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

Lesley A Smyth[1,2*], Lucy Meader[1], Fang Xiao[1], Martin Woodward[3] Hugh J Brady[4], Robert Lechler [1] and Giovanna Lombardi[1]

[1] Medical Research Council (MRC) Centre for Transplantation, King's College 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.

[2] School of Health, Sports and Biosciences, University of East London, London, UK.

[3] Peter Gorer Department of Immunobiology, 2nd Floor, Borough Wing Guy's Hospital, King's College London.

[4]Immunology and Infection Section, Division of Cell and Molecular Biology, Sir Alexander Fleming Building, Imperial College, London, UK.

Corresponding author details

Dr Lesley Ann Smyth

School of Health, Sport and Bioscience

University of East London

Stratford Campus

Water Lane

London

E15 4LZ

Tel +44(0)208 223 4067

Email; L.Smyth@uel.ac.uk

Short title:- A1 expression in EC confers transplant protection

Key Words: endothelial, anti-apoptotic

Abbreviations

EC(s) = Endothelial(s) cells

TNF-α = Tumor Necrosis Factor

Act D = Actinomycin D

MTT = (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

r.Ad. = recombinant adenovirus

BAEC = bovine aortic ECs

HMEC = human dermal microvascular endothelial cells

HUVEC = human umbilical vein endothelial cells

Abstract

Anti-apoptotic genes including those of the Bcl-2 family have been shown to have dual functionality in as much 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 ICAM-2 promoter. Constitutive expression of A1 in murine vascular ECs conferred protection from cell death induced by the pro-inflammatory cytokine 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 maybe 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.

Introduction

Endothelial cells (ECs) lining blood vessels function at the interface between the blood stream and tissue. EC activation and death is 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, heme 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, in as much as, they inhibit cell death but are also act as anti-inflammatory mediators. For example, A20 has been shown to inhibit NF-kB activation to a number of stimuli, including oxidative stress and inflammatory cytokines [6,7]. Recently, over expression of A20 in the vasculature, both ECs and smooth muscle cells (SMCs), using recombinant A20 adenovirus, reduced transplant arteriosclerosis in a mouse aorta, to carotid artery, allograft model [8]. Protection correlated with decreased graft inflammation, with the infiltrating cells being predominantly regulatory T cells (Tregs), suggesting that maintaining the endothelium in a damage free state may help 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 rapidly induced in ECs following stimuli from pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and IL-1β [15]. NF-kB 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-kB activation given that expression of full length A1 in bovine aortic ECs (BAEC) inhibited TNF-α-mediated IKBα degradation and subsequently NF-kB 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 constitutively expressed in the vascular endothelium. To achieve this, we generated mice expressing the mouse A1 gene under the control of the human 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). Anesthesia used was Isoflurane Euthanasia was 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, Australia) and the murine A1-a cDNA containing pBluesK plasmid was kindly provided by Prof. Michael B Prystowskyt (Albert Einstein College of Medicine, USA). Murine A1 was excised from the plasmid using HindIII and BamH1 restriction digestion before being ligated into the ECOR1 cloning site of pBluescript II SK+ plasmid, upstream of the ICAM-2 promoter and the universal intron. A 1.6kb 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 (1.6Kb) was then separated by agarose gel electrophoresis, followed by purification through sequential Wizard mini columns (Promega, Madison, WI) and CHROMA SPIN 400 (CLONTECH, Palo Alto, CA). The purified fragment was microinjected at a concentration of 10µg/ml in phosphate-buffered saline into fertilized CBA×C57BL6 mouse oocytes (Karolinska Institute, Sweden). Founder progeny were screened for transgene integrity by Southern Blot analysis using the ^{32}P-labeled 1600bp injection fragment as a probe. In addition, mice were screened using PCR using ICAM-A1 forward primer (5'-TTTGCAGAAGGCTAGGGAT-3')

and the reverse primer (5'- TTCAACTTCCTTCTGAACGG-3'). The resulting 356 base pair product was resolved by electrophoresis in 1% agarose. Transgenic founders were breed onto a C57BL/6 (B6) background for more than ten generations. Mice were kept under sterile conditions.

EC isolation and cell culture

Murine ECs were purified from murine heart tissue using a method previously described [22]. In brief, murine hearts were digested with collagenase (from clostrium hostolyticum Type IV, Sigma Aldrich) and trypsin (Invitrogen Paisley, UK) to obtain a single cell suspension. ECs were isolated following incubation with rat anti-CD31, anti-CD105 (BD Biosciences) and biotinylated Isolectin B4 (Vector Laboratories, Peterborough, UK) specific antibodies and anti-rat Ig and streptavidin coupled bead selection, using MS columns (Miltenyi Biotech, Germany). ECs were then cultured for 7-10 days in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FCS (GE Healthcare), 2mM glutamine and Penicillin/Streptomycin, 50mM 2-Mercaptoethanol, 1% Non-essential amino acids, 1mM Sodium Pyruvate, 20mM Hepes (Invitrogen, Paisley, UK) and EC growth factors (Sigma Aldrich) at 37^{o}C/5% CO_2. Once the cells had reached 90% confluence they were harvested using trypsin and re-plated. For functional assays, ECs were used between passage 3 and 4. ECs at a 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-PE labeled antibodies before being analysed by flow cytometry (BD Calibur). Subsequent analysis was accomplished with FlowJo

software (TreeStar, USA). To test A1 mRNA expression in the cultured ECs, cells were lysed using Trizol and RNA extracted using an RNeasy mini kit (Qiagen, USA). A one step RT-PCR kit (Promega) was used with the following primers 5'-AACTTCCACAAGAGCAGATTGCC-3' and 5'-TCAGCCAGCCAGATTTGGGTTC-3' to amplify A1 mRNA.

MTT cell survival assay

$2.5x10^4$ ECs 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 1μg/ml of Actinomycin D (Act D, Sigma Aldrich). Control cells were incubated with Act D alone. A MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed after 18 hours following manufactures instructions (Invitrogen).

Heart Transplantation

Intra-abdominal heterotopic heart transplantation (either ICAM-2/A1 or non-transgenic grafts) was performed in CBA mice (Harlan), as previously described [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 250µgs of anti-CD8 antibody, one day prior to and after transplantation, via intraperitoneal injection. Hearts were isolated on day 100, sectioned and stained with H&E.

Measuring expression of adhesion molecules

$2.5x10^4$ ECs were grown to 90% confluent in 6 well plates at 37°C/5% CO_2. 10ngs/ml of TNF-α was added for 24 hours. Cells were removed from the plate using Accutase before being incubated with Fc block (anti-CD16/32 antibody, eBioscience, UK) for 30 minutes at 4°C. Cells were then stained with anti-ICAM-1 or anti-VCAM-1 FITC antibodies or isotype controls (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 *t* tests [24]. Mean survival time of heart allografts was assessed by Logrank 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 expressing murine A1 in ECs (ICAM-2/A1 mice, Figure 1 A and B). We used the human ICAM-2 promoter to target A1 to ECs. This promoter has previously been shown 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] (Figure 1C, top panels), isolated and expanded from the hearts of ICAM-2/A1 mice expressed A1 at the mRNA level (Figure 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 day 6 and 7.5 for non-transgenic and ICAM-2/A1 transgenic donor hearts, respectively, $p=0.08$, Figure 2A). We have previously shown 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 one day prior to and after heart transplantation, constitutive expression of A1 on ECs lead to significant heart transplant survival as compared to control hearts (mean survival time was 86 days versus 17 for ICAM-2/A1 hearts versus non-transgenic littermates, $p<0.001$ and Figure 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 significantly improved transplant outcome.

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 pro-inflammatory 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 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 to 100ngs/ml) in the presence of Act D (1μg/ml) for 24 hours. 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 hours of treatment with either 0.01 or 0.1ng/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 100ngs/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 (Figure 3A). Suggesting that constitutive A1 expression in heart ECs provides protection from low levels of pro-inflammatory 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 pro-inflammatory 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 significantly increased on capillary endothelia, whilst 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 pro-inflammatory 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 (10ngs/ml) and the

expression of the aforementioned molecules were assessed via flow 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 2 to 5-fold increase (Figure 3B). However, although not significant, reduced ICAM-1 and VCAM-1 expression was observed when A1 was expressed in ECs, in all three experiments (Figure 3B). Next, to analyse whether this reduction in adhesion molecules expression lead 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 ICAM-2/A1 heart but not non-transgenic heart where still palpitating. An intact heart structure, with little leukocyte 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 (Figure 3C, increased magnification shown in insert box). To rule out that the diminished infiltration seen was due to A1 expressing 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 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 pro-inflammatory cytokine mediated cell death and activation,

both of which may have lead 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 protects 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 TNFα-inducible anti-apoptotic molecule. In ECs, under normal conditions, A1 is either expressed at low levels, or not at all, but it is induced via NF-kB activation in responses to inflammatory stimuli. Overexpression 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 maybe 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

pro-inflammatory 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-kB activation in ECs [10] suggesting that this molecule could have an anti-inflammatory function in ECs. Interestingly however, Guedes et al (2013) observed that when human HUVEC and HDMEC were transduced with recombinant adenovirus (rAd.) expressing full length human A1 (rAd.A1), TNF-induced upregulation of ICAM-1 in ECs was not inhibited, reflecting a lack of inhibited of NF-kB 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-kB 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 upregulation in response to TNF-α [35]. They suggested that cleavage of A1 at the C-terminal domain maybe 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 minimal and not

statistically significance, 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 (2013) 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 vs endogenous ICAM-2 promoter driven expression) is at the moment unknown.

Given that A1 expression increased the mean survival time of transplanted murine hearts from 17 to 86 days, 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 just ECs. Several promoters have been used to target EC in *vivo*. Lei et al (2007) 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 4 of 22 donor grafts, 3 days following transduction with 10^{10} pfu 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 maybe 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.

Disclosures

The authors declare that there is no conflict of interest in manuscript preparation or funding.

Figure Legends

Figure 1. Constitutive expression of A1 in ECs protects from cytokine mediated cell death *in vitro*.

(A) Map of the 1.6Kb Xho1-Not1 microinjection construct, showing the ICAM-2 promoter, universal intron (UI), A1 cDNA, and 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 PCR. Founder transgenic mice, two shown, are indicated by an (*), were identified by Southern Blotting using a ^{32}P labeled 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 RT-PCR using two A1 specific primers and a control β actin primer (C, lower panels). A1 mRNA is indicated using an (*).

Figure 2. Expression of A1 in murine ECs prolongs graft survival.

CBA mice received hearts from either 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 (250μgs/mouse). Results plotted show the MST of allografts and n= number of animals. P values are shown.

Figure 3. Reduced VCAM-1 and ICAM-1 expression in 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 TNF-α (0.01-10ngs/ml) in the presence of 1μgs/ml of Act D. Control ECs received Act D only. After 24 hours cell survival was measured using an MTT assay. The percent survival was measured by comparing TNF-α stimulated ECs to control Act D, which were deemed 100% viable. Data represents the pool of 5 experiments and the Mean +/SEM 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 10ngs/ml of TNFα for 24 hours. Expression of ICAM-1 and VCAM-1 were analysed via flow cytometry using specific antibodies and the fold changed of expression comparing to non-stimulated ECs is shown. Data represents the pooled mean +/- SEM of 3 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 H&E staining (C). Insert picture shows leucocyte infiltration around a blood vessel highlighted in the black box.

References

1. Denton MD, Davis SF, Baum MA, Melter M, Reinders ME, et al. (2000) 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 4: 252-260.
2. Bach FH, Ferran C, Hechenleitner P, Mark W, Koyamada N, et al. (1997) Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. Nat Med 3: 196-204.
3. Hancock WW, Buelow R, Sayegh MH, Turka LA (1998) Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. Nat Med 4: 1392-1396.
4. Salama AD, Delikouras A, Pusey CD, Cook HT, Bhangal G, et al. (2001) Transplant accommodation in highly sensitized patients: a potential role for Bcl-xL and alloantibody. Am J Transplant 1: 260-269.
5. Dorling A, Jordan W, Brookes P, Delikouras A, Lechler RI (2001) "Accomodated" pig endothelial cells promote nitric oxide-dependent Th-2 cytokine responses from human T cells. Transplantation 72: 1597-1602.
6. Kelly C, Shields MD, Elborn JS, Schock BC (2011) A20 regulation of nuclear factor-kappaB: perspectives for inflammatory lung disease. Am J Respir Cell Mol Biol 44: 743-748.
7. Oropeza M, Petersen B, Carnwath JW, Lucas-Hahn A, Lemme E, et al. (2009) Transgenic expression of the human A20 gene in cloned pigs provides protection against apoptotic and inflammatory stimuli. Xenotransplantation 16: 522-534.
8. Siracuse JJ, Fisher MD, da Silva CG, Peterson CR, Csizmadia E, et al. (2012) A20-mediated modulation of inflammatory and immune responses in aortic allografts and development of transplant arteriosclerosis. Transplantation 93: 373-382.
9. Choi SS, Park SH, Kim UJ, Shin HS (1997) Bfl-1, a Bcl-2-related gene, is the human homolog of the murine A1, and maps to chromosome 15q24.3. Mamm Genome 8: 781-782.
10. Stroka DM, Badrichani AZ, Bach FH, Ferran C (1999) Overexpression of A1, an NF-kappaB-inducible anti-apoptotic bcl gene, inhibits endothelial cell activation. Blood 93: 3803-3810.
11. Gonzalez J, Orlofsky A, Prystowsky MB (2003) A1 is a growth-permissive antiapoptotic factor mediating postactivation survival in T cells. Blood 101: 2679-2685.
12. Werner AB, de Vries E, Tait SW, Bontjer I, Borst J (2002) Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. J Biol Chem 277: 22781-22788.
13. D'Sa-Eipper C, Chinnadurai G (1998) Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities. Oncogene 16: 3105-3114.

14. D'Sa-Eipper C, Subramanian T, Chinnadurai G (1996) bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Res 56: 3879-3882.
15. Karsan A, Yee E, Kaushansky K, Harlan JM (1996) Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. Blood 87: 3089-3096.
16. Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C (1999) The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. Genes Dev 13: 382-387.
17. Hu X, Yee E, Harlan JM, Wong F, Karsan A (1998) Lipopolysaccharide induces the antiapoptotic molecules, A1 and A20, in microvascular endothelial cells. Blood 92: 2759-2765.
18. Kunter U, Daniel S, Arvelo MB, Choi J, Shukri T, et al. (2005) Combined expression of A1 and A20 achieves optimal protection of renal proximal tubular epithelial cells. Kidney Int 68: 1520-1532.
19. Fisicaro N, Aminian A, Hinchliffe SJ, Morgan BP, Pearse MJ, et al. (2000) The pig analogue of CD59 protects transgenic mouse hearts from injury by human complement. Transplantation 70: 963-968.
20. Herman MD, Nyman T, Welin M, Lehtio L, Flodin S, et al. (2008) Completing the family portrait of the anti-apoptotic Bcl-2 proteins: crystal structure of human Bfl-1 in complex with Bim. FEBS Lett 582: 3590-3594.
21. Simmons MJ, Fan G, Zong WX, Degenhardt K, White E, et al. (2008) Bfl-1/A1 functions, similar to Mcl-1, as a selective tBid and Bak antagonist. Oncogene 27: 1421-1428.
22. Marelli-Berg FM, Peek E, Lidington EA, Stauss HJ, Lechler RI (2000) Isolation of endothelial cells from murine tissue. J Immunol Methods 244: 205-215.
23. Smyth LA, Ratnasothy K, Moreau A, Alcock S, Sagoo P, et al. (2013) Tolerogenic Donor-Derived Dendritic Cells Risk Sensitization In Vivo owing to Processing and Presentation by Recipient APCs. J Immunol 190: 4848-4860.
24. Lemstrom K, Koskinen P, Hayry P (1995) Induction of adhesion molecules on the endothelia of rejecting cardiac allografts. J Heart Lung Transplant 14: 205-213.
25. Cowan PJ, Shinkel TA, Aminian A, Romanella M, Wigley PL, et al. (1998) 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 5: 184-190.
26. Cowan PJ, Shinkel TA, Witort EJ, Barlow H, Pearse MJ, et al. (1996) Targeting gene expression to endothelial cells in transgenic mice using the human intercellular adhesion molecule 2 promoter. Transplantation 62: 155-160.
27. de Fougerolles AR, Stacker SA, Schwarting R, Springer TA (1991) Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 174: 253-267.
28. Hoffmann MW, Wonigeit K, Steinhoff G, Herzbeck H, Flad HD, et al. (1993) Production of cytokines (TNF-alpha, IL-1-beta) and endothelial cell activation in human liver allograft rejection. Transplantation 55: 329-335.

29. McKee CM, Defina R, He H, Haley KJ, Stone JR, et al. (2002) Prolonged allograft survival in TNF receptor 1-deficient recipients is due to immunoregulatory effects, not to inhibition of direct antigraft cytotoxicity. J Immunol 168: 483-489.
30. Pober JS, Cotran RS (1990) Cytokines and endothelial cell biology. Physiol Rev 70: 427-451.
31. Myers CL, Wertheimer SJ, Schembri-King J, Parks T, Wallace RW (1992) Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after downregulation of PKC. Am J Physiol 263: C767-772.
32. Mattila P, Majuri ML, Mattila PS, Renkonen R (1992) TNF alpha-induced expression of endothelial adhesion molecules, ICAM-1 and VCAM-1, is linked to protein kinase C activation. Scand J Immunol 36: 159-165.
33. Detmar M, Imcke E, Ruszczak Z, Orfanos CE (1990) Effects of recombinant tumor necrosis factor-alpha on cultured microvascular endothelial cells derived from human dermis. J Invest Dermatol 95: 219S-222S.
34. Duriez PJ, Wong F, Dorovini-Zis K, Shahidi R, Karsan A (2000) A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. J Biol Chem 275: 18099-18107.
35. Guedes RP, Rocha E, Mahiou J, Moll HP, Arvelo MB, et al. (2013) The C-terminal domain of A1/Bfl-1 regulates its anti-inflammatory function in human endothelial cells. Biochim Biophys Acta 1833: 1553-1561.
36. Rocha E, Mahiou J, Badrichani AZ, Stroka DM, Ferran C (2001) The BH4 domain of A1, an anti-apoptotic bcl family gene, is necessary and sufficient for its antiinflammatory function in endothelial cells. Transplant Proc 33: 314.
37. Pizarro TT, Malinowska K, Kovacs EJ, Clancy J, Jr., Robinson JA, et al. (1993) Induction of TNF alpha and TNF beta gene expression in rat cardiac transplants during allograft rejection. Transplantation 56: 399-404.
38. Lei J, Vodovotz Y, Billiar TR (2007) Endothelial specifcity of ICAM2, Flt-1, and Tie2 promoters In vitro and In vivo/. In J Med, Health, Pharm Biomed Eng 5: 244-248.