Residual methylprednisolone suppresses human T-cell responses to spleen, but not islet, extracts from deceased organ donors

Max Joffe¹, Andra S. Necula¹, Rochna Chand¹, Brett C. McWhinney², Balasubramanian Krishnamurthy¹, Tom Loudovaris¹, David Goodman³, Helen E. Thomas¹,4, Thomas W. H. Kay¹,4 and Stuart I. Mannering¹,4

¹Immunology and Diabetes Unit, St Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia
²Department of Chemical Pathology, Pathology Queensland, Brisbane 4029, Queensland, Australia
³Department of Nephrology, St Vincent's Hospital, Fitzroy, Victoria 3065, Australia and ⁴Department of Medicine, University of Melbourne, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia

Correspondence to: S. I. Mannering, Immunology and Diabetes Unit, St Vincent’s Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia; E-mail: smannering@svi.edu.au

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Abstract

Pancreatic islets, transplanted into recipients with type 1 diabetes, are exposed to allogenic and autoimmune T-cell responses. We set out to develop an assay to measure these responses using PBMC. Our approach was to prepare spleen extract from the islet donors (allo-antigen) and islet extracts (auto-antigen). To our surprise, we found that spleen extracts potently inhibited the proliferation of human T cells driven by antigen (tetanus toxoid) and mitogen (anti-CD3 mAb, OKT3), whereas extracts prepared from pancreatic islets from the same donor did not suppress T-cell proliferation. Suppression mediated by spleen extracts was unaffected by blocking mAbs against the IL-10R, transforming growth factor-β or CD152 (CTLA-4). It was also unaffected by denaturing the spleen extracts by heating, exposing to reducing agents or protease digestion. Because deceased organ donors are commonly given the immunosuppressive glucocorticoid methylprednisolone prior to death, we hypothesized that suppression was due to residual methylprednisolone in the spleen extracts. Methylprednisolone could be detected by mass spectrometry in spleen extracts at concentrations that suppress T-cell proliferation. Finally, the glucocorticoid receptor antagonist mifepristone completely reversed the suppression caused by the spleen extracts. We conclude that extracts of human spleen, but not islets, from deceased organ donors contain sufficient residual methylprednisolone to suppress the proliferation of T-cells in vitro.

Keywords: deceased organ donors, human spleen, methylprednisolone, mifepristone, T-cells

Introduction

Transplantation of pancreatic islets into recipients with T1D exposes the islets to the destructive forces of the immune system on two fronts (1). First, being a tissue from an unrelated donor, the islets may be targeted by an allogeneic immune response (2). This response may be exacerbated by multiple transplants, particularly if the donors share HLA alleles foreign to the recipient. Second, because the recipient’s pancreatic beta cells have been destroyed by an autoimmune T-cell response, they may have a pool of memory T cells primed to respond to beta-cell antigens leading to rapid destruction of the transplanted beta cells (3).

We set out to develop an assay that could measure T-cell responses against allo-antigens and islet auto-antigens using peripheral blood from transplant recipients. Unfortunately, current assays do not reliably measure human T-cell responses to islet antigens (4, 5). One of the problems hampering the development of T-cell assays for islet antigens is our poor knowledge of the antigens recognized by autoreactive T cells in T1D. To circumvent this problem, we decided to use islet extracts as a source of antigen reasoning that an extract of human islets would contain most, if not all, the relevant beta-cell antigens. To this end, we developed a method for extracting tissue antigens in a format that is compatible with in vitro assays of human T-cell function (6). To measure allogenic T-cell responses, we used the same protocol to prepare extracts of human spleen tissue from
the deceased organ donors from whom the islets were isolated. In this way, we aimed to measure T-cell proliferation to islet auto-antigens, and splenic allo-antigens, using a sensitive 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-based proliferation assay (5, 7).

When spleen extracts were tested against PBMC from healthy subjects, we found that the spleen extracts potently inhibited human T-cell proliferation. Here, we show that suppression of T-cell proliferation, measured by 3H-thymidine incorporation or CFSE dilution assays, is caused by residual methylprednisolone given to the organ donors prior to organ retrieval.

Methods

Protein extraction

Spleen and islet proteins were extracted in a mixture of butanol, acetonitrile and water as described previously (6). Spleen tissue samples were obtained from deceased organ donors with informed consent from one of kin (approved by the St Vincent’s Hospital Human Research Ethics Committee, protocol No. HREC-A 011/04). Small pieces of spleen (500–2500 mg) were snap frozen in liquid nitrogen and stored at –80°C. To prepare tissue extracts, samples were homogenized in 3–10 ml of extraction solution and centrifuged at 4000 r.p.m. (3452 × g) for 10 min. The supernatant was collected and the concentration of protein in the supernatant was determined by Bicinchoninic Acid Assay (Fierce; Thermo Fischer Scientific, Rockford, IL, USA). Spleen extracts were dispensed into sterile 5 ml tubes (BD, Franklin Lakes, NJ, USA), lyophilized and stored at –80°C.

Antibodies and inhibitors

The following mAbs were added to the proliferation assays: anti-IL-10R (Clone 3F9; Biolegend, San Diego, CA, USA), anti-CTLA-4 (clone 14D3, eBioscience, San Diego, CA, USA) and anti-transforming growth factor (TGF)-β (Clone 9016.2; Thermo Scientific) were used at the final concentrations indicated in the figures. Isotype-matched control mAbs were included at the same concentrations. The indoleamine 2,3 dioxygenase (IDO) inhibitor, 1-methyl tryptophan, arginine and Indomethacin were all purchased from Sigma–Aldrich (Castle Hill, New South Wales, Australia). Mifepristone (Invitrogen Life Technologies, Carlsbad, CA, USA) was dissolved in DMSO (Sigma–Aldrich) and used at the concentrations indicated in the figures. In the DMSO, only controls DMSO was added to give the same final DMSO concentration as in the indicated assays. In the DMSO, only controls DMSO was added to give the same final DMSO concentration as in the indicated assays. Isotype-matched control mAbs were included at the same concentrations.

Cell culture and proliferation assays

All assays were performed in RPMI 1640, medium (Glutamax, Gibco, Rockville, MA, USA), supplemented with 5% pooled male human serum, 2 mM glutamine (Glutamax, Gibco), penicillin (100 U ml−1), streptomycin (100 mg ml−1) and 100 mM nonessential amino acids (all from Gibco), referred to as RPMI/5% pooled human serum (PHS). Blood was collected from healthy volunteers after informed consent (approved by St Vincent’s Hospital Human Research Ethics Committee, protocol No.135/08, and the Royal Melbourne Hospital Human Research Ethics Committee, protocol number: 2009.026). PBMC were isolated by Ficoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient centrifugation and washed. For 3H-thymidine assays fresh, or thawed PBMC that had been cryopreserved, were cultured with or without anti CD3 (OKT3, 10 ng ml−1) and the spleen or PHS extracts at the indicated concentrations, in 96-well round-bottom plates. Proliferation was measured by 3H-thymidine incorporation during the final 18 h of a 72-hour culture, as described previously (7, 8).

The CFSE proliferation assays were performed as described previously (5, 9). Briefly, PBMC were labeled with 0.1 μM CFSE (Invitrogen, Grand Island, NY, USA) and cultured (1 × 106 cells in 1.0 ml were cultured in sterile 5.0 ml tubes) with or without antigen and OKT3. After 7 days of culture, the cells were washed in PBS and stained on ice with anti-human CD4-AlexaFluor-647 (clone OKT4, conjugated in-house). Optimal compensation and gain settings for the flow cytometer were determined for each experiment based on unstained and single-stained samples. Propidium iodide was used to exclude dead cells. CD4+ T-cell proliferation was measured by determining the number of CD4+, CFSE−dim cells for every 5000 CD4+ CFSEbright cells. The results are presented as a cell division index which is the ratio of the number of CD4+ cells that have proliferated in the presence of antigen: without antigen (7).

Measurement of methylprednisolone in spleen extract

Methylprednisolone was measured in 100 μg of spleen extract using the method of McWhinney et al. (10). Briefly, 100 μl of internal standard (500 nmol l−1 d2-methylprednisolone) was added to each tube containing 100 μg of spleen extract. The tube was dried under nitrogen at 50°C. The samples were reconstituted in 125 μl of 40% methanol, transferred to vials and 20 μl was directly injected onto the Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC/MS/MS) system. The eluant was chromatographed using a Waters ACQUITY BEH C18 column (2.1 × 50 mm) with a water/methanol/ammonium acetate gradient. A Waters Premier XE mass spectrometer (Waters Corp, Milford, MA, USA) set in positive electrospray mode was used to quantify methylprednisolone by monitoring two transitions. Results were processed using MassLynx 4.1 software (Waters Corp). The chromatography time was 3 min per sample. The concentration of methylprednisolone was determined by comparison with a multi-level calibrator (10–2000 nmol l−1).

Statistical analysis

Differences in protein yield were analyzed by two-sided Student’s t test. P < 0.05 was considered to be a significant difference.

Results

Human spleen extracts potently suppressed the anti-CD3 (OKT3) driven proliferation of human PBMC (Fig. 1). As little as 10 ng ml−1 of spleen extract almost completely inhibited T-cell proliferation. In contrast, PHS, prepared in a similar manner, had little effect on T-cell proliferation. Spleen extracts also suppressed proliferation in response to the
Spleen extracts suppress human T-cell responses in vitro. (A) Spleen and pooled human extracts were added to each tube to give the final concentration of protein indicated after reconstitution in culture medium. The following treatments were included: PHS alone (no CD3), solid diamonds (observed); PHS with anti-CD3 (OKT3), open diamonds; spleen extract alone, filled circles; spleen extract with anti-CD3 (open circles); PBMC alone (crosses); PBMC with anti-CD3 only (solid stars). (B) Spleen extract inhibits anti-CD3 mAb and antigen driven T-cell responses. CFSE-labeled PBMC were cultured with up to 100 μg ml⁻¹ of spleen extract and OKT3 (filled circles) or tetanus toxoid (open squares). The number of cells that proliferated, measured by CFSE dilution, was calculated for each spleen extract concentration with anti-CD3 mAb or tetanus toxoid. Results are expressed as the mean percentage suppression of triplicate samples. In the presence of anti-CD3 mAb, an average of 22 286 T cells proliferated per 5000 undivided CD4⁺ T cells. In the presence of tetanus toxoid 10 974 CD4⁺ T cells proliferated, per 5000 undivided CD4⁺ T cells. Spleen extracts are not toxic (C). PBMC were cultured with PHA (5 μg ml⁻¹) for 2 days, washed, CFSE labeled and cultured with spleen extract (filled squares) or PHS ‘extract’ (open squares) at the concentrations indicated in the presence of IL-2 (20 U ml⁻¹). Proliferation was measured by CFSE dilution after a further 3 days of culture. One representative of two independent experiments is shown. In all experiments, each point represents the number of CD4⁺, CFSEbright events per 5000 CD4⁺, CFSEbright events for triplicate. Each point is the mean of triplicate samples and representative of three independent experiments.

To determine if suppression of human T-cell proliferation by spleen extract required IL-10 signaling, TGFβ or CD152 (CTLA-4), blocking mAbs to these molecules, or isotype controls, were added to the cultures. None of these mAbs reversed the suppression of T-cell proliferation (Fig. 2A–C). The IDO inhibitor, 1-methyl tryptophan, did not reverse the spleen extract suppression (Fig. 2D) indicating that IDO-mediated depletion of tryptophan was not inhibiting T-cell responses. Suppression was also unaffected by: the cyclooxygenase inhibitor indomethacin, exogenous arginine (Supplementary Figure 1, available at International Immunology Online) or exogenous tryptophan (Supplementary Figure 2, available at International Immunology Online).

To determine if denatured spleen proteins could suppress T-cell responses, the extracts were denatured by: heating, treatment with the reducing agent dithiothreitol or reconstituting in 8 M urea. None of these treatments had any effect on the response to spleen extract (Fig. 3A–C). To determine if the effect depended upon intact spleen proteins, we digested the spleen extracts with proteinase K. Following this treatment, the spleen extracts maintained their capacity to suppress human T-cell responses driven by OKT3, whereas pooled human serum treated in the same manner had no impact upon the OKT3-driven proliferation (Fig. 3C). We concluded that the suppression of T-cell proliferation was not dependent upon protein(s) in the spleen extract.

In light of these data, we hypothesized that the suppressive effect we observed may be due to residual methylprednisolone in the spleen extracts. This is possible because deceased organ donors are commonly given the immunosuppressive glucocorticoid, methylprednisolone, prior to death and the collection of their organs to prevent death associated inflammation (11–13). To address this, we compared suppression by spleen extracts from four deceased organ donors who received methylprednisolone to suppression mediated by spleen extracts from two donors who had not received methylprednisolone. All spleen extracts from the methylprednisolone treated donors suppressed OKT3-driven T-cell proliferation (Fig. 4A). In contrast, spleen extracts from two deceased organ donors, who did not receive methylprednisolone prior to organ collection, did not suppress T-cell proliferation (Fig. 4A).

Next, we sought to determine how much methylprednisolone was required to suppress human anti-CD3 driven human T-cell proliferation in vitro. Titration experiments revealed that the IC50 of methylprednisolone in these assays was ~65 ng ml⁻¹ (Fig. 4B). Analysis of spleen samples from five organ donors who had received methylprednisolone revealed that they contained from 23 to 90 ng per 100 μg of spleen extract. No methylprednisolone was detected in the spleen lysate from an organ donor who did not receive methylprednisolone. The level
of suppression correlated with the concentration of methylprednisolone detected in each extract (Fig. 4C).

To confirm the role of methylprednisolone, we added the glucocorticoid receptor antagonist, mifepristone, to cultures containing spleen extracts from organ donors who had received methylprednisolone. Mifepristone caused a dose-dependent reversal of the spleen-extract-mediated suppression of T-cell proliferation (Fig. 5). This confirmed that the residual methylprednisolone mediates the suppression of T-cell responses observed in our cultures. In contrast, an extract prepared from pancreatic islets did not suppress the proliferation of human T cells in vitro, while the same concentration of spleen extract, from the same donor, markedly suppressed proliferation in a mifepristone dependent manner (Fig. 5B). This is consistent with our earlier observation that pancreatic islet extracts prepared using our protocol can stimulate human insulin-specific CD4+ T-cell clones (6, 8, 14).

Discussion
Here, we show that sufficient methylprednisolone accumulates in the spleen of organ donors to suppress T-cell responses in vitro. Our conclusion, that methylprednisolone mediates the suppression, comes from several lines of evidence. First, we showed that the spleen extracts inhibited both antigen- and mitogen-driven proliferation but were not toxic to the cells. Second, we showed that the suppression was not immunological or mediated by amino acid deficiency. Third, we showed that intact protein was not required, implicating a small molecule. Fourth, extracts from deceased organ donors who were not administered methylprednisolone did not suppress T-cell proliferation. Fifth, we show that methylprednisolone is found at concentrations that suppress human T-cell proliferation in spleen extracts from organ donors who were treated with methylprednisolone. Finally, suppression of T-cell proliferation was reversed when mifepristone was added to the cultures.

Our results raise the question: does residual methylprednisolone enhance the acceptance of grafts, at least in the short term, after transplantation? Methylprednisolone is administered to organ donors before their death and the collection of their organs (13, 15, 16). This treatment prevents organ damage caused by inflammatory responses that occur when a patient dies improving the quality of the...
recovered organs (11). It is not known if methylprednisolone has any beneficial effect after transplantation. We found that extracts of spleen tissue, but not islets, suppressed antigen- and mitogen-driven T-cell proliferation. These observations suggest that more methylprednisolone accumulates in the spleen than the islets. We have not tested other tissues so it remains to be determined whether other tissues accumulate methylprednisolone at similar concentrations to the spleen. Traces of methylprednisolone may help to prevent graft rejection immediately after transplantation, but based on our data, it seems unlikely that there would be sufficient methylprednisolone in the islets to confer any clinical benefit to the islet transplant recipient.

The novel finding from this work is that methylprednisolone accumulates in the spleen of deceased organ donors who received it prior to death. This observation has important implications for the use of material isolated from human spleen. In our case, attempts to use human spleen extracts as a source of antigen were thwarted by the presence of residual methylprednisolone. The analysis of immune cells derived from human spleen may also be affected by methylprednisolone. Indeed, the phenotype of dendritic cell isolated from the spleens of deceased organ donors has been shown to be affected by methylprednisolone treatment (17). We found no evidence that methylprednisolone accumulates in the pancreatic islets at concentrations high enough to suppress human T-cell proliferation in vitro (≥10 ng per 100 mg of extract). Nonetheless, it remains to be determined if methylprednisolone is present in other tissues at concentrations similar to those found in the spleen (~20–100 ng per 100 mg of extract). Based on our results, analysis of cells or extracts from methylprednisolone-treated donors should take into account the possibility that methylprednisolone may affect the results.

Our original goal of measuring allo- and auto-immune responses using spleen and islet extracts as allo- and auto-antigens respectively needs to be modified to account for the presence of methylprednisolone in the tissue extracts. Most deceased organ donors receive methylprednisolone prior to organ collection so simply using tissue that has not been exposed to methylprednisolone is not feasible. Blocking the effects of methylprednisolone is the alternative approach. Because we found that addition of mifepristone completely reversed the inhibitory effect of spleen lysates on human T-cell proliferation, mifepristone could be added to the cultures to counteract the effects of methylprednisolone. Indeed, the phenotype of dendritic cell isolated from human spleen may also be affected by methylprednisolone. The analysis of immune cells derived from human spleen has been shown to be affected by methylprednisolone treatment (17).

Our organic solvent-based extraction protocol may have enriched methylprednisolone in the tissue extracts. This protocol was chosen because it gives the greatest yield of proteins of the solvent mixtures tested (6). Detergents were avoided because of the technical problems associated with removing these chemicals from the tissue extract, whereas organic solvents used in our extraction protocol can be removed by lyophilization.
In conclusion, we show that methylprednisolone accumulates in the spleens of deceased organ donors to concentrations that inhibit human T-cell proliferation. The presence of immunosuppressive drugs in tissue extracts should be considered in studies using deceased organ-donor-derived extracts as antigens in assays of immune function.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

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