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Donor IL-4-treatment induces alternatively activated liver macrophages and IDO-expressing NK cells and promotes rat liver allograft acceptance

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ABSTRACT

Most approaches to transplant tolerance involve treatment of the recipient to prevent rejection. This study investigates donor treatment with IL-4 for its effect on subsequent rat liver allograft survival. Rat orthotopic liver transplants were performed in rejecting (PVG donor to Lewis recipient) or spontaneously tolerant (PVG to DA) strain combinations. Donors were untreated or injected intraperitoneally with IL-4 (30,000 U/day) for 5 days. Tissue infiltrates and gene expression were examined by immunohistochemistry and real-time quantitative PCR. IL-4 induced a marked leukocyte infiltrate in donor livers prior to transplant. Macrophages comprised the major population, although B cells, T cells and natural killer (NK) cells also increased. IL-4-induced liver macrophages had an alternatively activated phenotype with increased expression of mannose receptor but not inducible nitric oxide synthase (NOS2). IL-4 also induced IDO and IFN-gamma expression by NK cells. Donor IL-4-treatment converted rejection to acceptance in the majority of Lewis recipients (median survival time >96 days) and did not prevent acceptance in DA recipients. Acceptance in Lewis recipients was associated with increased donor cell migration to recipient spleens and increased splenic IL-2, IFN-gamma and IDO expression 24 h after transplantation. Donor IL-4-treatment increased leukocytes in the donor liver including potentially immunosuppressive populations of alternatively activated macrophages and IDO-expressing NK cells. Donor treatment led to long-term acceptance of most livers in association with early immune activation in recipient lymphoid tissues.

1. Introduction

IL-4 plays a central role in humoral and cell-mediated immunity where it promotes B cell activation and immunoglobulin class-switching, as well as T cell survival and differentiation (reviewed in [1]). In addition, it exerts a range of effects on other immune cells including alternative activation of macrophages (reviewed in [2]), dendritic cell modulation [3] and natural killer (NK) cell activation [4]. Non-haematopoietic cells including endothelial cells and hepatocytes are also responsive to IL-4 signalling [5]. The role of IL-4 in organ transplantation can vary significantly. Depending on the experimental model, timing and nature of IL-4 administration, IL-4 can either play a protective role, or enhance graft rejection (reviewed in [6]). In neonatal rodents, IL-4 alone or in combination with IL-13, is required for transplant tolerance [7,8], although it is not critical for transplant tolerance in adults [9]. It has long been held that IL-4 is associated with graft protection, as a number of tolerance-inducing therapies result in sustained expression of Th2 cytokines, including IL-4, and reduced levels of Th1 cytokines (reviewed in [10]). In support, systemic IL-4-treatment prolongs neonatal cardiac graft survival in adult rats [11]. However, intragraft overexpression of IL-4 alone is insufficient to induce long-term graft acceptance [12]. Instead, there is now evidence that IL-4 and Th2-type responses in some circumstances can promote rejection [13–15].

We have been investigating the role of IL-4 in rat liver transplantation. Livers transplanted across some rat strain combinations, for example, from PVG strain donors to DA strain recipients (PVG to DA) are spontaneously accepted without immunosuppression [16], a phenomenon which is also described in all mouse strains, outbred pigs and some primates. Spontaneous liver transplant tolerance may be due to release of soluble MHC class I from the liver or the unique antigen-presenting functions of liver sinusoidal endothelia (reviewed in [17]). Previous studies have also implicated liver passenger leukocytes [18,19] in tolerance induction, which was paradoxically

Abbreviations: CHO, Chinese hamster ovary; γc, cytokine receptor common γ chain; CTLA4, cytotoxic T-lymphocyte antigen 4; Foxp3, Forkhead-box p3; HO-1, heme oxygenase-1; IDO, indoleamine dioxygenase; NOS2, inducible nitric oxide synthase; NK, natural killer; ManR, macrophage mannose receptor; MST, median survival time; PVG, Piebald Virol Glaxo; TNF, tumour necrosis factor.

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associated with early abortive immune activation in the recipients, demonstrated by an early increase in IL-2 and IFN-γ production [20,21].

2. Objectives

In a previous study, we showed that IL-4 administered to DA recipients of PVG livers shortly after transplantation converted spontaneous liver allograft tolerance to rejection [15]. The mechanism of conversion was associated with increased infiltration of macrophages into the liver. As IL-4 can induce liver pathology via both leukocyte-dependent [22] and independent mechanisms [23], the approach of the current study was to first examine the direct effects of IL-4-treatment on the liver of untransplanted animals. IL-4 administration induced a marked leukocyte infiltrate, with a major proportion consisting of macrophages. IL-4-treatment also induced expression of indoleamine dioxygenase (IDO) and markers of alternative macrophage activation. As these can play a role in transplant acceptance (reviewed in [24]), we examined the effect of IL-4-treatment of the donor in liver allograft rejection and tolerance.

3. Materials and methods

3.1. Animals

Male inbred PVG ($RT1^c$), Lewis ($RT1^l$) and DA ($RT1^a$) strain rats weighing 200 g–300 g (Animal Resources Centre, Perth, Australia) were used. These strains are completely mismatched at the MHC and minor histocompatibility loci. Experiments were performed with the approval of the Sydney South West Area Health Service Animal Welfare Committee.

3.2. Donor treatment

Production of recombinant rat IL-4 in transfected Chinese Hamster Ovary (CHO) cells and measurement of IL-4 bioactivity by MHC class II upregulation on B cells has been described [25]. PVG donors were treated with five daily intraperitoneal injections of recombinant rat IL-4 (30,000 U/day) in 0.75 ml of CHO supernatant. This is the dosage schedule that has been used previously to obtain a biological effect in rats [15]. Control PVG rats were untreated or treated with five daily intraperitoneal injections of 0.75 ml of supernatant from untransfected CHO cells.

3.3. Orthotopic liver transplantation and tissue harvest

Orthotopic liver transplantation was performed according to the method of Kamada and Calne [26]. Donor livers from untreated or IL-4-treated PVG strain rats were transplanted into Lewis or DA strain recipients. In some experiments, recipients of IL-4-treated livers were implanted subcutaneously at the time of transplantation with 1 g of 1-methyl tryptophan 7-day slow release pellets (4×250 mg pellets/rat) (Innovative Research of America, Sarasota, FL) or with four placebo pellets from the same suppliers. In separate experiments, the effect of 1-methyl tryptophan pellets and placebo pellets on Lewis recipients of syngeneic liver grafts was examined. For survival experiments, recipients were euthanased when they showed signs of morbidity combined with >15% weight loss. For tissue analyses, recipients were sacrificed on days 1, 5, 7 and 10 post-transplantation. Liver and spleen specimens were either frozen in liquid nitrogen or embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) and stored at −70 °C.

3.4. Immunohistochemical analysis

Frozen sections of 6 μm thickness were cut onto Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany) and fixed in acetone before indirect immunoperoxidase staining [27]. Primary antibodies used were to rat TCRαβ (R73, BD Pharmingen, San Diego, CA), IgG1 (RG11/39.4, Pharmingen), IgD (MARD-3, AbD Serotec, Oxford, UK), IgM (MARM-4, Serotec), IgE (G53-238, Pharmingen) and PVG strain-specific MHC class I (MRC OX27, Serotec). Tissue culture supernatants from hybridomas producing monoclonal antibodies to rat MHC class II (OX6) and MHC class I (OX18), CD45RC (OX22), CD11b/c (OX42), a kind gift from Dr. J. Sedgwick (Eli Lilly, Indianapolis, IN) were also used. The isotype control used was MOPC-21 (Sigma-Aldrich, Sydney, Australia). Primary antibody binding was detected with horseradish peroxidase-conjugated rabbit–anti-mouse IgG secondary antibody (Dako, Copenhagen, Denmark) and colour developed with diaminobenzidine (Dako). Sections were counterstained with Mayer's haematoxylin (Sigma). Three to six digital images covering 2.4 mm^2 were obtained for each section at 100× magnification (Provis AX-70 microscope with StudioLite software, Olympus, Tokyo, Japan). The brown diaminobenzidine-stained cells were counted using image analysis software (AxioVision 4.4, Carl Zeiss, Oberkochen, Germany).

3.5. Preparation of leukocyte subsets from liver or spleen

Liver or spleen tissue was dissociated by mashing through a 100 μm-mesh steel sieve. Liver leukocytes were purified by centrifugation of the washed cell suspension on 1.05 g/ml Percoll (Pharmacia Biotech, Uppsala, Sweden) for 15 min at 840×g. The leukocyte-containing fraction was washed and red blood cells lysed with NH_4Cl buffer as described [28]. Cells were stained with fluorescein isothiocyanate-conjugated anti-IgM (MARM-4-FITC, Serotec), phycoerythrin-conjugated anti-CD11b/c (OX42-PE, Pharmingen), biotinylated anti-TCRαβ (R73-bio, Serotec) plus streptavidin–allophycocyanin (Invitrogen, Carlsbad, CA) or a mouse monoclonal antibody against the rat NK cell marker NKR.P1 (10.78, Pharmingen) plus FITC-conjugated anti-mouse IgG (F2266, Sigma). Populations of interest were obtained by fluorescence-activated cell sorting using the FACS Vantage cell sorter (Becton Dickinson, NSW, Australia), excluding dead cells identified by propidium iodide (1 μg/ml) staining.

3.6. Quantification of mRNA expression by real-time quantitative PCR

Gene expression in liver and spleen tissue or in purified leukocytes was measured as previously described [29]. Briefly, total RNA was isolated (RNAqueous, Ambion, Applied Biosystems, Austin, TX) and 1 μg of RNA reverse-transcribed into cDNA with Superscript III (Invitrogen). cDNA was amplified in SensiMix dT (Quantace, London, UK) with primers and fluorogenic-probes designed using Primer Express (Applied Biosystems). Real-time quantification was performed using a Model 7700 Sequence detector (Applied Biosystems) or Rotor-Gene™ 6000 (Corbett Life Science, Sydney, Australia). The levels of mRNA expression were calculated from standard curves prepared from 4-fold dilution series of samples with high expression for each gene. Expression was normalised to expression of the constitutively-expressed gene rat histone H3 according to the formula: expression of gene of interest/expression of histone H3×1000. Primers and probe sequences for IL-2, IL-4, IL-10, Foxp3, IDO, IFN-γ, NOS2 and TNF-α have been published [29,30]. Other probes and primers used were: rat histone H3 (probe) 5′ 6FAM-CTC ACT TGC CTC CTG CAA AGC ACC AA-BHQ 3′; (forward) 5′ CAG ACC TGC GCT TCC AGA GT 3′; (reverse) 5′ TTC AAA AAG GCC AAC CAG ACA 3′. Macrophage mannose receptor (ManR) (probe) 5′ 6FAM-AGC ACC TGT GAC AGT AAA CAA GGC TAT ATA TGC CA-BHQ 3′; (forward) 5′ ACG AAG ATG TTG ACT GTG TTG TTG T 3′; (reverse) 5′ GGT TGG AGA GAT AGG CAC AGA AG 3′. Common gamma chain (γc) (probe) 5′ 6FAM-ATT TGT TCC GTC CAG CTT CGA TCT CG 3′; (forward) 5′ TGG TGC AGT ACC GGA GCA A