Antigen persistence and time of T-cell tolerization determine the efficacy of tolerization protocols for prevention of skin graft rejection

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We studied antigen-specific T-cell tolerization therapy using skin transplantation across a defined minor histocompatibility antigen difference. Specific tolerization protocols using short-lived peptide or long-lived spleen cells presenting the peptide as antigen prevented graft rejection without immunosuppression when started before or as long as 10 days after transplantation. Peptide-induced T-cell tolerance was transient, and antigen presentation by the graft was not sufficient to maintain tolerance. In contrast, transfer of antigen-expressing lymphoid cells induced long-lasting tolerance correlating with donor cell chimerism. These findings show that antigen-specific tolerization can induce graft acceptance even when begun after transplantation and that long-term graft survival depends on persistence of the tolerizing antigen.

Induction of antigen-specific peripheral T-cell tolerance is a potentially useful therapeutic approach to specifically prevent or treat immunopathologies mediated by T cells, such as autoimmune diseases or transplant rejections. It has been shown that adoptive transfer of antigen-expressing lymphoid cells[1–5], infusion of proteins[6–9] and even the administration of free synthetic peptides[10–14] can effectively induce T-cell tolerance in an antigen-specific fashion.

In contrast to the previously demonstrated successful T-cell tolerization of naive mice, it is unclear to what extent already ongoing immune responses mediated by T cells are still susceptible to specific tolerance induction[15–18]. We have recently shown that peptide therapy resulting in T-cell tolerance in naive mice activates T cells in sensitized mice and may consequently induce rather than prevent immunopathology[15]. Whether an ongoing immune response mediated by T cells can still be specifically tolerized is of essential importance for the clinical application of T-cell tolerization therapies. This is particularly true for the treatment of autoimmune diseases, but is also relevant to organ transplantation. Usually the tissue donor is not known in advance and, consequently, treatment to induce donor-specific tolerance can only be begun with or soon after grafting. It is therefore essential to determine whether antigen-specific T-cell tolerance can still be induced after transplantation.

Another clinically important aspect of tolerization protocols is the mechanism of long-term maintenance of T-cell unresponsiveness. It is unclear whether antigen presentation by the graft itself and/or lymphoid cells emigrating from the graft are sufficient for this task[19–21]. In some experimental models, transplant tolerance can be induced in non-immunosuppressive conditions by additional administration of the donor antigen (for example, in the form of lymphoid cells) (refs. 1–5). It is essential to define the relative importance of these additional donor cells versus antigen presentation by the graft itself for long-term graft acceptance. Although several studies have addressed the role of lymphoid donor cell persistence[22–26], previous experimental protocols involved immunosuppression or partial effector-cell depletion. A clear demonstration of the importance of antigen persistence, comparing long-lived antigens with short-lived antigens without additional manipulation of the immune system, is still lacking.

To study rejection versus tolerance of foreign grafts, we established a model of skin transplantation across an antigenic difference defined by a single MHC class I-presented peptide. Skin grafts obtained from mice ubiquitously expressing a cytotoxic T lymphocyte (CTL) epitope derived from the glycoprotein (gp) of lymphocytic choriomeningitis virus (LCMV; gp33, presented by H-2D^b) (ref. 27) were transplanted on naive or immunized C57BL/6 recipients. Gp33-specific T-cell tolerization therapy was begun before or after transplantation using either synthetic gp33 peptide (short-lived antigen) or donor spleen cells presenting this peptide (long-lived antigen) to determine whether tolerization protocols can still prevent graft rejection when begun after transplantation, and what the role of antigen persistence is in long-term maintenance of tolerance and graft survival.

A single CTL epitope elicits skin graft rejection

Skin from a donor mouse ubiquitously expressing the well characterized LCMV glycoprotein epitope gp33–41 (H8 mice[27]) as a transgene was used to study the immunological response to skin transplants with a defined antigenic difference. This short transgene did not encode any additional T-helper-cell epitopes. Naive C57BL/6 recipient mice were grafted with skin from sex-matched H8 mice or transgene-negative control littermates. Gp33-expressing H8 skin grafts were rejected spontaneously 70–120 days after transplantation (Fig. 1*a*), whereas all control grafts remained intact for the observation period of 200 days. The rejection response

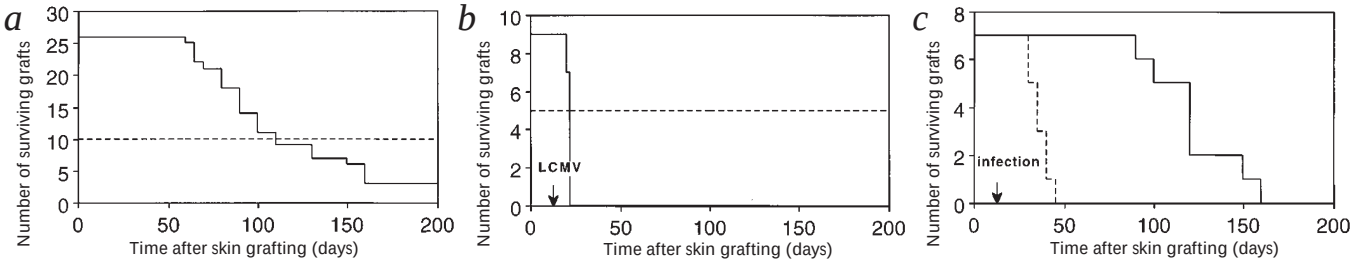

Fig. 1 Spontaneous and virus-triggered rejection of H8 skin grafts. ***a*** and ***b***, Naive C57BL/6 mice were grafted with skin from H8 mice (solid lines) or transgene-negative control littermates (dashed lines) and monitored for skin graft survival. *a*, Data are pooled from three independent experiments. *b*, Mice were infected with LCMV 10 days after skin transplantation. ***c***, C57BL/6 mice grafted with skin from H8 mice were infected with vaccinia WR (Vacc WR; solid line) or with vaccinia recombinant expressing the LCMV glycoprotein (Vacc G2; dashed line) and monitored for skin graft survival.

was considerably accelerated when the recipient mice were infected with LCMV after transplantation: all skin grafts from H8 donor mice were rejected 10–15 days after immunization (Fig. 1*b*). Three of twenty-six mice did not spontaneously reject their H8 grafts until 200 days after transplantation (Fig. 1*a*). However, when these mice were challenged with LCMV at this late time point, all rejected the grafts (data not shown), indicating that the grafts had not tolerized the gp33-specific CTL precursors.

To determine whether virus-induced accelerated graft rejection was a result of antigen-specific immunity or non-specific mechanisms, we tested a group of C57BL/6 mice, grafted with H8 skin, by infecting them with vaccinia virus or with a recombinant vaccinia virus expressing the LCMV glycoprotein. Although wild-type vaccinia virus infection did not influence the kinetics of spontaneous graft rejection, expression of the relevant antigen after infection with recombinant vaccinia virus enhanced rejection considerably, demonstrating an antigen-specific mechanism of graft rejection (Fig. 1*c*).

H8 grafts were also rapidly rejected when the recipient mice were immunized with LCMV 60 days before skin transplantation (Fig. 2*a*). 'LCMV memory mice' have an increased precursor frequency of gp33-specific CTLs (in the range of $1{:}10^3$, compared with $1{:}10^6$ for naive mice[28]). Moreover, these memory CTLs are in a different functional state[29,30], with a different recirculation behavior[31,32]. To determine which of these factors contributed to the accelerated rejection response, we grafted H8 skin onto TCR318 mice, which have an increased frequency of functionally naive gp33-specific CTL precursors. H8 skin was spontaneously rejected by TCR318 mice 10–15 days after transplantation (Fig. 2*b*). This indicates that an increased CTL precursor frequency alone can contribute to an accelerated rejection response.

CTL tolerization is effective before and after skin grafting

The characterization of the rejection response to skin grafts expressing a single CTL epitope allowed us to investigate how therapeutic approaches to tolerize CTL responses can influence the rejection process. For this we used the well established antigen-specific tolerization protocol of three intraperitoneal injections of synthetic gp33 peptide in Incomplete Freund's Adjuvans[14] (IFA). To exclude repopulation of gp33-specific CTL precursors by the thymus, thymectomized recipients were used in initial experiments. Peptide-specific tolerization of gp33-specific $CD8^+$ T cells in C57BL/6 mice begun two weeks before transplantation of H8 skin prevented graft rejection induced by LCMV infection in six of seven recipients (Fig. 3*a*). In contrast, all five control mice treated with IFA alone rejected H8 skin grafts 10–20 days after LCMV challenge infection.

Next, we investigated whether effective antigen-specific tolerization of $CD8^+$ T cells is still possible after the skin had been transplanted. Peptide-specific T-cell tolerization was still able to prevent virus-accelerated graft rejection, if begun 10 days after skin grafting (Fig. 3*b*). If, however, peptide treatment was started 30 days after transplantation, rejection was considerably slower (mean rejection time of 50 versus 15 days), but could not be prevented (Fig. 3*b*). Similar results were obtained when, instead of peptide, transfusion of gp33-expressing spleen cells from H8 mice was used to tolerize the gp33 specific CTL response. A single transfer of 1×10^7 spleen cells from sex-matched H8 mice 10 days after skin grafting prevented the virus-induced rejection of H8 skin grafts (Fig. 3*c*), whereas the same treatment 30 days after transplantation was unsuccessful in preventing this rejection.

Persistence of tolerogen determines the duration of tolerance

Although the *in vivo* half-life of synthetic peptides mixed with a mild adjuvans is relatively short (presumably in the range of days), adoptive transfer of spleen cells could produce long-term antigen

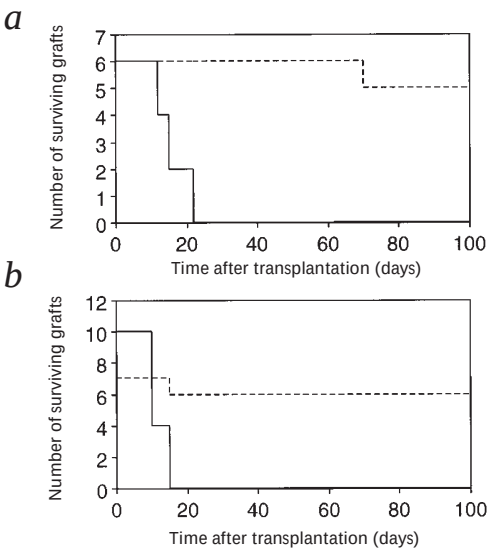

Fig. 2 Rejection of H8 skin grafts by virus-immune mice and mice transgenic for (an LCMV gp33-specific) T-cell receptor. ***a***, C57BL/6 mice were immunized with LCMV and grafted with skin from H8 mice (solid lines) or transgene-negative control littermates (dashed lines) 60 days after immunization and monitored for skin graft survival. ***b***, Naive mice transgenic for an LCMV gp33-specific T-cell receptor (TCR318 mice) were grafted with skin from H8 mice (solid lines) or transgene-negative control littermates (dashed lines) and monitored for skin graft survival. Data are representative of two independent experiments.

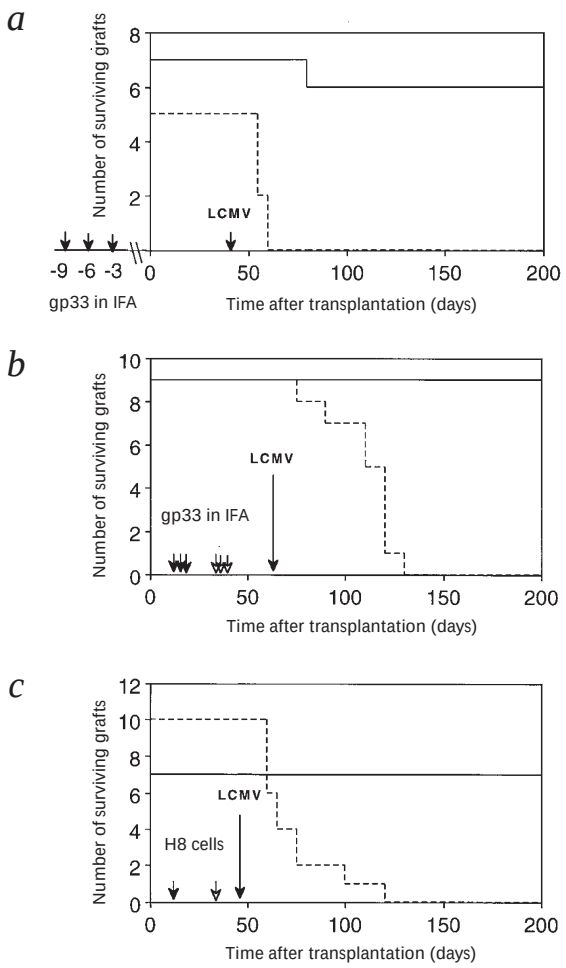

Fig. 3 Peptide-specific T-cell tolerization can prevent skin graft rejection. ***a***, Thymectomized C57BL/6 mice were injected three times in three day intervals with gp33 peptide in IFA (solid lines) or with IFA alone (dashed lines). Three days later, they received skin grafts from H8 mice. Forty days after transplantation, mice were challenged with LCMV and monitored for skin graft survival. ***b*** and ***c***, Naive thymectomized C57BL/6 mice were grafted with skin from H8 mice. Ten days (solid arrows and lines) or thirty days (open arrows and dashed lines) later, they were either tolerized with gp33 peptide (*b*) or injected with a single dose of spleen cells from H8 mice (*c*). Forty (*c*) or sixty (*b*) days after transplantation, mice were challenged with LCMV and monitored for skin graft survival. All experiments were repeated twice. Similar results were obtained whether the mice were infected 10 or 60 days after completion of the tolerization protocol.

persistence in the form of donor-cell chimerism. To study the persistence of H8 donor cells in the tolerized recipients, spleen cells from H8 mice were labelled with the flourescent dye CFSE before transfer into sex-matched recipients. After transfer of 7×10^7 H8 cells, donor cells represented about 6% of the circulating lymphocyte population during the first few days, and slowly decreased thereafter (Fig. 4*a*). Donor cells persisted at about 1.5% of the circulating blood lymphocytes as long as 55 days after transfer; at later time points, the fluorescent intensity of the dye had decreased to amounts that could not be reliably discriminated from background. Syngeneic C57BL/6 control cells had a similar decline (Fig. 4*a*). The steady-state decline of H8 cells between day 10 and day 55 indicates a donor cell half-life of about 40 days; by conservative extrapolation, by day 300 about 0.5–1.0% of the original number of donor cells should still persist in the host.

To determine the effect of antigen persistence on tolerization of CTLs and graft survival, we compared the two tolerization protocols in long-term experiments. Euthymic and thymectomized C57BL/6 mice were tolerized either by three intraperitoneal injections of gp33 in IFA or by transfer of 1×10^7 H8 spleen cells. At various times later, they were infected with 200 plaque-forming units (PFU) of LCMV, and eight days after infection, the specificity of the CTL response was determined. Sixty days after peptide tolerization, the gp33-specific CTL response had been fully reconstituted in euthymic mice, but not in thymectomized mice (Fig. 4*b*). This was dependent on the presence of an intact thymus. In contrast, gp33-specific tolerance remained firmly established for more than 300 days in euthymic mice that had been tolerized with H8 spleen cells (Fig. 4*b*). This important role for antigen persistence in the maintenance of T-cell tolerance was confirmed when the durations of the therapeutic effects of the two tolerization protocols were compared in terms of skin graft survival. As before, euthymic mice transplanted with H8 skin were treated either three times with gp33 in IFA or once with 1×10^7 H8 cells, beginning 10 days after transplantation. By day 320 after skin grafting, seven of nine mice tolerized with peptide had rejected their grafts, whereas seven of eight mice tolerized by donor spleen cells retained healthy grafts (Fig. 4*c*).

Discussion

Here, we assessed $CD8^+$ T-cell tolerization protocols in a skin transplantation model, in which both the antigen and the immunological effector population are defined at the molecular level. We found that antigen-specific tolerization is still possible within a short time after transplantation and that the duration of the therapeutic effect depends on persistence of the tolerizing antigen.

In our experimental model, skin transplantation across a single MHC class I-restricted antigenic difference (the gp33 peptide) was sufficient to elicit graft rejection. Spontaneous rejection of H8 skin grafts occurred within 70–120 days after transplantation, but this could be accelerated to rejection within 10–15 days, if the recipients were challenged with the transplantation antigen in the context of a virus infection. These findings contrast with those of a recent study, in which skin from mice expressing a defined tumor CTL epitope as a transgene was found to induce tolerance rather than graft rejection[33]. Skin rejection in that model could only be elicited when the recipient mice had been primed with antigen-expressing spleen cells before grafting and if the graft expressed additional T-helper-cell epitopes[33]. Differences in the expression level of the transgenic CTL epitope, the precursor frequency of the responding T cells or the size and location of the skin graft source (tail skin versus full-size abdominal skin graft) may contribute to these discrepancies.

The different kinetics of graft rejection in the various recipients and the acceleration of rejection after LCMV infection demonstrate some important factors determining graft rejection in this model. First, antigen must be transported from the graft to lymphoid tissue in sufficient amounts to initiate a CTL response[34–36]. The slow rejection kinetics in naive skin graft recipients can probably be explained by the fact that this process is slow and the quantitative requirements for CTL induction are relatively high. LCMV infection considerably accelerated graft rejection by introducing high amounts of the relevant gp33 antigen into lymphoid tissue. This is comparable to the previously described rapid induction of diabetes after LCMV infection of mice expressing full-length LCMV gp as a transgene under the rat insulin promoter in pancreatic islet cells[37,38]. Second, a critical number of CTLs must be primed in lymphoid tissue and migrate to the site of the graft[39]. Memory CTLs

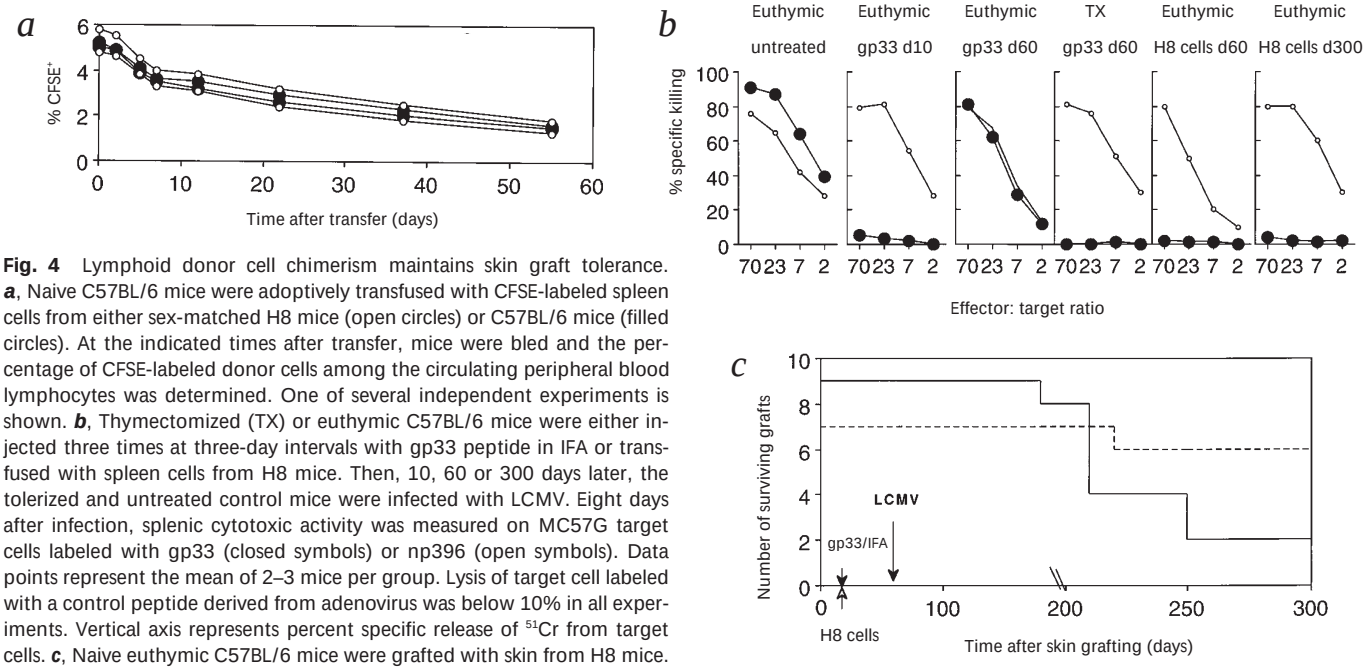

Fig. 4 Lymphoid donor cell chimerism maintains skin graft tolerance. ***a***, Naive C57BL/6 mice were adoptively transfused with CFSE-labeled spleen cells from either sex-matched H8 mice (open circles) or C57BL/6 mice (filled circles). At the indicated times after transfer, mice were bled and the percentage of CFSE-labeled donor cells among the circulating peripheral blood lymphocytes was determined. One of several independent experiments is shown. ***b***, Thymectomized (TX) or euthymic C57BL/6 mice were either injected three times at three-day intervals with gp33 peptide in IFA or transfused with spleen cells from H8 mice. Then, 10, 60 or 300 days later, the tolerized and untreated control mice were infected with LCMV. Eight days after infection, splenic cytotoxic activity was measured on MC57G target cells labeled with gp33 (closed symbols) or np396 (open symbols). Data points represent the mean of 2–3 mice per group. Lysis of target cell labeled with a control peptide derived from adenovirus was below 10% in all experiments. Vertical axis represents percent specific release of ^{51}Cr from target cells. ***c***, Naive euthymic C57BL/6 mice were grafted with skin from H8 mice. Ten days later, they were either injected three times in three-day intervals with gp33 peptide in IFA (solid line) or injected with a single dose of spleen cells obtained from H8 mice (dashed line). Fifty-five days after transplantation, mice were challenged with LCMV and monitored for skin graft survival.

specific for gp33 peptide in LCMV immune mice have both an elevated precursor frequency[28] and a different migration behavior[32]. Both of these factors probably contribute to the accelerated graft rejection by mice immune to LCMV. However, the experiments using mice transgenic for the LCMV-gp33-specific T-cell receptor show that an (artificial) increase in the number of naive T cells specific for the gp33 peptide may be sufficient.

Our experiments showed that antigen expression by the 1.5×1.5 cm, full-thickness skin graft itself was not sufficient to induce T-cell tolerance or to maintain long-term unresponsiveness after an initial tolerization. In contrast, both peptide-mediated as well as cell transfer protocols were able to induce functionally complete $CD8^+$ T-cell tolerance to the skin graft even when begun 10 days after transplantation. Tolerance could not be broken even by a subsequent LCMV infection. A comparison of the two therapeutic protocols showed the importance of antigen persistence outside the graft for long-term maintenance of transplantation tolerance. The half-life of free peptide is hours to days, which explains the full regeneration of gp33-specific CTL responsiveness within 60 days, probably a result of export of new CTL precursors from the thymus. In fact, thymectomy before tolerization prevented re-emergence of gp33-specific CTLs by day 60 (Fig. 4*b*). In contrast, adoptive transfer of spleen cells expressing the gp33 epitope leads to long-term donor-cell chimerism. Recirculating donor cells were found by flow cytometry for at least two months after transfer at levels about 15–20% of those originally injected, indicating that relevant amounts of lymphoid donor cells persist considerably longer. These cells seem necessary and sufficient to maintain the gp33-specific T-cell tolerance still present almost one year after cell transfusion.

Whether induction of antigen-specific T-cell tolerance is still possible at the time of or after transplantation is a clinically important question, as the organ donor is often not known before transplantation. In immunological terms, this problem leads to the question of whether activated or memory CTLs can be tolerized as efficiently as naive $CD8^+$ T cells[15–18]. We have recently shown that peptide tolerization protocols established for naive $CD8^+$ T cells may lead to T-cell activation in immune mice with the consequence of severe lymphoid immunopathology[15]. Neither peptide treatment nor spleen cell transfer were able to induce antigen-specific tolerance in antigen-primed mice (data not shown). The two tolerization protocols, however, protected against both spontaneous and virus-induced graft rejection when begun 10 days, but not 30 days, after skin grafting. These results indicate that there is a period of time between skin transplantation and the activation of a sufficient number of graft-rejecting effector T cells during which successful therapeutic intervention can still happen. This is probably influenced by the kinetics of antigen transport from the graft to the local lymph node, as well as by the number of antigen-specific T cells.

Results of experiments using a transgenic T-cell epitope as a model transplantation antigen have some limitations. Both the antigens recognized as well as the effector cells involved are much more complex in fully allogeneic transplant situations. Furthermore, the requirements for the induction of tolerance may be more demanding[23,40] and usually require additional immunosuppression, particularly if T cells primed by cross-reactive antigens are already present before transplantation. Nevertheless, the precise definition of both the antigen as well as the involved effector CTL population in our model allows identification of the basic rules of $CD8^+$ T-cell tolerization in an antigen-specific system, which may be relevant for both transplantation medicine and the treatment of autoimmune diseases. Antigen-specific CTL tolerization protocols can be effective if started within a short time after transplantation; this could allow therapeutic intervention early after grafting. After the recipient is fully sensitized, either by the graft or by previous exposure to the antigen, tolerance can not be induced with these protocols. Comparison of tolerization protocols using peptide or donor lymphoid cells (that is, short-lived or long-lived antigens) showed that each strategy can induce initial T-cell tolerance. However, long-term graft survival requires persistence of the tolerizing antigen. These findings support the idea that an efficient means to achieve transplant acceptance should be to establish sufficient levels of persistent lymphoid donor cell chimerism[41–43].

Methods

Mice. C57BL/6 mice were obtained from the Institut für Labortierkunde (University of Zürich, Switzerland). The transgenic mice expressing the LCMV gp33–41 epitope sequence under control of the H-2 K^b promoter (H8 mice; official nomenclature TgN (LCMVGP33)224Zbz) (ref. 27) and mice expressing a Va2/Vb8.2 T-cell receptor specific for amino acids 33–41 of LCMV gp1 in association with $H2D^b$ (TCR318 mice) (ref. 13,44) have been described.

Virus. LCMV WE was originally obtained from F. Lehmann-Grube (Heinrich-Pette-Institut für Experimentelle Virologie, University of Hamburg, Germany) and was propagated on L 929 fibroblast cells. Vaccinia virus WR was grown on BSC 40 cells. Recombinant vaccinia virus expressing the LCMV glycoprotein (Vacc G2) was obtained from D.H. Bishop (University of Oxford, Oxford, England).

$CD8^+$ T-cell tolerization protocols. LCMV glycoprotein peptide GP33–41 (KAVYNFATM) was purchased from Neosystem Laboratoire (Strasbourg, France). For T-cell tolerization, the peptide was dissolved in balanced salt solution (BSS), emulsified 1:1 (vol/vol) in IFA (Difco Laboratories, Detroit, Michigan), and three doses of 500 μg in a total volume of 200 μl were injected intraperitoneally at 3-day intervals[14]. For tolerization by adoptive transfer of H8 spleen cells, single-cell suspensions were prepared from spleens of donor mice, washed twice, counted and injected intravenously in a volume of 500 μl BSS into the recipient mice. For fluorescence labeling, spleen cells were incubated at a concentration of 5×10^6 cells/ml in BSS containing 0.5 μM CFSE (Molecular Probes, Eugene, Oregon) for 10 min at 37 °C. The cells were washed once in BSS 1% FCS and injected intravenously. The percentage of donor cells among recipient peripheral blood lymphocytes was determined by FACS analysis. LCMV gp33-specific cytotoxicity after $CD8^+$ T-cell tolerization was tested eight days after LCMV challenge infection in a 5-hour ^{51}Cr-release assay as described[45]. Percent specific release from target cells = (sample cpm – background cpm)/(total cpm – background cpm) × 100.

Skin grafting. Skin grafting was done using the method of Billingham and Medawar[1]. Full-thickness skin (~1.5 × 1.5 cm) from the belly of a donor mouse was engrafted onto the right side of the thorax of a recipient mouse. The graft was covered with gauze and plaster that were removed on day nine. Grafts were scored daily for the first two weeks and every four to five days thereafter until rejection (defined as loss of >80% of the grafted tissue), or until the time points after transplantation indicated in the figures (100–320 days).

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