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Immunology

Virus-induced delayed-type hypersensitivity reaction is sequentially mediated by CD8$^+$ and CD4$^+$ T lymphocytes

(virus infection/lymphocytic choriomeningitis virus/virus clearance/control of virus infection)

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ABSTRACT **After subcutaneous inoculation into the hind foot of a mouse, the lymphocytic choriomeningitis (LCM) virus multiplies locally, attaining 10^7–10^8 mouse infectious units per g of tissue; elimination commences around day 7. About 1 day earlier, the foot begins to swell, which is regarded as a delayed-type hypersensitivity (DTH) reaction. To answer the question of whether the local inflammatory response is involved in virus clearance, we needed to know what cells mediate both these phenomena. With three different procedures—namely, depletion *in vivo* of defined cells by treatment of mice with monoclonal antibodies ("serologic surgery"), adoptive immunization with negatively selected cells, and adoptive immunization with cells from mice differing at the major histocompatibility gene complex—it is shown that the LCM virus-induced local DTH reaction consists of two phases that are sequentially mediated by (first) class I-restricted cytotoxic/suppressive CD8$^+$ and (second) class II-restricted helper/inducer CD4$^+$ T lymphocytes. In contrast, for virus elimination only the former subset of T lymphocytes was found to be needed. Thus, an association may exist between the CD8$^+$ cell-mediated component of the local DTH response and control of the infection, but the CD4$^+$ cell-mediated part appears to be of doubtful antiviral relevance.**

Local delayed-type hypersensitivity (DTH) reactions seem to be caused by different mechanisms, but the central event is generally assumed to be interaction between helper/inducer T lymphocytes and eliciting antigen that is presented by specialized cells together with major histocompatibility gene complex (MHC)-encoded class II molecules. As a consequence, lymphokines are released and these cause the influx of inflammatory cells (1–3). While this simplified outline is probably correct in many cases, it does not adequately describe what happens when the antigen is infectious. Use of MHC recombinant mice has disclosed for several viruses that DTH reactions may be mediated by both class II- as well as class I-restricted T lymphocytes (4–7). In the case of infection of the mouse with lymphocytic choriomeningitis (LCM) virus, it has been demonstrated that K and/or D region-restricted cells are the effectors (8). We now show with three procedures that in the LCM virus-infected mouse the DTH reaction consists of two components that are sequentially mediated by CD8$^+$ and CD4$^+$ T lymphocytes, respectively.

MATERIALS AND METHODS

Mice. C57BL/6NCr1BR (B6) ($K^bI^bD^b$) mice were purchased from Charles River Wiga (Sulzfeld, F.R.G.), and B10.BR/Ola//Hsd (B10.BR) ($K^kI^kD^k$), B10.D2/n/Ola//Hsd (B10.D2) ($K^dI^dD^d$), B10.G/Ola//Hsd (B10.G) ($K^qI^qD^q$), and B10.AQR/Ola//Hsd (B10.AQR) ($K^qI^kD^d$) mice from Harlan Olac (Blackthorn, U.K.). All mice were female, 8–12 weeks old, and maintained for at least 2 weeks under conditions prevailing during the experiments. BALB/cJ mice and [LOU × DA]F_1 rats of either sex came from Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.). According to the producers, all animals were specific pathogen-free and were thus kept in our animal quarters.

Virus. WE strain LCM virus (9) was plaque purified three times; it was produced in L cells and titrated as plaque-forming units in L cells, which were converted to mouse infectious units (IU) by multiplication with the factor 10 (10).

Adoptive Immunization. Donor mice were infected intravenously (i.v.) with 10^3 IU and 8 days later (day-8 immune) their spleen cells were dispersed and counted as "living" on the basis of trypan blue exclusion. Defined numbers were inoculated i.v. into recipient mice that had been infected by subcutaneous footpad inoculation of 10^5 or 10^7 IU, and at intervals swelling was measured as detailed below.

Monoclonal Antibodies (mAbs). For depletion *in vivo* ("serologic surgery") of Thy-1$^+$, CD4$^+$ (L3T4$^+$), or CD8$^+$ (Lyt-2$^+$) cells, we used the mAbs Thy 1.2C (mouse, IgG2a) (11), YTS 191.1 (rat, IgG2b) (12), and YTS 169.4 (rat, IgG2b) (12), respectively; negative selection *in vitro* of these cells was achieved by antibodies Lö-2.2 (mouse, IgM) (established in our laboratory by Cornelius Löliger), LICR.LAU.RL172.4 (rat, IgM) (13), and HO-2.2 (mouse, IgM) (14), respectively. The (irrelevant) Thy-1.1 mAb HO-22-1 (15) was used for control purposes. Mouse and rat antibodies were produced as ascitic fluids in pristane-primed BALB/c mice and (LOU × DA)F_1 rats, respectively, partially purified by precipitation with 50% saturated $(NH_4)_2SO_4$ solution followed by dialysis against phosphate-buffered saline, adjusted to a concentration of 10 mg of protein per ml (≈2 mg of antibody per ml), and stored at −20°C. Protein was determined according to Lowry *et al.* (16).

Serologic Surgery. The mice were inoculated once i.v. with mAb directed against Thy-1, CD4, or CD8, which effectively depletes T lymphocytes or their subsets (11, 12, 17–19).

Negative Selection *in Vitro*. Day-8 immune spleen cells were dispersed, counted as living on the basis of trypan blue exclusion, and kept for 30 min at 4°C with suitably diluted mAb. They were washed with phosphate-buffered saline and incubated for 30 min with suitably diluted rabbit serum as complement source, which had been selected for high lytic and low toxic potential; the procedure was repeated once. Finally, the cells were taken up in the initial volume, meaning that they were not adjusted for losses during treatment.

Measurement of LCM Virus-Specific Cell-Mediated Immunity. Cytotoxic T cell (CTL) activity was quantified according

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Abbreviations: CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; IU, infectious unit; LCM virus, lymphocytic choriomeningitis virus; mAb, monoclonal antibody; MHC, major histocompatibility gene complex.

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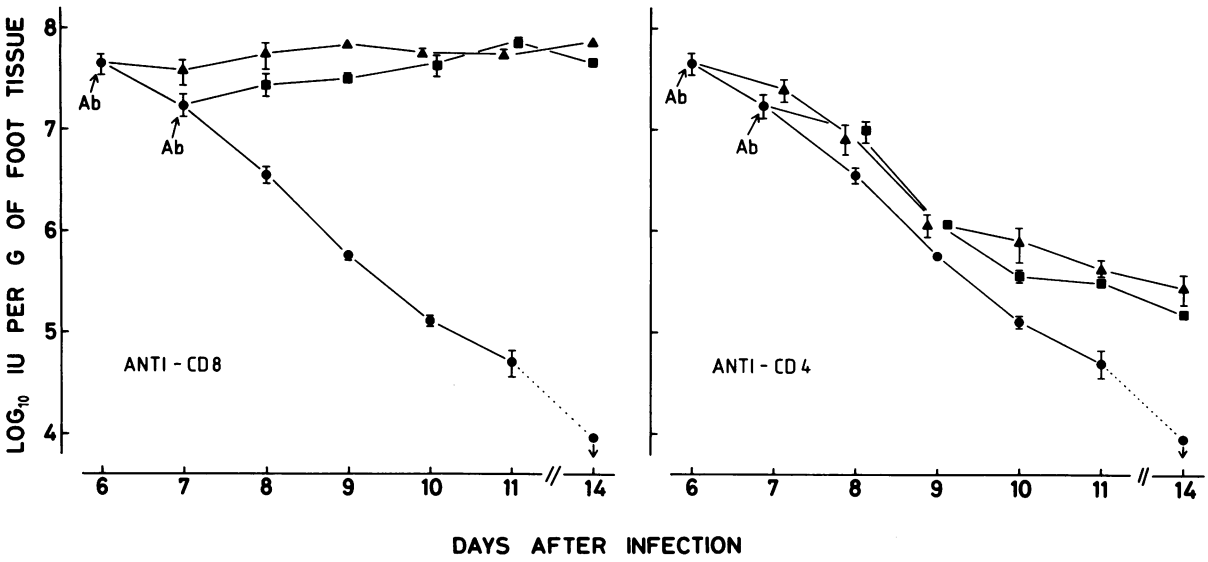

FIG. 1. Effect of treatment of mice with mAb directed against subsets of T lymphocytes on elimination of LCM virus from feet. B6 mice were infected into right hind feet by subcutaneous injection of 10^5 IU and inoculated once i.v. 6 or 7 days later with (per g) 80 μg of specific CD4 rat mAb YTS 191.1 (12) or 80 μg of specific CD8 rat mAb YTS 169.4 (12). At intervals, feet were weighed and homogenized with known volumes of balanced salt solution containing 1% heated calf serum, and virus concentrations were determined. Data points and bars signify, respectively, means ± SEM (IU) per g of tissue for five mice. ●, No antibody (virus only); ▲, treatment 6 days after infection; ■, treatment 7 days after infection.

to Brunner *et al.* (20) with minor modifications. Target cells were from a continuously growing line of B6 origin (C57-SV), which was established by infecting primary fetal fibroblasts with simian virus 40, and the incubation time in microcultures was 4 hr at 37°C.

DTH was determined as local swelling after subcutaneous inoculation of virus into the right hind footpad (21). At intervals, the dorsoventral thicknesses of both hind feet were measured with dial calipers (Oditest, especially equipped for this purpose with a soft spring; H. C. Kröplin, Schlüchtern, F.R.G.), and swelling is expressed as the factor with which the thickness of the inoculated foot exceeded the thickness of the contralateral uninoculated foot (22).

RESULTS

After subcutaneous intraplantar inoculation of 10^5 IU, the LCM virus replicates locally to $>10^7$ IU per g of tissue; this concentration is retained until about day 7 when elimination sets in. Swelling of the foot commences about 1 day earlier and reaches a maximum on days 7–8. The subsequent decline

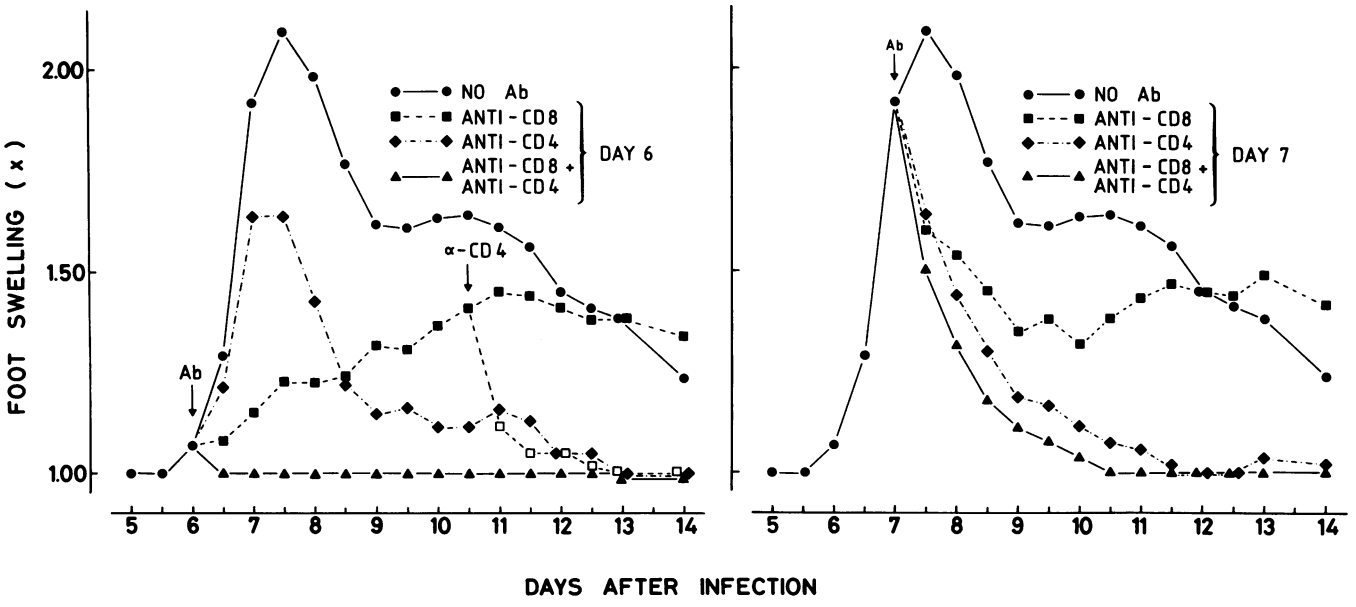

FIG. 2. Effect of treatment of mice with mAb directed against subsets of T lymphocytes on LCM virus-specific DTH measured as foot swelling. B6 mice were infected as described for Fig. 1 and treated 6 or 7 days later with mAb (see Fig. 1). On day 10 after infection, half the mice that had been depleted of $CD8^+$ cells were inoculated with CD4 mAb. At intervals of 12 hr, dorsoventral thicknesses of inoculated and uninoculated contralateral feet were measured, and swelling is expressed as the factor by which the thickness of the inoculated foot exceeded the thickness of the uninoculated foot. Data points denote means of determinations in 10 mice, the standard errors being so small that they were not included.

is slow and may be incomplete as late as 14 days after infection (23).

Treatment of footpad-infected mice during the early part of the effector phase with mAb Thy-1.2C, known to remove all peripheral T lymphocytes (11), resulted in blockade and reversal of virus elimination (data not shown); the same was observed when the mice were depleted of $CD8^+$ cells by injection of CD8 mAb (Fig. 1). Treatment with CD4 mAb slightly (but reproducibly) diminished the animals' ability to clear the virus (Fig. 1).

Removal of T lymphocytes by injection on day 6 with Thy-1.2 mAb prevented all swelling; if the antibody was inoculated later—i.e., during the time when thicknesses were increasing—the feet rapidly resumed their normal dimensions (data not shown). The same was observed when the animals were depleted of both major subsets of T lymphocytes by injecting concomitantly two mAbs directed against CD4 and CD8, respectively (Fig. 2). If treatment was with a CD4 antibody (leaving behind the $CD8^+$ cells), foot swelling was less marked and the subsequent decline was much more rapid than in untreated controls (Fig. 2). If, on the other hand, the mice were depleted of $CD8^+$ cells, the swelling was delayed and exhibited the characteristic tailing. That this reaction was indeed mediated by $CD4^+$ cells is evidenced by its quick disappearance after injection of CD4 mAb (Fig. 2).

Mutatis mutandis, the same results were obtained in experiments in which footpad-infected mice were adoptively immunized by i.v. transfusion of negatively selected immune spleen cells (Fig. 3). Transfer of unaltered splenocytes led to swelling that commenced within 48 hr. This reaction was LCM virus specific, because it was not seen in LCM virus-infected mice transfused with vesicular stomatitis virus-immune spleen cells or in vesicular stomatitis virus-infected mice transfused with LCM virus-immune spleen cells. The accelerated LCM virus-specific swelling was but slightly affected by treatment of cells with (irrelevant) Thy-1.1 mAb plus complement. Elimination of $CD4^+$ cells from the immune splenocytes prior to cell transfer resulted in a reduced but not delayed response, whereas removal of $CD8^+$ cells led to its retardation as well as its diminution. As before, the characteristic tailing of the response attributed by us to the activity of $CD4^+$ cells was quickly terminated by treatment of the mice with CD4 mAb. As a control, the effect of negative selection of immune spleen cells on their cytotoxic activity *in vitro* was determined. Neither the mAb against Thy-1.1 nor the mAb against CD4 reduced the cells' cytolytic potential, whereas treatment with Thy-1.2 or CD8 mAb (always together with complement) completely abolished lysis of target cells.

As a third approach, intraplantarly infected mice were adoptively immunized with immune spleen cells from H-2 recombinant donors. One such experiment is presented in Fig. 4; it shows that the local swelling was mediated by cells restricted by class I (identity in K and D) as well as class II (identity in I) molecules. The swelling in B10.AQR mice transfused with immune I region-compatible B10.BR cells

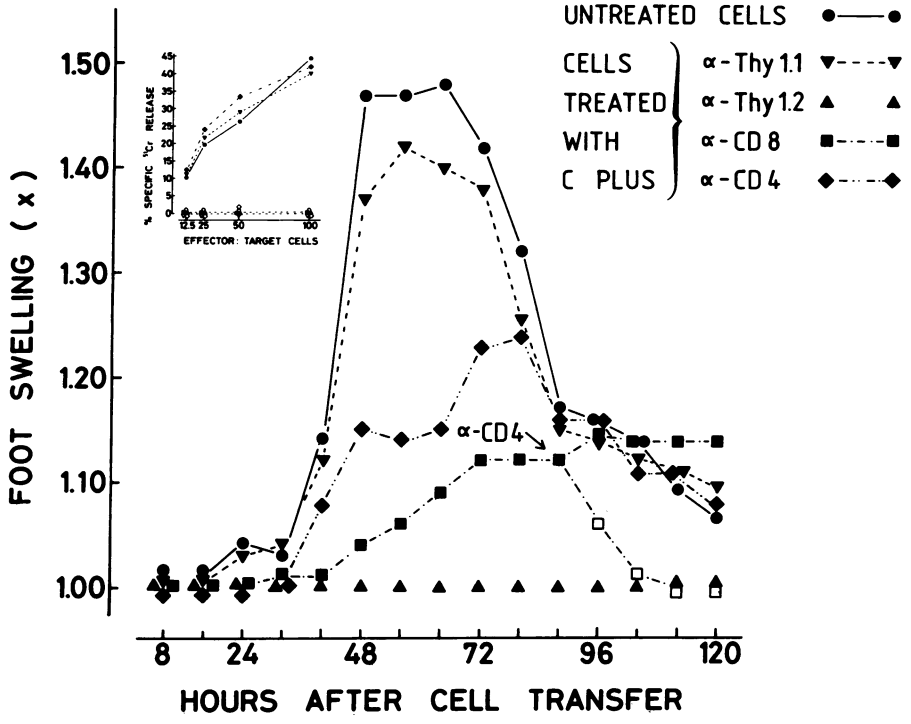

FIG. 3. Effect of adoptive immunization with negatively selected T lymphocyte subsets on DTH in LCM virus-infected syngeneic recipient mice. B6 donor mice were infected by i.v. inoculation of 10^3 IU of LCM virus. Eight days later, their dispersed spleen cells were counted as living on the basis of trypan blue exclusion. Aliquots were kept for 30 min at 4°C with suitably diluted IgM mAb directed against Thy-1.1 HO-22-1 (15), Thy-1.2 Lö-2-2 (our own hybridoma), CD4 LICR.LAU.RL172.4 (13), or CD8.2 HO-2.2 (14), whereupon the cells were washed with phosphate-buffered saline and incubated for 30 min with suitably diluted rabbit serum as complement source; the procedure was repeated once. The sample that had been treated with (irrelevant) Thy-1.1 mAb was counted again and adjusted to 1 ml containing 2×10^8 trypan blue-excluding nucleated cells, and untreated as well as the Thy-1.2-, CD4-, and CD8-treated samples were then adjusted to the same volume (meaning not corrected for losses of cells due to selective removal). Recipient mice were infected by intraplantar inoculation of 10^5 IU and 24 hr later were injected i.v. with 2×10^8 Thy-1.1-treated or volume equivalents of untreated or negatively selected immune splenocytes. Eighty-eight hours after transfer, five mice that had received $CD4^+$ cells (spleen cells treated with CD8 mAb) were depleted of $CD4^+$ cells by i.v. inoculation of CD4 mAb as specified for Fig. 1. Thicknesses of feet were determined (see Fig. 2) at 8-hr intervals, and data points signify means of five mice. Treated and untreated day-8 immune spleen cells were tested in parallel for cytotoxic activity in a 4-hr chromium release assay using LCM virus-infected simian virus 40-transformed B6 fibroblastic target cells. Results thus obtained are shown in the *Inset*. Open symbols, values obtained with uninfected target cells.

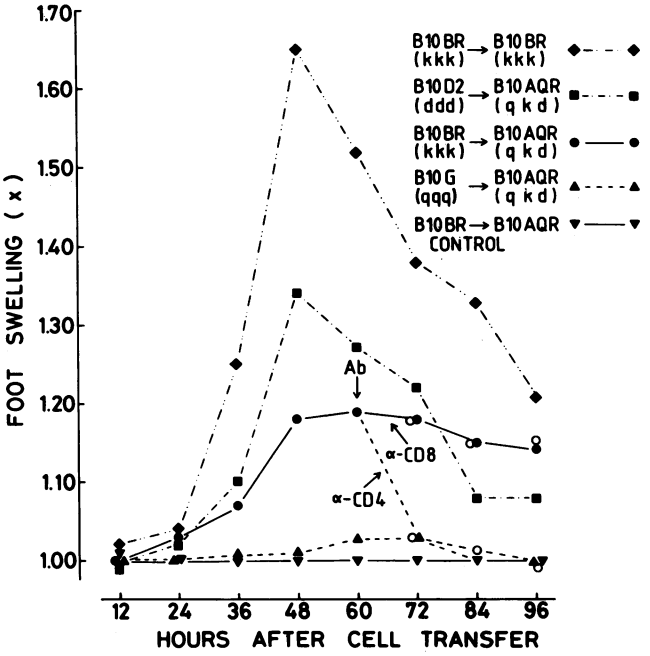

FIG. 4. Effect on virus-specific DTH in LCM virus-infected recipient mice of adoptive immunization with immune spleen cells from mice differing at H-2. Donors were infected by i.v. inoculation of 10^3 IU. Eight days later, their dispersed spleen cells were counted as viable on the basis of trypan blue exclusion. Recipient mice were infected by intraplantar inoculation of 10^7 IU and 24 hr later were inoculated i.v. with 2×10^8 immune splenocytes. Sixty hours after cell transfer, B10.AQR mice that had received B10.BR cells (I region compatibility only) were subdivided and left untreated or depleted *in vivo* of $CD4^+$ or $CD8^+$ cells by i.v. inoculation of CD4 or CD8 mAb, respectively (see Fig. 1). Thicknesses of feet were determined at 12-hr intervals, and data points signify means of 10 individual measurements.

was precipitously reversed by injection of CD4 mAb, whereas no such effect was seen after treatment with CD8 mAb. The relatively low response when compatibility was at K had previously been seen by Zinkernagel and his colleagues (24).

DISCUSSION

Although the local DTH response is among the oldest immunologic phenomena ever recognized (25–28), the biologic function of the underlying state is uncertain. The findings reported here demonstrate that in the LCM virus-infected mouse, $CD8^+$ as well as $CD4^+$ cells independently of each other mediate a local response, which together with previous observations (4–8) suggests that DTH is not as distinct an immunologic phenomenon as generally assumed. Probably the immune system's main task is to protect against infectious agents, which may be accomplished by humoral or cell-mediated mechanisms, in which B lymphocytes and T lymphocytes, respectively, are centrally involved. For recovery from acute virus infections, cell-mediated immunity is assumed to be of supreme importance (29). The extent to which $CD4^+$ helper/inducer cells participate seems to vary. Antibody production against a variety of antigens (30–32) including viruses (12, 19, 33–35) requires $CD4^+$ T lymphocytes, but antibodies are often not needed for terminating a primary infection (although they may protect against reinfection). Furthermore, examples have become known suggesting that the development *in vivo* of antiviral CTLs does not necessarily depend on $CD4^+$ cells (33, 35, 36). For the elimination of LCM virus from the organs of acutely infected adult mice, CTLs are decisive (37, 38), and the same is apparently true with regard to the foot (this work). In contrast, the helper/inducer T lymphocytes are apparently not needed for clearing the virus from spleen and liver (19), although in the foot they may play some role, because virus elimination was suboptimal in $CD4^+$ cell-depleted mice (Figs. 1 and 2; unpublished observations). However, it appears that these cells exert their antiviral effects indirectly by fulfilling some accessory function (unpublished data).

If the $CD8^+$ CTL is the critical antiviral element, its activation may be regarded as the main event, whereas activation of $CD4^+$ helper/inducer cells seems to be an accompanying circumstance. In the foot as in other tissues of the mouse, the LCM virus infects cells expressing MHC-encoded molecules of class I as well as class II (Jürgen Löhler, personal communication), which may function as antigen-presenting cells, thereby activating (or maintaining activity) of $CD4^+$ as well as $CD8^+$ T lymphocytes. That swelling follows in either case is perhaps not surprising, if one accepts the idea that it occurs whenever the appropriate lymphokines are released irrespective of the T lymphocytes' class restriction and function.

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