppressor factor (SF) (Tandon *et al.* 1979; Chaturvedi & Shukla 1981). SF acts via live macrophages to recruit a second generation of T suppressor cells (Ts_2) which secrete a soluble factor similar to prostaglandin (PG) which we have termed SF_2 (Shukla & Chaturvedi 1981, 1982, 1983; Chaturvedi *et al.* 1981, 1982). In the present study an effort was made to investigate whether SF_2 acts directly on B lymphocytes to mediate suppression of IgM PFC or needs an intermediary cell. The findings demonstrate that SF_2 acts via a third generation of T lymphocytes to mediate suppression.

Materials and methods

Animals. Adult Swiss albino mice were used, obtained from the mouse colony maintained in this department.

Virus. Dengue type 2 virus (DV), strain P23085, was used in the form of adult mouse brain suspension in a dose of 1000 LD_{50}. Details of the virus have been decribed elsewhere (Chaturvedi *et al.* 1977, 1978, 1980*a*, *b*). Japanese encephalitis virus (JEV) strain 78668A (Mathur *et al.* 1982) was used in the form of mouse brain suspension.

Preparation of SF_2: SF_2 was prepared by treating normal mouse spleen cells with SF as described elsewhere (Chaturvedi & Shukla 1981; Chaturvedi *et al.* 1981; Shukla & Chaturvedi 1981, 1983); 1.0 ml SF (1:10) was incubated with 1×10^7 spleen cells for 1 h at 37°C. The cells were centrifuged at 3000 r/min, the supernatant discarded and the cells washed three times with Eagle's minimum essential medium (MEM) containing 5% fetal calf serum (FCS). The cells were then cultured in 3.0 ml MEM containing 10% FCS at 37°C for 24 h in an atmosphere of 5% CO_2. The cell suspension was centrifuged at 3000

* Correspondence: U.C. Chaturvedi, Medical College Quarters, 50 Jagat Narain Road, Lucknow 226 003, India.

r/min and the supernatant collected and stored at −20°C. This supernatant was used as SF_2.

Depletion of T lymphocytes. T lymphocytes were depleted by the technique of Golub (1971). A spleen-cell suspension was treated with monoclonal anti-Thy 1.2 antibody (New England Nuclear) and complement as described elsewhere (Tandon *et al.* 1979).

Depletion of macrophages. Macrophages were depleted by treating the spleen cell suspension with carbonyl iron (Lymphocyte separator reagent, Technicon Instruments Corp. NY) at 37°C for 1 h. The iron-containing macrophages were removed with a magnet.

Depletion of Ly1$^+$ and Ly2$^+$ subpopulations of T lymphocytes. Spleen cells were treated with anti-Ly 1.2 or anti-Ly2.2 antisera (provided by Dr F. Y. Liew) and complement by the technique of Liew & Simpson (1980) to deplete Ly $1^+2^3^-$ (Ly 1) and Ly $1^-2^3^+$ (Ly 2) subpopulations of the T lymphocytes respectively. Normal mouse sera were used as controls.

Preparation of spleen-cell cultures. Normal mouse spleen cells were cultured in MEM-HEPES containing 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol; 5-cm glass petri dishes were seeded with 4-ml cell suspensions (5×10^6 cells/ml) and incubated at 37°C in an atmosphere of 5% CO_2.

Assay of suppressor activity. Spleen cell cultures were stimulated with 1000 LD_{50}DV at '0 hour' followed, 24 h later, by inoculation of 0.2 ml SF_2 (1:10 dilution). On the third and fourth day of culture DV-specific IgM PFC were counted by the haemolysis-in-gel technique of Jerne & Nordin (1963) as described elsewhere (Tandon & Chaturvedi 1977). In the specificity experiments PFC against DV, as well as JEV, were counted. From each culture multiple slides were prepared. The PFC counts on the 2 days of the experiment (days 3 and 4) are similar and therefore the values have been considered together. The results are expressed as mean values with standard deviations after correcting for background PFC. The data have been analysed by Student's *t*-test for *P* values.

Results

Suppressor activity of SF_2

Suppressor activity of various dilutions of SF_2 was screened *in vitro*. As shown in Fig. 1 significant suppression of PFC (52–31%) was observed with 10^{-1} to 10^{-4} dilutions of SF_2. With higher dilutions of SF_2 there was no significant suppression (9%; $P > 0.05$).

Antigen specificity of SF_2

Antigen-specific suppressor activity of SF_2 in DV- and JEV-stimulated spleen-cell cultures was examined in a PFC assay. Fig. 2 shows that DV-induced SF_2 suppressed PFC against

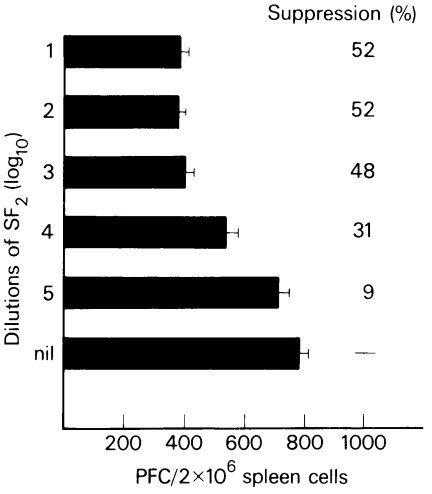

Fig. 1. Suppressor activity of SF_2; normal mouse spleen-cell cultures were stimulated with 10^3 LD_{50} of DV, followed 24 h later by 0.2 ml of various Log_{10} dilutions of SF_2. For controls, stimulated cultures were inoculated with diluent. DV-specific IgM PFC were counted on the third and fourth day of culture. Each column represents mean value ±SD.

DV (52%). Cultures stimulated with JEV gave little suppression of PFC (2%).

To confirm these findings, suppressor activity of SF_2 was screened in spleen-cell cultures stimulated simultaneously with both DV and JEV. In this experiment suppression of PFC against JEV by SF_2 was still less than 5%, while that against DV resulted in a > 50% decrease in response (Fig. 2).

Target of SF_2

To identify the target cell of SF_2, DV-stimulated cultures were depleted of macrophages or T lymphocytes, before inoculation of SF_2.

In the first experiment, spleen-cell cultures stimulated with DV 24 h earlier were depleted of macrophages and were inoculated with SF_2. Data presented in Fig. 3 indicate that depletion of macrophages from the cultures has no effect on the suppressor activity of SF_2 as the suppression of PFC was 45%.

In the second experiment, spleen-cell cultures stimulated with DV 24 h earlier were depleted of T lymphocytes and were then inoculated with SF_2. As shown in Fig 3, suppression of PFC by SF_2 was abrogated in cultures depleted of T lymphocytes.

Ly phenotype of target cells

Normal mouse spleen cells were treated with anti-Ly 1 or anti-Ly 2 antisera and complement. Cells were then cultured and stimulated with DV, followed 24 h later by inoculation of SF_2. As shown in Fig. 4, SF_2 suppressed PFC 20% in cultures depleted of Ly 1 cells, while suppression of PFC in Ly-2-depleted cells was 50%.

In the second set of experiments, DV-stimulated spleen-cell cultures were treated 24 h later with Ly 1 or Ly 2 antisera and complement. The cells were cultured and inoculated with SF_2. DV-specific IgM-PFC were counted in different sets. Data presented in Fig. 4 also indicate that the suppressor activity of SF_2 in Ly-1-depleted

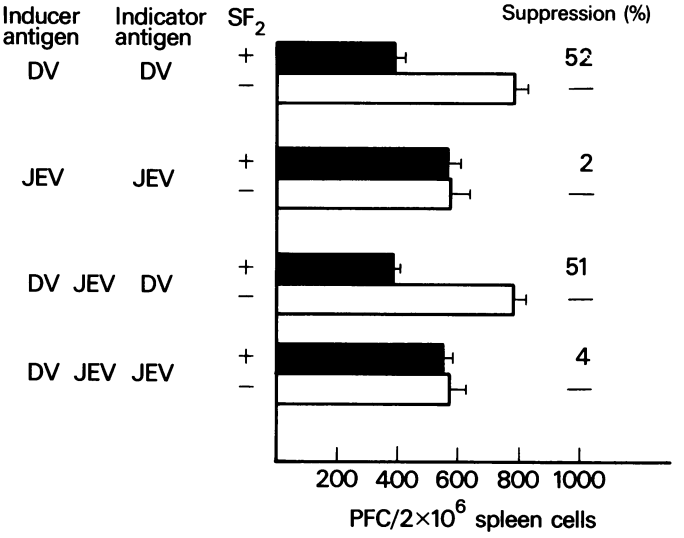

Fig. 2. Antigen specificity of SF_2 activity: normal mouse spleen-cell cultures were stimulated with 10^3 LD_{50} of DV or 10^3 LD_{50} of JEV or by both the viruses at 0 hour, followed 24 h later, with 0.2 ml SF_2 (■) or the diluent for control (□). On the third and fourth day of culture DV- and JEV-specific IgM-PFC were counted. Each column represents mean value ±SD.

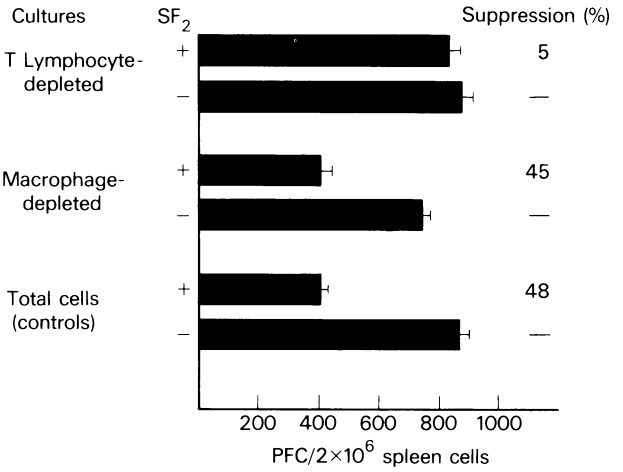

Fig. 3. Suppresor activity of SF_2 in cell-depleted cultures: normal mouse spleen-cell cultures were inoculated with 10^3 LD_{50} of DV at 0 hour. After 24 h either T lymphocytes or macrophages were depleted from the cultures; 0.2 ml SF_2 was inoculated into the cultures with diluent used as a control. DV-specific IgM PFC were counted on the third and fourth day of culture. Each column represents mean value ±SD.

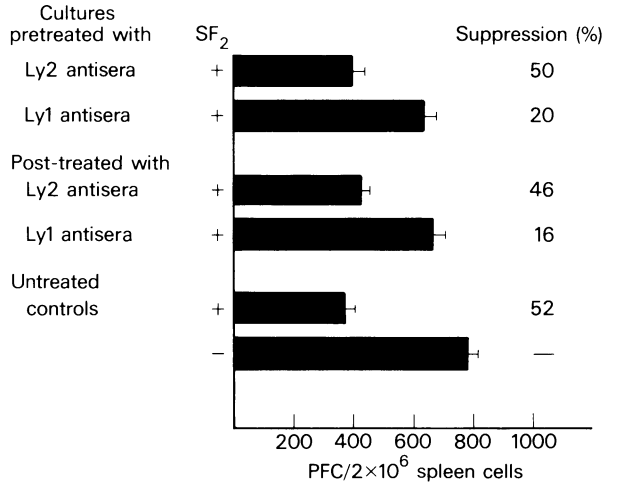

Fig. 4. SF_2 activity in cells treated with anti Ly antisera: normal mouse spleen-cell cultures were treated with anti-Ly 1.2 or Ly 2.2 antisera and complement just before, or 24 h after, stimulation with 10^3 LD_{50} of DV; 24 h after virus inoculation, 0.2 ml SF_2 was added to test cultures and diluent to the controls. DV-specific IgM-PFC were counted on the third and fourth day and mean vaue ±SD have been presented.

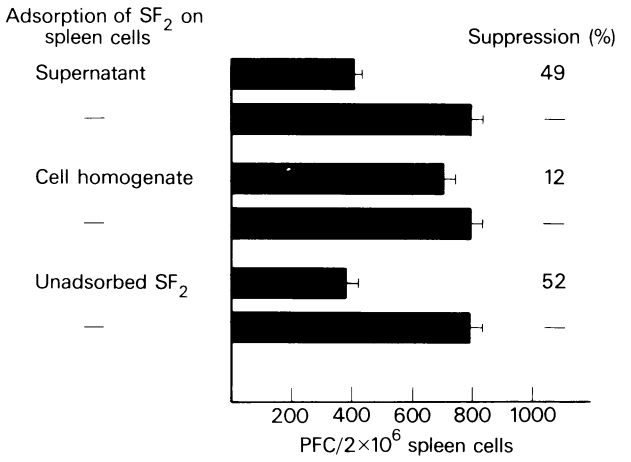

Fig. 5. Effect of adsorption of SF_2 with spleen cells on its suppressor activity: SF_2 was adsorbed on 1×10^7 spleen cells for 1 h at 37°C. The suspension was centrifuged at 3000 r/min. Suppressor activity was screened in the supernatant and cell homogenate by inoculating them in spleen-cell cultures stimulated with 10^3 LD_{50} of DV. DV-specific IgM-PFC were counted on the third and fourth day and mean value ±SD have been presented.

spleen-cell cultures was significantly less (16%, $P<0.001$) than that in Ly-2-depleted spleen-cell cultures (46%).

Adsorption of SF_2 on total spleen cells

This experiment was done to see whether or not SF_2 is adsorbed on the target cells. SF_2 (1 ml) was adsorbed on 1×10^7 spleen cells for 1 h at 37°C and then the supernatant was collected by centrifugation at 3000 r/min for 10 min. The cells were washed three times and then homogenized. The suppressor activities of the supernatant and of the cell homogenate were compared. Fig. 5 shows that the supernatant suppressed PFC (49%) whereas the cell homogenate was less suppressive (12%; $P>0.05$).

Discussion

The present study of the pathway of suppressor signals in the DV–mouse model indicates that (i) the target of SF_2 is a subpopulation of splenic T lymphocytes; (ii) the signal is antigen-specific; and (iii) it does not attach to the target cell.

SF_2 is a suppressive product of the Ts_2 population. This product could act either directly on IgM-producing B lymphocytes or via an intermediary cell which, in turn, could be either a macrophage or a T lymphocyte. Depletion of macrophages had no effect on the transmission of the suppressor signal, but depletion of T lymphocytes from the cultures by treatment with anti-Thy 1.2 antibody and complement abolished the suppressor activity of SF_2, thereby indicating that the signal is mediated through a T lymphocyte population.

The next question addressed was whether the SF_2 is adsorbed onto the target cells or their precursors. The activity of SF_2 is not lost when incubated with normal mouse spleen cells. Since SF_2 is very potent and has significant suppressor activity, even at a dilution of 10^{-4}, if only a small amount of it were to be adsorbed onto the cells, the cell homogenate should show some suppressor activity. As this was not found it suggests that SF_2 may recruit the T_3 cells without

becoming attached to their precursor cells. We reported previously that the SF and SF_2 mediate antigen-specific suppression of PFC (Chaturvedi & Shukla 1981; Shukla & Chaturvedi 1981); the current findings show that the suppressor signal maintains antigen-specificity throughout the pathway.

The phenotype of the target T cell is Ly 1^+23^-. Thus we suggest that in the transmission of suppressor signal three subpopulations of T lymphocytes are involved:

DV→T (LY?)→Ts_1→(Ly 1^-2^+)→SF→Mϕ→T (Ly 1^+2^+)→Ts_2→(Ly 1^-2^+)→SF_2→T_3→(Ly 1^+2^-)→B cell (suppresses IgM PFC).

As we do not know the functional nature of this cell we have referred to it as a T_3 cell. It has two interesting possible roles. One is that it is a helper–inducer of suppression and the other is that it could be another suppressor cell in a series.

T lymphocytes of Ly 1^+ phenotype have been shown to perform several helper functions. These include induction of suppressor T cells for optimum function; induction of macrophages etc. to participate in delayed hypersensitivity reactions; induction of killer-cell activity; and induction of B cells to produce antibody (Cantor & Boyse 1977; Cantor & Gershon 1979). Among these functions the suppressor activity is induced by I–J^+ Ly 1^+ cells (Eardley *et al.* 1980), but we could not test for the presence of I–J subregion gene products.

Recently Sy *et al.* (1981) have described a third generation of suppressor T cells (Ts_3) in the *P*-azobenzenearsonate system. These Ts_3 cells are generated by induction through a second generation of T suppressor cells (Ts_2), have a surface phenotype of Ly 1^-23^+ and are necessary for the manifestation of suppressor function of Ts_2 cells and their product, namely the TsF_2 (Sy *et al.* 1981). Is our T_3 cell similar to the Ts_3 cell of Sy *et al.* (1981)? The two are different in surface phenotype and further Ts_3 are generated in antigen-primed cells, while T_3 cells can be induced also in unstimulated spleen-cell cultures, as the depletion of Ly 2^+ cells before priming with DV abolishes suppressor activity. Most suppressor T cells have an Ly 2^+ phenotype *in vitro*, but the primary suppressor cells for delayed hypersensitivity against horse erythrocytes, influenza virus and *Leishamania tropica* have Ly 1^+ surface phenotype (Ramshaw *et al.* 1976; Liew 1977; Liew & Russell 1980). Thus Feldmann & Kontiainen (1981) suggested that Ly 1^+ cells which mediate suppression may be suppressor–inducers or amplifier cells and not actual suppressor cells *per se*. Further, Tada (1977) has shown the requirement for a T cell from antigen-primed mice in order to show that a T cell-derived suppressor factor acts on T helper cells. A similar T auxiliary cell has been reported by Sy *et al.* (1979). Additional studies are needed to establish the functional activity of T_3 cells in our model, but the possibility is raised that it is an intermediate suppressor–inducer T cell.

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References

CANTOR H. & BOYSE E.A. (1977) Regulation of cellular and humoral immune responses by T-cell subclasses. *Cold Spring Harbor Symp. quant. Biol.* **41**, 23–28.

CANTOR H. & GERSHON R.K. (1979) Immunological circuits: cellular composition. *Fed. Proc.* **38**, 2058–2064.

CHATURVEDI U.C., TANDON P. & MATHUR A. (1977) Effect of immunosuppression on dengue virus infection in mice. *J. gen. Virol.* **36**, 449–458.

CHATURVEDI U.C., TANDON P., MATHUR A. & KUMAR A. (1978) Host defence mechanism against dengue virus infection of mice. *J. gen. Virol.* **39**, 293–302.

CHATURVEDI U.C., BHARGAVA A. & MATHUR A. (1980*a*) Production of cytotoxic factor in the spleen of dengue virus-infected mice. *Immunology* **40**, 665–671.

CHATURVEDI U.C., DALAKOTI H. & MATHUR A. (1980*b*) Characterization of the cytotoxic factor produced in the spleen of dengue virus-infected mice. *Immunology* **41**, 387–392.

CHATURVEDI U.C. & SHUKLA M.I. (1981) Characterization of the suppressor factor produced in the spleen of dengue virus-infected mice. *Annls Immun. (Pasteur Inst.)* **132C**, 245–255.

CHATURVEDI U.C., SHUKLA M.I. & MATHUR A. (1981) Thymus dependent lymphocytes of the dengue virus-infected mice spleen mediate suppression through prostalandin. *Immunology* **42**, 1–6.

CHATURVEDI U.C., SHUKLA M.I. & MATHUR A. (1982) Role of macrophages in transmission of dingue virus-induced suppressor signal to a subpopulation of T lymphocytes. *Annls Immun. (Pasteur Inst.)* **133C**, 83–96.

EARDLEY D.D., MURPHY D.B., KEMP J.D., SHEN F.W., CANTOR H. & GERSHON R.K. (1980) Ly-1 inducer and Ly-1,2 acceptor T cells in the feed back suppression circuit bear an I–J subregion controlled determinant. *Immunogenetics* **11**, 549–557.

FELDMANN M. & KONTIAINEN S. (1981) The role of antigen-specific T-cell factors in the immune response. *Lymphokines* **2**, 87–123.

GOLUB E.S. (1971) Brain-associated theta antigen: reactivity of rabbit anti-mouse brain serum with mouse lymphoid cells. *Cell. Immun.* **2**, 353–361.

JERNE N.K. & NORDIN A.A. (1963) Plaque formation in agar by single antibody-producing cells. *Science* **140**, 405–407.

LIEW F.Y. (1977) Regulation of delayed type hypersensitivity. I. Suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. *Eur. J. Immun.* **7**, 714–718.

LIEW F.Y. & RUSSELL S.M. (1980) Delayed type hypersensitivity to influenza virus. Induction of antigen-specific suppressor T cells for delayed-type hypersensitivity to haemagglutinin during influenza virus infection in mice. *J. exp. Med.* **151**, 799–806.

LIEW F.Y. & SIMPSON E. (1980) Delayed-type hypersensitivity responses to H–Y: characterisation and mapping of Ir genes. *Immunogenetics* **11**, 255–262.

MATHUR A., CHATURVEDI U.D., TANDON H.O., AGRAWAL A.K., MATHUR G.P., NAG D., PRASAD A. & MITTAL V.P. (1982) Japanese encephalitis epidemic in Uttar Pradesh, India during 1978. *Indian J. med. Res.* **75**, 161–169.

RAMSHAW I.A., BRETCHER P.A. & PARISH C.R. (1976) Regulation of the immune reponse. I. Suppression of delayed type hypersensitivity by T cells from mice expressing humoral immunity. *Eur. J. Immun.* **6**, 674–678.

SHUKLA M.I. & CHATURVEDI U.C. (1981) Dengue virus-induced suppressor factor stimulates production of prostaglandin to mediate suppression. *J. gen. Virol.* **56**, 241–249.

SHUKLA M.I. & CHATURVEDI U.C. (1982) In vivo role of macrophages in transmission of dengue virus-induced suppressor signal to T lymphocytes. *Br. J. exp. Path.* **63**, 522–530.

SHUKLA M.I. & CHATURVEDI U.C. (1983) Transmission of dengue virus-induced suppressor signal from macrophage to lymphocyte occurs by cell contact. *Br. J. exp. Path.* **64**, 87–92.

SY M.S., MILLER S.D., MOORHEAD J.W. & CLAMAN H.N. (1979) Active suppression of 1-fluoro-2-4-dinitrobenzene-immune T cells. Requirement of an auxiliary T cell induced by antigen. *J. exp. Med.* **149**, 1197–1207.

SY M.S., NISONOFF A., GERMAIN R.N., BENACERRAF B. & GREENE M.I. (1981) Antigen- and receptor driven regulatory mechanisms. VIII suppression of idiotype-negative, p-Azobenzene arsonate-specific T cells results from the interaction of an anti-idiotypic second-order T suppressor cell with a cross-reactive-idiotype-positive, p-Azobenzenearsonate-primed T cell target. *J. exp. Med.* **153**, 1415–1425.

TADA T. (1977) Regulation of the antibody response by T cell products determined by T subregions. In *Proceedings from ICN-UCLA Symposium on the immune System: Regulatory Genetics.* Eds E. Sercarz, L.A. Herzenberg & C.F. Fox. New York: Academic Press Inc. pp. 345–355.

TANDON P. & CHATURVEDI U.C. (1977) Antibody forming cell response of mice to dengue virus given by different routes. *Curr. Sci.* **46**, 43–44.

TANDON P., CHATURVEDI U.C. & MATHURA. (1979) Dengue virus-induced thymus-derived suppressor cells in the spleen of mice. *Immunology* **38**, 653–658.