Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12931

Constitutive expression of the anti-apoptotic Bcl-2 family member A1 in murine endothelial cells leads to transplant tolerance

L. A. Smyth,*† L. Meader,* F. Xiao,* M. Woodward,‡ H. J. M. Brady,§ R. Lechler* and G. Lombardi*
*Medical Research Council (MRC) Centre for Transplantation, King's College London, London, UK, National Institute for Health Research (NIHR) Comprehensive Biomedical Research Centre, Guy's and St Thomas' NHS Foundation Trust and King's College London, London, UK, †School of Health, Sports and Biosciences, University of East London, London, UK, ‡Peter Gorer Department of Immunobiology, Borough Wing, Guy's Hospital, King's College, London, UK, and §Immunology and Infection Section, Division of Cell and Molecular Biology, Sir Alexander Fleming Building, Imperial College, London, UK

Accepted for publication 6 January 2017
Correspondence: L. A. Smyth, School of Health, Sport and Bioscience, University of East London, Stratford Campus, Water Lane, London E15 4LZ, UK.
E-mail: L.Smyth@uel.ac.uk

Summary

Anti-apoptotic genes, including those of the Bcl-2 family, have been shown to have dual functionality inasmuch as they inhibit cell death but also regulate inflammation. Several anti-apoptotic molecules have been associated with endothelial cell (EC) survival following transplantation; however, their exact role has yet to be elucidated in respect to controlling inflammation. In this study we created mice expressing murine A1 (Bfl-1), a Bcl-2 family member, under the control of the human intercellular adhesion molecule 2 (ICAM-2) promoter. Constitutive expression of A1 in murine vascular ECs conferred protection from cell death induced by the proinflammatory cytokine tumour necrosis factor (TNF)-α. Importantly, in a mouse model of heart allograft transplantation, expression of A1 in vascular endothelium increased survival in the absence of $CD8^+$ T cells. Better graft outcome in mice receiving an A1 transgenic heart correlated with a reduced immune infiltration, which may be related to increased EC survival and reduced expression of adhesion molecules on ECs. In conclusion, constitutive expression of the anti-apoptotic molecule Bfl1 (A1) in murine vascular ECs leads to prolonged allograft survival due to modifying inflammation.

Keywords: anti-apoptotic, endothelial

Introduction

Endothelial cells (ECs) lining blood vessels function at the interface between the bloodstream and tissue. EC activation and death are believed to be key initiating events in the development of transplant vascular disease [1]. This idea is supported by the strong correlation between graft survival and expression of protective genes in vascular ECs. In animal models, where long-term allograft survival was established, expression of anti-apoptotic molecules A20, haem oxygenase-1 (HO-1), Bcl-xL and Bcl-2 were observed in graft ECs [2–5]. Some of these molecules have a dual cytoprotective role, inasmuch as they inhibit cell death but also act as anti-inflammatory mediators. For example, A20 has been shown to inhibit nuclear factor kappa B (NF-κB) activation to a number of stimuli, including oxidative stress and inflammatory cytokines [6,7]. Recently, overexpression of A20 in the vasculature, both ECs and smooth muscle cells (SMCs), using recombinant A20 adenovirus, reduced transplant arteriosclerosis in a mouse aorta to a carotid artery allograft model [8]. Protection correlated with decreased graft inflammation, with the infiltrating cells being predominantly regulatory T cells (T_{regs}), suggesting that maintaining the endothelium in a damage-free state may help to tip the balance towards transplant tolerance [8].

A1, known as Bfl-1 in the human setting [9], is another anti-apoptotic molecule with dual cytoprotective abilities [9–14]. Unlike Bcl-2 and Bcl-xL, the expression of A1 is induced rapidly in ECs following stimuli from proinflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)−1β [15]. NF-κB activation is required for the induction of A1 in ECs [10,16]. Expression of A1, following retrovirus transduction, protected both human dermal microvascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVEC) against TNF-α−induced apoptosis [15,17,18]. Induced expression of A1 not only inhibited ECs death but also their activation

(reduction of E-selectin levels and IL-8 production), making this molecule a potential regulator of inflammation [10]. A1 may regulate inflammation in ECs by inhibiting NF-κB activation, given that expression of full-length A1 in bovine aortic ECs (BAEC) inhibited TNF-α-mediated I kappa Bα (IKBα) degradation and subsequently NF-κB activation [10,16].

Due to the anti-apoptotic and anti-inflammatory nature of A1, we investigated the role of this molecule in graft outcome when expressed constitutively in the vascular endothelium. To achieve this, we generated mice expressing the mouse A1 gene under the control of the human intercellular adhesion molecule 2 (ICAM-2) promoter [19] and assessed the role of this molecule in a vascular transplant setting. We observed that expression of A1 in ECs conferred protection in a murine heart transplant setting due to maintaining ECs in a non-inflammatory state [20,21].

Materials and methods

Animal studies were carried out in accordance with UK Research Councils and Medical Research Charities guidelines on responsibility in the use of animals in Bioscience Research, under a UK Home Office Licence (PPL70/7302). The anaesthesia used was isoflurane, euthanasia via CO_2 inhalation.

ICAM-2/A1 transgenic mice

The human ICAM-2 containing pBluescript II SK+ plasmid was kindly provided by Dr Peter Cowan (St Vincent's Hospital, Sydney, Australia), and the murine A1 cDNA containing pBluesK plasmid was kindly provided by Professor Michael B. Prystowskyt (Albert Einstein College of Medicine, New York, NY, USA). Murine A1 was excised from the plasmid using *Hin*dIII and *Bam*H1 restriction digestion before being ligated into the *Eco*R1 cloning site of pBluescript II SK+ plasmid, upstream of the ICAM-2 promoter and the universal intron. A 1·6 kb microinjection construct containing both the ICAM-2 promoter and A1 gene was excised from the vector by Xho1 and Not1 enzyme restriction digestion. The injection fragment (1·6 Kb) was then separated by agarose gel electrophoresis, followed by purification through sequential Wizard mini columns (Promega, Madison, WI, USA) and CHROMA SPIN 400 (Clontech, Palo Alto, CA, USA). The purified fragment was microinjected at a concentration of 10 μg/ml in phosphate-buffered saline into fertilized CBA × C57BL6 mouse oocytes (Karolinska Institute, Stockholm, Sweden). Founder progeny were screened for transgene integrity by Southern blot analysis using the ^{32}P-labelled 1600 base pairs (bp) injection fragment as a probe. In addition, mice were screened using polymerase chain reaction (PCR) using ICAM-A1 forward primer (5′-TTTGCAGAAGGCTAGGGAT-3′) and reverse primer (5′-TTCAACTTCCTTCTGAACGG-3′). The resulting 356 bp product was resolved by electrophoresis in 1% agarose. Transgenic founders were bred onto a C57BL/6 (B6) background for more than 10 generations. Mice were kept under sterile conditions.

EC isolation and cell culture

Murine ECs were purified from murine heart tissue using a method described previously [22]. In brief, murine hearts were digested with collagenase (from *Clostridium hostolyticum* Type IV; Sigma Aldrich, St Louis, MO, USA) and trypsin (Invitrogen, Paisley, UK) to obtain a single-cell suspension. ECs were isolated following incubation with rat anti-CD31, anti-CD105 (BD Biosciences, San Jose, CA, USA) and biotinylated isolectin B4 (Vector Laboratories, Peterborough, UK)-specific antibodies and anti-rat immunoglobulin (Ig) and streptavidin-coupled bead selection, using MS columns (Miltenyi Biotech, Bergisch Gladbach, Germany). ECs were then cultured for 7–10 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS) (GE Healthcare, Cardiff, UK), 2 mM glutamine and penicillin/streptomycin, 50 mM 2-mercaptoethanol, 1% non-essential amino acids, 1 mM sodium pyruvate, 20 mM Hepes (Invitrogen) and EC growth factors (Sigma Aldrich) at 37°C/5% CO_2. Once the cells had reached 90% confluence they were harvested using trypsin and replated. For functional assays, ECs were used between passages 3 and 4. ECs at 90% confluence were used for all experiments. To check that the cultured cells were indeed ECs, cells were harvested using Accutase (Invitrogen) and stained with anti-CD105-phycoerythrin (PE)-labelled antibodies before being analysed by flow cytometry (BD FACSCalibur; BD Biosciences). Subsequent analysis was accomplished with FlowJo software (TreeStar, Inc., Ashland, OR, USA). To test A1 mRNA expression in the cultured ECs, cells were lysed using Trizol and RNA extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA). A one-step reverse transcription–polymerase chain reaction (RT–PCR) kit (Promega) was used with the following primers 5′-AACTTCCACAAGAGCAGATTGCC-3′ and 5′-TCAGCCAGCCAGATTTGGGTTC-3′ to amplify A1 mRNA.

MTT [3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide] cell survival assay

ECs, $2{\cdot}5 \times 10^4$, were added to each well of a 96-well plate in complete media lacking Phenol red (Invitrogen). Various concentrations of TNF-α (First Link UK Ltd, Brierley Hill, UK) were added with or without 1 μg/ml of actinomycin D (Act D; Sigma Aldrich). Control cells were incubated with Act D alone. An MTT assay was performed after 18 h following the manufacturer's instructions (Invitrogen).

Heart transplantation

Intra-abdominal heterotopic heart transplantation (either ICAM-2/A1 or non-transgenic grafts) was performed in

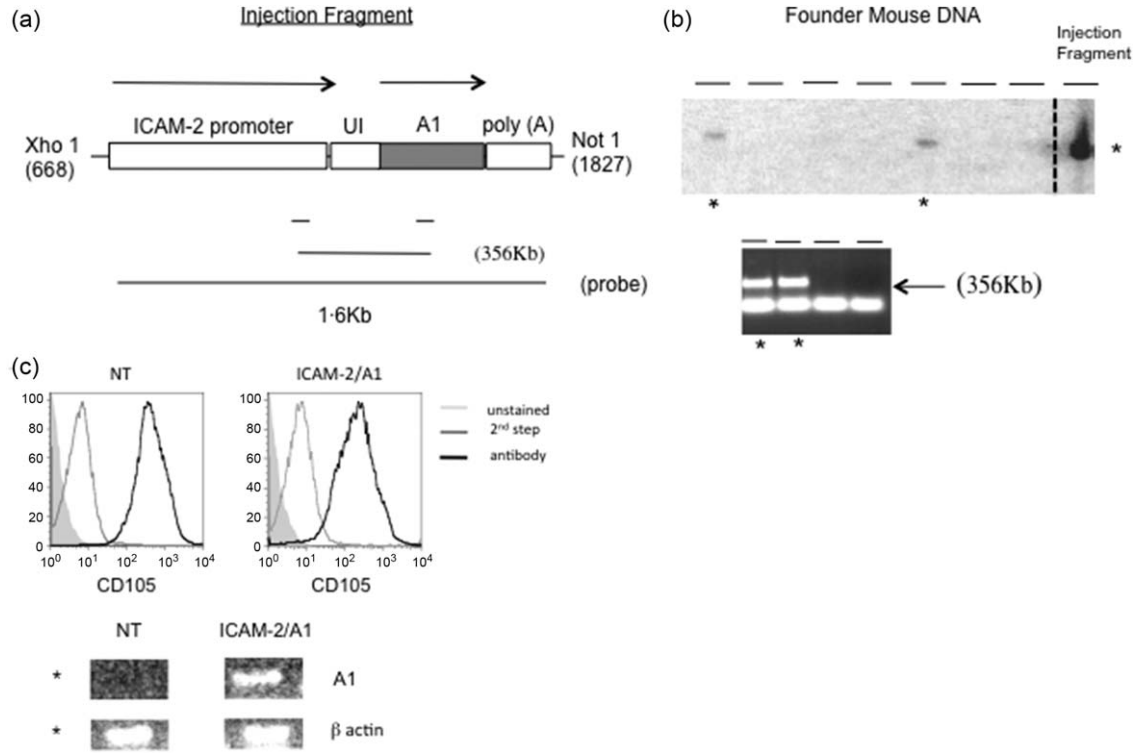

Fig. 1. Constitutive expression of A1 in endothelial cells (ECs) of ICAM-2/A1 mice. (a) Map of the 1.6 Kb Xho1–Not1 microinjection construct, showing the intercellular adhesion molecule 2 (ICAM-2) promoter, universal intron (UI), A1 cDNA and simian virus 40 (SV40) early polyadenylation signal sequence [poly(A)]. The location of probe used for Southern blot analysis is shown, as well as the location of primers used for polymerase chain reaction (PCR). Founder transgenic mice, two shown, are indicated by (*), were identified by Southern blotting using a ^{32}P-labelled injection fragment as a probe (b, top panel) and by PCR using specific primers (b, lower panel). ECs were isolated from the hearts of the ICAM-2/A1 and non-transgenic (NT) littermates and grown *in vitro* for 7 days before the expression levels of CD105 were assessed using specific conjugated antibodies and flow cytometry (c, upper panels). Histograms representing the expression of CD105 in ECs are shown compared to unstained or secondary antibody-stained ECs. Expression of A1 at the mRNA level was assessed using reverse transcription–polymerase chain reaction (RT–PCR) using two A1-specific primers and a control β actin primer (c, lower panels).

CBA mice (Harlan Laboratories, Indianapolis, IN, USA), as described previously [23]. Heart allograft survival was assessed by direct abdominal palpation, where rejection was defined by complete cessation of cardiac impulses. Some mice were treated with 250 μg of anti-CD8 antibody, 1 day prior to and after transplantation, via intraperitoneal injection. Hearts were isolated on day 100, sectioned and stained with haematoxylin and eosin (H&E).

Measuring expression of adhesion molecules

ECs, $2{\cdot}5 \times 10^4$, were grown to 90% confluence in six-well plates at 37°C/5% CO_2; 10 ng/ml of TNF-α was added for 24 h. Cells were removed from the plate using Accutase before being incubated with Fc block (anti-CD16/32 antibody; Affymetrix eBioscience, Hatfield, UK) for 30 min at 4°C. Cells were then stained with anti-ICAM-1 or anti-vascular cell adhesion protein 1 (VCAM-1) fluorescein isothiocyanate (FITC) antibodies or isotype controls (Affymetrix eBioscience) before being analysed by flow cytometry and FlowJo software.

Statistics

Statistical comparisons for experiments assessing *in-vitro* proliferation were performed using unpaired two-tailed Student's *t*-tests [24]. Mean survival time of heart allografts was assessed by log-rank test. Statistical significance is expressed as (*) and is defined as *P*-values lower than 0·05.

Results

Constitutive expression of A1 in ECs prolongs the survival of transplanted hearts

As both anti-apoptotic molecules Bcl-2 and Bcl-xL have been implicated in protecting graft ECs during transplantation, we addressed whether the expression of A1 in ECs also has this capacity. To this end, we generated mice

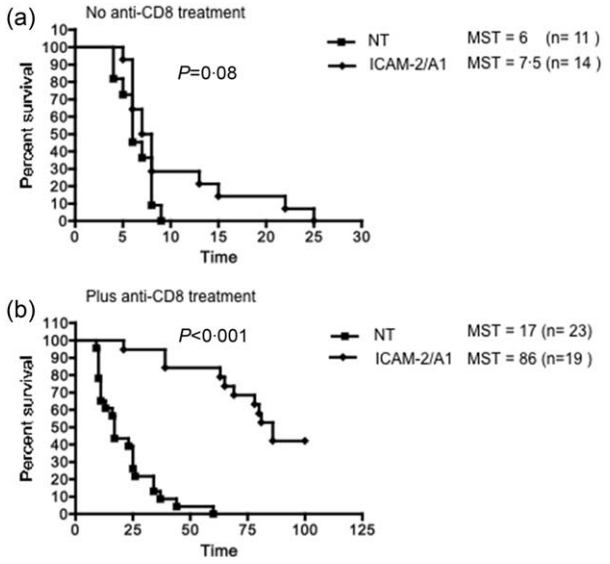

Fig. 2. Expression of A1 in murine endothelial cells (ECs) prolongs graft survival. CBA mice received hearts from either intercellular adhesion molecule 2 (ICAM-2)/A1 or non-transgenic (NT) littermates in the absence (a) or presence (b) of anti-CD8 antibody given 1 day before and after transplantation (250 μg/mouse). Results plotted show the mean survival time (MST) of allografts and n = number of animals. *P*-values are shown.

expressing murine A1 in ECs (ICAM-2/A1 mice, Fig. 1a, b). We used the human ICAM-2 promoter to target A1 to ECs. This promoter has been shown previously to target human CD59 expression to the endothelium of all tissues [25,26] due to the high expression of ICAM-2 on all vascular ECs, including those of the heart [27]. Indeed ECs, identified by their expression of CD105 [22] (Fig. 1c, top panels), isolated and expanded from the hearts of ICAM-2/A1 mice, expressed A1 at the mRNA level (Fig. 1c, lower panels). In comparison, ECs isolated from non-transgenic mice did not, therefore confirming the expression of the A1 gene in ECs isolated from these transgenic mice.

To assess whether constitutive A1 protected the endothelium *in vivo* from immune-mediated damage elicited during transplantation, we transplanted hearts derived from ICAM-2/A1 mice (on a B6 background) or non-transgenic littermates into CBA/Ca recipients. We observed rapid rejection of donor hearts in recipient mice regardless of the expression of the A1 gene (mean survival time was days 6 and 7·5 for non-transgenic and ICAM-2/A1 transgenic donor hearts, respectively, $P = 0{\cdot}08$, Fig. 2a). We have shown previously that treatment of recipient mice with anti-CD8 antibody, to remove the contribution of $CD8^+$ T cells to graft rejection, allowed prolongation of heart transplants in a complete mismatched transplant model [23]. In conjunction with anti-CD8 therapy treatment, given 1 day prior to and after heart transplantation, constitutive expression of A1 on ECs led to significant heart transplant survival compared to control hearts (mean survival time was 86 *versus* 17 days for ICAM-2/A1 hearts *versus* non-transgenic littermates, $P < 0{\cdot}001$ and Fig. 2b). Overall, these observations suggest that expression of this anti-apoptotic gene did not protect ECs against direct CD8-mediated killing; however, when anti-CD8 antibody was used, A1 expression by the graft improved transplant outcome significantly.

Murine vascular ECs expressing A1 are less susceptible to cytokine- mediated activation

Given the transplant survival data, it was important to understand how constitutive expression of A1 contributes towards graft protection. The proinflammatory cytokine TNF-α has been shown to be an important mediator of allograft rejection [28], with prolongation of allograft hearts survival being reported in recipient mice lacking the TNF receptor [29]. As over-expression of A1, using retrovirus transduction, protected human ECs against TNF-α-induced apoptosis [15], we tested whether or not constitutive expression of A1 protected murine ECs from TNF-α-induced cell death. ECs isolated from the hearts of ICAM-2/A1 transgenic mice were treated with different concentrations of TNF-α (0·01–100 ng/ml) in the presence of Act D (1 μg/ml) for 24 h. Cell viability was measured using an MTT assay. No significant EC death was evident in the presence of Act D (data not shown), and ECs treated with Act D only were deemed to be 100% viable. We observed that after 24 h of treatment with either 0·01 or 0·1 ng/ml of TNF-α, in combination with Act D, ECs isolated from ICAM-2/A1 transgenic mice were less susceptible to cytokine-induced cell death than ECs from non-transgenic mice (*P*-values were 0·003 and 0·023, respectively). However, treatment with higher doses, 1, 10 and 100 ng/ml TNF-α plus Act D, induced similar levels of cell death in ECs isolated from either ICAM-2/A1 transgenic or non-transgenic mice (Fig. 3a), suggesting that constitutive A1 expression in heart ECs provides protection from low levels of proinflammatory cytokines.

A1 expression may modulate immune recruitment

In addition, A1 has been described as a potential anti-inflammatory molecule [10]. Therefore, it is feasible that constitutive expression of this molecule in the murine endothelium protects against both cell death and proinflammatory cytokine-mediated activation by inhibiting/reducing expression of key inflammatory adhesion molecules required to recruit lymphocytes. In rat allografts, ICAM-1 and VCAM-1 expression are increased significantly on capillary endothelia, while only VCAM-1 expression was observed on arteries and arterioles during acute transplantation rejection [24]. As expression of both ICAM-1 and VCAM-1 on ECs are increased by proinflammatory cytokines such as TNF-α [30–33], we treated ECs isolated from the hearts of ICAM-2/A1 transgenic or non-transgenic littermates with TNF-α (10 ng/ml), and expression of the aforementioned molecules was assessed via flow

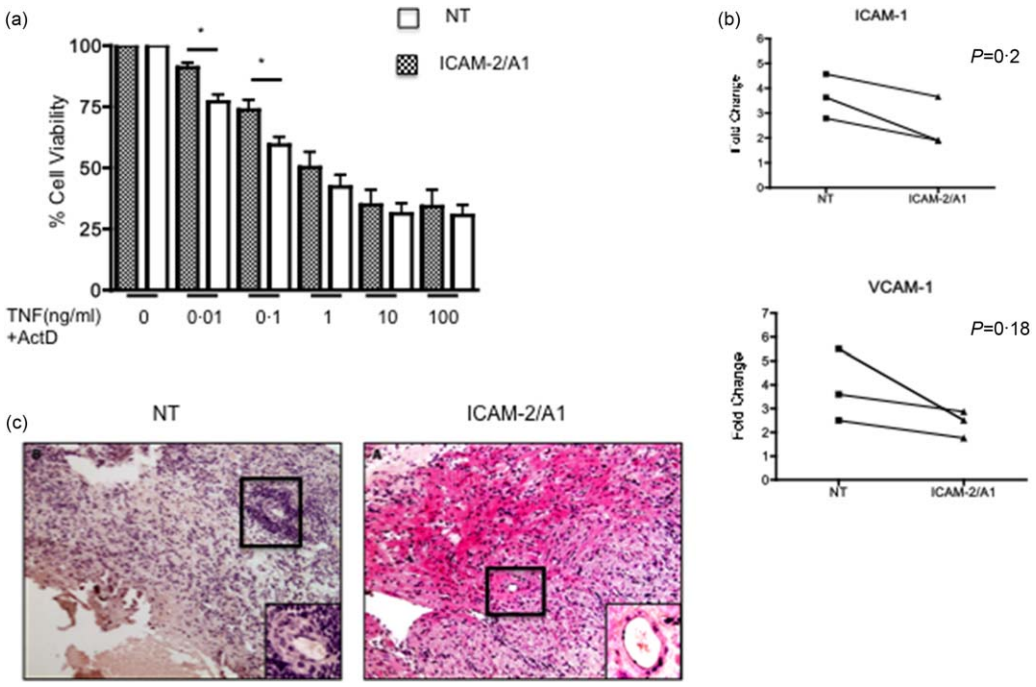

Fig. 3. Reduced vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-2) expression in endothelial cells (ECs) expressing A1 may reduce leucocyte infiltration. ECs isolated from ICAM-2/A1 (filled bars) and non-transgenic (open bars) littermates were stimulated with increasing concentrations of tumour necrosis factor (TNF)-α (0·01–10 ng/ml) in the presence of 1 μg/ml of actinomycin D (Act D). Control ECs received Act D only. After 24 h cell survival was measured using a 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay. The percentage survival was measured by comparing TNF-α-stimulated ECs to control, no TNF plus ActD, which were deemed 100% viable. Data represent the pool of five experiments and the mean ± standard error of the mean (s.e.m.) is shown (a). Statistical significance is shown using (*). ECs isolated from ICAM-2/A1 (black bars) and non-transgenic (open bars) littermates were stimulated with 10 ng/ml of TNF-α for 24 h. Expression of ICAM-1 and VCAM-1 were analysed via flow cytometry using specific antibodies and the fold changed of expression compared to non-stimulated ECs is shown. Data represent the pooled fold change of three experiments; lines indicate the comparison between ICAM-2/A1 and non-transgenic mice per individual experiment (b). Heart transplants were explanted on day 100 and sections taken for haematoxylin and eosin (H&E) staining (c). Insert picture shows leucocyte infiltration around a blood vessel, highlighted in the black box. [Colour figure can be viewed at wileyonlinelibrary.com].

cytometry. ECs isolated from both non-transgenic littermates and ICAM-2/A1 mice expressed ICAM-1 and VCAM-1 at low levels (data not shown). As expected, TNF-α induced expression of ICAM-1 and VCAM-1 on ECs isolated from non-transgenic mice by a two- to fivefold increase (Fig. 3b). However, although not significant, reduced ICAM-1 and VCAM-1 expression was observed when A1 was expressed in ECs in all three experiments (Fig. 3b). Next, to analyse whether this reduction in adhesion molecules expression led to an altered cell infiltration, cardiac grafts were harvested from mice receiving an ICAM-2/A1 heart or a non-transgenic heart day 100 post-transplantation, and tissue sections were stained with H&E. At this time-point the ICAM-2/A1 heart, but not the non-transgenic heart, was still palpitating. An intact heart structure, with little leucocyte infiltration, was observed in the A1-expressing heart and there was no evidence of cardiac allograft vasculopathy in coronary vessels. In contrast, diffuse myocardial inflammation with extensive myocyte damage, haemorrhage, fibrosis and vasculitis was seen in the non-transgenic heart (Fig. 3c, increased magnification shown in the insert box). To rule out that the diminished infiltration seen was due to A1-expressing dendritic cells (DCs) having a reduced capacity to activate T cells, antigen-specific CD4 and CD8 T cells were stimulated with antigen-pulsed DCs isolated from ICAM-2/A1 or non-transgenic mice and T cell proliferation was measured. DCs isolated from ICAM-2/A1 mice stimulated both CD4 and CD8 T cell responses to the same extent as DCs isolated from control mice (data not shown). In conclusion, constitutive expression of A1 may protect murine ECs from proinflammatory cytokine-mediated cell death and activation, both of which may have led to the prolonged allograft survival observed *in vivo*.

Discussion

During transplantation EC dysregulation is the key initiator of graft rejection, and inhibiting EC activation is an effective way to prevent graft loss. Over-expressing anti-apoptotic genes such as HO and A20 in ECs protect ECs from cytokine-mediated activation and cell death [2,3]. In addition, anti-apoptotic molecules are expressed in 'accepted' graft tissues [2]. In this study we extend these findings to Bfl-1/A1, a TNF-α-inducible anti-apoptotic

molecule. In ECs under normal conditions, A1 is expressed either at low levels or not at all, but is induced via NF-κB activation in response to inflammatory stimuli. Over-expression of this molecule has been shown previously to inhibit ECs activation [10], making it a promising anti-inflammatory and cytoprotective molecule for these cells.

It has been reported that A1, like other Bcl-2 family members, is located in the mitochondria, where it binds to Bid and tBid to inhibit the activation of the pro-apoptotic Bcl-2 family members, Bak and Bax [12,34]. A1 has four Bcl-2 homology (BH) domains in addition to a C-terminal domain containing an amphipathic tail. Expression of full-length A1 by recombinant adenovirus (rAd) has been shown to protect human ECs from staurosporine [35] and TNF-induced cell death [15]. This may be due to both the BH4 and C-terminal domains of this molecule, both of which have been reported to be involved in the anti-apoptotic function of A1 [36]. Here, we show that constitutive expression of A1 can protect murine ECs from low levels of TNF-α *in vitro*. This proinflammatory cytokine has been shown to be an important mediator of allograft rejection [28]. In a rat cardiac transplant model, TNF-α protein levels within allografts were maximal 3 days post-transplant [37]. Whether A1 expression in the murine endothelium protects against this early *in-vivo* production of TNF-α is as yet unknown.

A1 has also been shown to inhibit NF-κB activation in ECs [10], suggesting that this molecule could have an anti-inflammatory function in ECs. Interestingly however, Guedes *et al*. [35] observed that when human HUVEC and HDMEC were transduced with recombinant adenovirus (rAd.) expressing full length human A1 (rAd.A1), TNF-induced up-regulation of ICAM-1 in ECs was not inhibited, reflecting a lack of inhibited of NF-κB activation, as measured by IKBα degradation. This result contradicted their earlier research on BAEC, where they expressed A1 via transfection with an A1 plasmid [18,35]. However, expression of full-length A1, using rAd.A1, in bovine ECs gave the same result as in human cells with respect to IKBα degradation [35]. These authors concluded that full-length A1 does not inhibit NF-κB activation. However, they observed that expressing only the C-terminal domain of A1, using rAd., in human ECs led to an anti-inflammatory effect, as shown by a lack of ICAM-1 up-regulation in response to TNF-α [35]. They suggested that cleavage of A1 at the C-terminal domain may be important for its anti-inflammatory effect, possibly by releasing A1 from the mitochondria, given that the C-terminus is crucial for anchoring A1 to this organelle. We found that constitutive expression of full-length murine A1 in murine vascular ECs appears to reduce, although minimally and not statistically significantly, the induction of both ICAM-1 and VCAM-1 following TNF-α stimulation. In addition, reduced immune infiltration was observed in heart allografts expressing A1 in murine vascular ECs, suggesting perhaps a limited expression of adhesion molecules on these cells. Whether the difference between our study and that of Guedes *et al*. [35] reflects a difference in species used and type of EC studied (HUVEC and HDMEC *versus* murine vascular ECs) or a level of expression difference (rAd. transduction *versus* endogenous ICAM-2 promoter-driven expression) is unknown at present.

Given that A1 expression increased the mean survival time of transplanted murine hearts from 17 to 86 days, the assessment of whether expression of Bfl-1 in human vascular endothelium also confers protection is warranted. Viral vectors to target molecules to vascular endothelium are limited by their lack of specificity to only ECs. Several promoters have been used to target EC *in vivo*. Lei *et al*. [38] used adenoviral vectors containing an ICAM-2 promoter linked to lacZ, to transduced murine hearts *ex vivo* [38]. These authors found high lacZ expression (22–57%) in ECs of the coronary arteries and veins in four of 22 donor grafts 3 days following transduction with 10^{10} plaque-forming unit virus [38]. Expression was ECs-specific, as no lacZ expression was observed in cardiomyocytes [38]. Given our data, we suggest that targeting anti-apoptotic molecules such as Bfl-1, perhaps through adenovirus infection to ECs, using the ICAM-2 promoter may be a promising targeting strategy for gene therapy. In conjunction with this, other therapies may also be required, as expression of A1 did not protect against direct pathway-activated $CD8^+$ T cells.

Acknowledgements

We would like to acknowledge Yanou Chi for his early contributions to the project. This project was funded by a Programme Grant from the British Heart Foundation. The research was also supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Disclosure

The authors declare that there are no disclosures.

References

1 Denton MD, Davis SF, Baum MA *et al*. The role of the graft endothelium in transplant rejection: evidence that endothelial activation may serve as a clinical marker for the development of chronic rejection. Pediatr Transplant 2000; **4**:252–60.

2 Bach FH, Ferran C, Hechenleitner P *et al*. Accommodation of vascularized xenografts: expression of 'protective genes' by donor endothelial cells in a host Th2 cytokine environment. Nat Med 1997; **3**:196–204.

3 Hancock WW, Buelow R, Sayegh MH, Turka LA. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. Nat Med 1998; **4**: 1392–6.

4 Salama AD, Delikouras A, Pusey CD *et al.* Transplant accommodation in highly sensitized patients: a potential role for Bcl-xL and alloantibody. Am J Transplant 2001; **1**:260–9.

5 Dorling A, Jordan W, Brookes P, Delikouras A, Lechler RI. 'Accommodated' pig endothelial cells promote nitric oxide-dependent Th-2 cytokine responses from human T cells. Transplantation 2001; **72**:1597–602.

6 Kelly C, Shields MD, Elborn JS, Schock BC. A20 regulation of nuclear factor-kappaB: perspectives for inflammatory lung disease. Am J Respir Cell Mol Biol 2011; **44**:743–8.

7 Oropeza M, Petersen B, Carnwath JW *et al.* Transgenic expression of the human A20 gene in cloned pigs provides protection against apoptotic and inflammatory stimuli. Xenotransplantation 2009; **16**:522–34.

8 Siracuse JJ, Fisher MD, da Silva CG *et al.* A20-mediated modulation of inflammatory and immune responses in aortic allografts and development of transplant arteriosclerosis. Transplantation 2012; **93**:373–82.

9 Choi SS, Park SH, Kim UJ, Shin HS. Bfl-1, a Bcl-2-related gene, is the human homolog of the murine A1, and maps to chromosome 15q24.3. Mamm Genome 1997; **8**:781–2.

10 Stroka DM, Badrichani AZ, Bach FH, Ferran C. Overexpression of A1, an NF-kappaB-inducible anti-apoptotic bcl gene, inhibits endothelial cell activation. Blood 1999; **93**:3803–10.

11 Gonzalez J, Orlofsky A, Prystowsky MB. A1 is a growth-permissive antiapoptotic factor mediating postactivation survival in T cells. Blood 2003; **101**:2679–85.

12 Werner AB, de Vries E, Tait SW, Bontjer I, Borst J. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. J Biol Chem 2002; **277**: 22781–8.

13 D'Sa-Eipper C, Chinnadurai G. Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities. Oncogene 1998; **16**:3105–14.

14 D'Sa-Eipper C, Subramanian T, Chinnadurai G. bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Res 1996; **56**: 3879–82.

15 Karsan A, Yee E, Kaushansky K, Harlan JM. Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. Blood 1996; **87**:3089–96.

16 Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. Genes Dev 1999; **13**:382–7.

17 Hu X, Yee E, Harlan JM, Wong F, Karsan A. Lipopolysaccharide induces the antiapoptotic molecules, A1 and A20, in microvascular endothelial cells. Blood 1998; **92**:2759–65.

18 Kunter U, Daniel S, Arvelo MB *et al.* Combined expression of A1 and A20 achieves optimal protection of renal proximal tubular epithelial cells. Kidney Int 2005; **68**:1520–32.

19 Fisicaro N, Aminian A, Hinchliffe SJ *et al.* The pig analogue of CD59 protects transgenic mouse hearts from injury by human complement. Transplantation 2000; **70**:963–8.

20 Herman MD, Nyman T, Welin M *et al.* Completing the family portrait of the anti-apoptotic Bcl-2 proteins: crystal structure of human Bfl-1 in complex with Bim. FEBS Lett 2008; **582**:3590–4.

21 Simmons MJ, Fan G, Zong WX *et al.* Bfl-1/A1 functions, similar to Mcl-1, as a selective tBid and Bak antagonist. Oncogene 2008; **27**:1421–8.

22 Marelli-Berg FM, Peek E, Lidington EA, Stauss HJ, Lechler RI. Isolation of endothelial cells from murine tissue. J Immunol Methods 2000; **244**:205–15.

23 Smyth LA, Ratnasothy K, Moreau A *et al.* Tolerogenic donor-derived dendritic cells risk sensitization *in vivo* owing to processing and presentation by recipient APCs. J Immunol 2013; **190**:4848–60.

24 Lemstrom K, Koskinen P, Hayry P. Induction of adhesion molecules on the endothelia of rejecting cardiac allografts. J Heart Lung Transplant 1995; **14**:205–13.

25 Cowan PJ, Shinkel TA, Aminian A *et al.* High-level co-expression of complement regulators on vascular endothelium in transgenic mice: CD55 and CD59 provide greater protection from human complement-mediated injury than CD59 alone. Xenotransplantation 1998; **5**:184–90.

26 Cowan PJ, Shinkel TA, Witort EJ *et al.* Targeting gene expression to endothelial cells in transgenic mice using the human intercellular adhesion molecule 2 promoter. Transplantation 1996; **62**:155–60.

27 de Fougerolles AR, Stacker SA, Schwarting R, Springer TA. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 1991; **174**:253–67.

28 Hoffmann MW, Wonigeit K, Steinhoff G *et al.* Production of cytokines (TNF-alpha, IL-1-beta) and endothelial cell activation in human liver allograft rejection. Transplantation 1993; **55**:329–35.

29 McKee CM, Defina R, He H *et al.* Prolonged allograft survival in TNF receptor 1-deficient recipients is due to immunoregulatory effects, not to inhibition of direct antigraft cytotoxicity. J Immunol 2002; **168**:483–9.

30 Pober JS, Cotran RS. Cytokines and endothelial cell biology. Physiol Rev 1990; **70**:427–51.

31 Myers CL, Wertheimer SJ, Schembri-King J, Parks T, Wallace RW. Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC. Am J Physiol 1992; **263**:C767–72

32 Mattila P, Majuri ML, Mattila PS, Renkonen R. TNF alpha-induced expression of endothelial adhesion molecules, ICAM-1 and VCAM-1, is linked to protein kinase C activation. Scand J Immunol 1992; **36**:159–65.

33 Detmar M, Imcke E, Ruszczak Z, Orfanos CE. Effects of recombinant tumor necrosis factor-alpha on cultured microvascular endothelial cells derived from human dermis. J Invest Dermatol 1990; **95**:219S–22S.

34 Duriez PJ, Wong F, Dorovini-Zis K, Shahidi R, Karsan A. A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. J Biol Chem 2000; **275**: 18099–107.

35 Guedes RP, Rocha E, Mahiou J *et al.* The C-terminal domain of A1/Bfl-1 regulates its anti-inflammatory function in human endothelial cells. Biochim Biophys Acta 2013; **1833**:1553–61.

36 Rocha E, Mahiou J, Badrichani AZ, Stroka DM, Ferran C. The BH4 domain of A1, an anti-apoptotic bcl family gene, is necessary and sufficient for its antiinflammatory function in endothelial cells. Transplant Proc 2001; **33**:314.

37 Pizarro TT, Malinowska K, Kovacs EJ *et al.* Induction of TNF alpha and TNF beta gene expression in rat cardiac transplants during allograft rejection. Transplantation 1993; **56**:399–404.

38 Lei J, Vodovotz Y, Billiar TR. Endothelial specifcity of ICAM2, Flt-1, and Tie2 promoters *in vitro* and *in vivo*. Int J Med, Health Pharm Biomed Eng 2007; **5**:244–8.