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ORIGINAL ARTICLE

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Intrathymic delivery of plasmid-encoding endoplasmic reticulum signal-sequence-deleted MHC class I alloantigen can induce long-term allograft survival

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Abstract Intrathymic (IT) delivery of donor alloantigen is a potent strategy to induce operational tolerance. In this study we determined whether this effect was dependent on direct allorecognition of the tolerogen. Ten microgrammes of plasmid, encoding either the wild-type major histocompatibility complex (MHC) class I molecule K^b or a truncated form in which the signal sequence for translocation into the endoplasmic reticulum was deleted, preventing cell surface expression and direct allorecognition of the tolerogen, was administered intrathymically to CBA.Ca ($H2^k$) recipients. In addition, recipients were treated with anti-CD4 antibody (YTA3.1) at the time of IT injection and underwent transplantation 28 days later with a fully mismatched C57BL/10 ($H2^b$) cardiac allograft. Wildtype, as well as truncated K^b genes, were able to induce long-term survival of the cardiac allografts, in contrast to empty control plasmid. Reverse-transcriptase PCR showed expression of the K^b genes for up to 28 days in thymus and spleen of pretreated recipients. These data show that direct allorecognition of the tolerogen was not required for the induction of long-term allograft survival following the introduction of plasmid-encoded MHC alloantigen into the thymus.

Keywords Thymus · Operational tolerance · Cardiac allograft

Introduction

Administration of alloantigen into the adult thymus is a powerful tool to induce operational tolerance in rodents [1, 2]. The interest in the thymic pathway to transplantation tolerance was renewed through the investigation by Posselt and colleagues, who demonstrated acceptance of allogeneic islets of Langerhans after intrathymic application in conjunction with a single dose of anti-lymphocyte serum [3]. Furthermore, the recipients subsequently also accepted peripheral islet allografts from the same donor strain [3]. The sources of antigen that

have since been used successfully for the induction of intrathymic tolerance range from donor-derived splenocytes [4], resting T- and B-cells [5, 6], soluble major histocompatibility complex (MHC) molecules [7, 8] to MHC-derived peptides [9].

In an interesting study Knechtle and colleagues could show, in a rat model, that intrathymic application of plasmid-encoding MHC class I alloantigen, in conjunction with a single dose of anti-lymphocyte serum, induced donor-specific unresponsiveness and effectively prolonged the survival of subsequent liver allografts [10]. The liver is generally considered an immune-privileged organ graft, since it has the potential to favour its immunological acceptance [11, 12] and can induce spontaneous tolerance [13]. Liver allografts may, consequently, be less prone to rejection than are other vascularised organ grafts.

In this study we therefore wanted to test whether intrathymic (IT) delivery of plasmid encoding a single donor MHC class I alloantigen, in combination with peripheral administration of depleting anti-CD4 antibody, could prolong the survival of fully mismatched allografts in a mouse model of cardiac transplantation.

Previous studies that had used soluble major MHC molecules [7, 8] or MHC-derived peptides [9] indicated that direct allorecognition of the tolerogen was not necessarily required in thymic tolerance induction. Hence, we were interested to determine whether cell surface expression, and, therefore, direct allorecognition of the injected donor MHC class I alloantigen, was required to prolong cardiac allograft survival in this model. We addressed this question by IT delivery of plasmid encoding a truncated gene of the MHC class I alloantigen which had a deletion of the endoplasmic reticulum signal sequence, preventing cell surface expression and direct allorecognition.

Material and methods:

Animals

CBA.Ca ($H2^k$) mice were used as recipients and C57BL/10 ($H2^b$) and NZW ($H2^z$) mice as fully allogeneic heart donors. The mice were bred and maintained in the Biomedical Services Unit at the John Radcliffe Hospital, Oxford. All mice were male, aged 6–10 weeks and weighed 20–30 g at the time of experimental use. All animals were treated in accordance with the principles of laboratory animal care and the Home Office Animals (Scientific Procedures) Act of 1986.

Treatment protocol and cardiac transplantation

The gene encoding the MHC class I antigen K^b was cloned into the expression vector pSVK3 (Invitrogen), under control of the SV40 promoter, generating pSV-K^b. A mutant form of the K^b gene was generated through deletion of the endoplasmic reticulum signal sequence that encodes a signal peptide required for translocation of newly synthesised proteins into the endoplasmic reticulum and subsequent expression at the cell surface [14]. Recently, we characterised this construct and showed that the deleted form of K^b was expressed in the cytosol but not on the cell surface of transduced cells [15]. The signal-sequence deleted mutant form of K^b ($SDELK^b$) was cloned into pSVK3, generating pSV-$SDELK^b$.

CBA.Ca ($H2^k$) recipients received 50 μg of depleting anti-CD4 antibody (YTA 3.1, hybridoma kindly provided by Prof. H. Waldman, Sir William Dunn School of Pathology, Oxford) on days 28 and 27 before transplantation. In addition, recipients received 10 μg per thymic lobe of either the empty plasmid pSVK3, pSV-K^b or pSV-$SDELK^b$, 27 days before transplantation. The plasmid was dissolved in one microlitre of normal saline per microgramme plasmid and injected in each of the lobes of the thymus, as described earlier [4]. Four weeks after pretreatment the mice were grafted with a heterotopic fully mismatched C57BL/10 ($H2^b$) or NZW ($H2^z$) heart transplant, principally as described [16]. Graft function was assessed by abdominal palpation, and rejection was confirmed by laparotomy. Using the log-rank test, we compared graft survival between the plasmid-treated groups.

Assessment of transgene expression

For the expression of the respective plasmid-encoded K^b gene to be analysed, recipients were given anti-CD4 antibody and an IT injection as described above. At days 7, 14 and 28 three animals per treatment group were killed, and the total RNA was isolated from thymus, spleen, and cervical lymph nodes. Five microgrammes of total RNA were reverse transcribed according to standard protocols. Two microlitres of the resulting 40 μl cDNA mix were amplified with K^b primers, as described earlier [15]. As a control the gene for hypoxanthine guanine phosphoribosyl transferase (HPRT) was amplified.

Results

Induction of long-term allograft survival

Untreated control CBA mice rejected C57BL/10 heart allografts acutely (Fig. 1; median survival time (MST) = 9 days, $n=5$). Recipients pretreated with anti-CD4 antibody alone rejected the allografts, with an MST of 24 days ($n=6$). Similarly, recipients receiving an additional IT injection of the empty control plasmid pSVK3

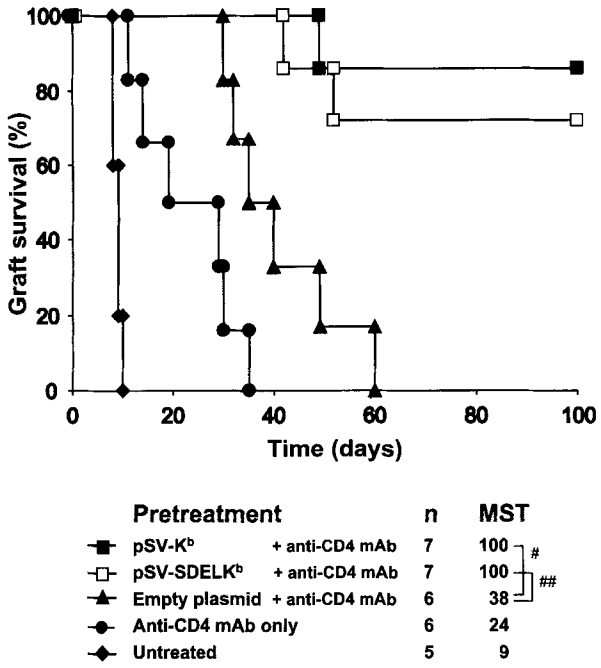

Fig. 1 Survival of fully mismatched C57BL/10 heart grafts ($H2^b$) in CBA recipients ($H2^k$). Pretreatment of recipients with intrathymic (IT) injection of plasmid encoding the single MHC class I alloantigen K^b (pSV-K^b) under the cover of depleting anti-CD4 antibody (YTA3.1) induced long-term survival in six out of seven fully allogeneic heart grafts (■; MST = 100 days). IT delivery of plasmid encoding the truncated intracellular form of K^b (pSV-SDELK^b) under the cover of depleting anti-CD4 antibody (YTA3.1) induced long-term survival in five out of seven fully allogeneic heart grafts (□; MST = 100 days). This prolongation of graft survival was significant when compared with recipients receiving anti-CD4 antibody and the empty control plasmid (▲ MST = 38 days, # $P < 0.001$, ## $P < 0.005$, log-rank test). Recipients in the untreated control group rejected their allografts acutely (♦; MST = 9 days, $n = 5$) and recipients receiving anti-CD4 antibody alone rejected the allografts, with an MST of 24 days (●; $n = 6$). *mAb* monoclonal antibody)

also rejected their heart transplant, albeit with a slightly delay when compared with anti-CD4 therapy alone (Fig. 1; MST = 38 days, $n = 6$).

In contrast, pretreatment with plasmid encoding either the full length K^b (pSV-K^b) or the signal-sequence-deleted K^b (pSV-SDELK^b) in combination with anti-CD4 antibody was able to induce long-term survival of C57BL/10 heart allografts in six of seven recipients for pSV-K^b (MST = 100 days, $n = 7$) and five of seven recipients for pSV-SDELK^b (MST = 100 days, $n = 7$; Fig. 1). This difference in graft survival was significant when compared with that of recipients receiving anti-CD4 antibody and the empty control plasmid ($P < 0.001$ for pSV-K^b, $P < 0.005$ for pSV-SDELK^b). The induction of long-term allograft survival through the K^b-encoding plasmids was antigen specific, as third-party control heart grafts (NZW, $H2^z$) were all rejected, with an MST of 35 days (pSV-K^b, n = *6*) and 37 days (pSV-SDELK^b, n = *6*), respectively. Untreated CBA recipients rejected NZW third-party control hearts with an MST of 10 days ($n = 4$).

It is interesting to note that anti-CD4 antibody treatment in combination with IT delivery of the empty control plasmid delayed graft rejection, when compared with anti-CD4 antibody treatment alone (MST = 38 days vs MST = 24 days, $P < 0.05$). This is most likely due to an unspecific effect of the IT plasmid injection. Following IT plasmid injection, we observed cellular depletion of the thymus, with a maximum on day 7 post-injection. On day 28, at the time of transplantation, the cellularity of the thymus was still not fully restored (data not shown). This suggests that IT plasmid injection delayed the restoration of the peripheral T-cell repertoire following transient CD4 T cell depletion, resulting in an unspecifically delayed rejection of the heart allografts in this control group. This finding is in accordance with earlier studies that showed a certain unspecific effect of IT injection [1, 10].

On the other hand, recipients receiving only IT injection of either one of the K^b-encoding plasmids without CD4 T cell depletion acutely rejected the C57BL/10 heart allograft (MST = 8 days for pSV-K^b; MST = 9 days for pSV-SDELK^b, $n = 4$ each). These data show that, in order to induce long-term allograft survival in our experimental system, both the anti-CD4 antibody treatment and IT delivery of the respective plasmid was required. The induction of long-term allograft survival was dose dependent, since 5 μg of K^b-encoding plasmid per thymic lobe was less efficient in prolonging graft survival (data not shown).

Persistence of transgene expression in vivo

The expression and distribution of the plasmid-encoded K^b genes, following IT injection under the cover of anti-CD4 antibody, was assessed by reverse transcriptase PCR of thymus, spleen, and cervical lymph nodes (Fig. 2). Expression of K^b, as well as of SDEL-K^b, was detected in all three recipients tested per time point until day 28 in the thymus and, to a lesser degree, in the spleen, with a further decline in expression towards day 28. In cervical lymph nodes only weak expression of either K^b construct was found on day 7 in one recipient per group, indicating that leakage of the injected plasmid from the thymus did not occur to any significant extent. We therefore assume that K^b expression in the recipient spleen was due to migration of cells that had taken up the plasmid in the thymus.

Discussion

The mechanisms of transplantation tolerance following IT antigen delivery are still not fully elucidated. Several studies have shown that deletion and apoptosis of thy-

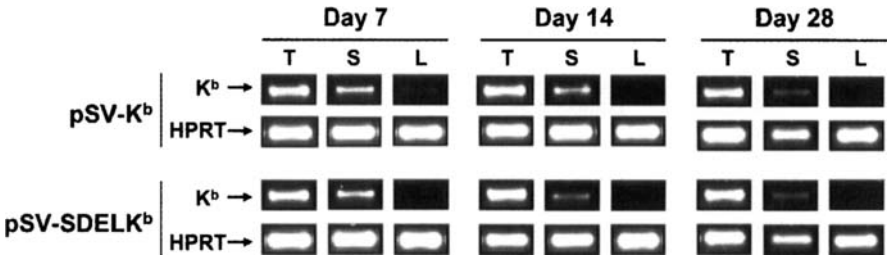

Fig. 2 Analysis of K^b expression following IT injection of plasmid encoding either wildtype K^b (pSV-K^b) or the truncated intracellular form of K^b (pSV-SDELK^b) under the cover of anti-CD4 antibody, using reverse transcriptase PCR of thymus, spleen, and cervical lymph nodes. Three recipients were tested per time point. Expression of K^b, as well as of SDEL-K^b, was detected in all recipients up to day 28 in the thymus and spleen. Weak expression of K^b was found in cervical lymph nodes only on day 7 in one recipient per group

mocytes is involved [4, 17]. However, there is also good evidence that non-deletional mechanisms, such as peripheral T-cell anergy and T-cell regulation, play a role in modulating the immune response following IT injection of antigen [17, 18, 19]. Indeed, several mechanisms may act simultaneously [10].

In our study IT injection of plasmid encoding a single MHC class I alloantigen (K^b) into both lobes of the thymus under the cover of anti-CD4 antibody was able to induce long-term survival of subsequently transplanted fully mismatched C57BL/10 cardiac grafts, a mechanism generally described as linked epitope suppression [20, 21]. This finding implies that regulatory mechanisms are involved, since mere deletion of thymocytes recognising the intrathymically injected single alloantigen would not account for the functional acceptance of grafts expressing additional MHC class I and II alloantigens. This interpretation is supported by a study that has shown that central deletion of alloreactive T cells prevented linked epitope suppression mediated by regulatory $CD4^+$ T cells [22].

It has been suggested that linked epitope suppression requires co-expression of the tolerogen and the "linked" third-party antigen on the same tissue or antigen-presenting cell [23]. In our study, this prerequisite was achieved when the gene for the wildtype K^b was applied, and, following transplantation of the fully mismatched cardiac graft, the tolerogen and the linked antigens were co-expressed on graft tissue and potential donor-derived passenger leukocytes.

Interestingly, IT injection of the gene encoding the cytosolic form of K^b was also able to induce long-term survival of subsequently transplanted fully mismatched C57BL/10 cardiac allografts. In this situation the prerequisite of co-expression of tolerogen and "linked" third-party antigen was not fulfilled. The cytosolic form of K^b could be recognised only by recipient T cells through the indirect pathway of allorecognition by presentation of K^b-derived peptides in the context of recipient MHC. This raises the question as to how linked epitope suppression can influence the immune response of direct-pathway T cells in this setting. Regulatory T cells, induced through the indirect pathway of allorecognition, might cross-react with donor MHC alloantigens on graft tissue or passenger leukocytes presenting K^b-derived peptides. On the other hand, it is likely that co-expression of tolerogen and "linked" antigen on the same cell is not a strict prerequisite for linked epitope suppression, as has also been shown by Yamada et al. [24].

The question as to how regulation is induced in our system requires further investigation. It might be possible that regulatory T cells are generated directly within the thymus, similarly to the naturally occurring regulatory T cells originating in the thymus and which can also promote transplantation tolerance [25]. On the other hand, K^b expression was detected up to day 28 after IT delivery, in the spleen, although to a lesser extent than in the thymus. However, this might also allow regulation to be induced outside the thymus. The generation of alloantigen-induced regulatory T cells in a thymus-independent process has recently been show in a study by Karim et al., members of our group [26].

In summary, we could show that IT delivery of plasmid encoding a single MHC class I alloantigen under the cover of anti-CD4 antibody was able to induce long-term survival of fully mismatched cardiac allografts. Furthermore, this effect was not dependent on the cell surface expression of the tolerogen. Further experiments will be required to address the mechanisms by which linked epitope suppression can influence the immune response of direct-pathway T cells without co-expression of the linked antigens.

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