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ORIGINAL ARTICLE

Inhibition of TNF-α reduces transplant arteriosclerosis in a murine aortic transplant model

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Summary

Experimental and clinical data provide evidence that TNF-α contributes to acute and chronic allograft rejection. In this study, we explored the effect of TNF-α blockade using the chimeric monoclonal antibody infliximab on the development of transplant arterisoclerosis in a fully mismatched aortic allograft model. Post-transplant treatment of CBA ($H2^k$) recipients with 250 μg infliximab (cumulative dose 1.25 mg) reduced luminal occlusion of C57Bl/6 ($H2^b$) aortic grafts on day 30 from 77 ± 5% in untreated controls to 52 ± 6%. Increasing the dose of anti-TNF-α antibody had no further beneficial effect. Treatment with human control immunoglobulin had no effect on intima proliferation. Under TNF-α blockade, ICAM-1 and PDGF mRNA expression within the grafts was strongly reduced, whereas iNOS expression was enhanced. The data show that TNF-α blockade using infliximab can reduce the development of transplant arteriosclerosis in fully mismatched murine aortic grafts.

Introduction

Tumour necrosis factor alpha (TNF-α) is a pleiotropic pro-inflammatory cytokine, which plays a major role in a variety of inflammatory processes, including septic shock, graft-versus-host disease and autoimmunity. TNF-α has also been implicated in the pathogenesis of atherosclerosis [1]. In transplantation, TNF-α has been identified as a mediator of ischaemia-reperfusion injury [2]. Preclinical studies suggest that ozone oxidative preconditioning [3] or application of antisense oligonucleotides against nuclear factor-κB reduces ischaemia-reperfusion induced TNF-α expression [4]. Increased systemic TNF-α levels were also detected during acute allograft rejection episodes [5]. The role of TNF-α in promoting acute allograft rejection is further substantiated by experimental data showing that blocking TNF-α prolongs heart allograft survival, whereas administration of TNF-α accelerates rejection [6]. However, the role of TNF-α in chronic rejection is less well established. High producer genotypes of the TNF-α gene were found to be associated with cardiac allograft vasculopathy and mortality in heart transplant recipients [7]. Upregulation of TNF-α expression was found in chronic allograft nephropathy [8] and may promote the development of transplant vasculopathy [9,10]. Persistent intragraft expression of TNF-α has also been suggested as a mediator of the development of cardiac allograft hypertrophy and fibrosis in clinical transplantation [11].

In this study, we investigated whether neutralizing TNF-α using infliximab might influence the development of transplant arteriosclerosis in a fully mismatched aortic allograft model. Infliximab is a chimeric monoclonal antibody composed of a murine variable and human constant regions [12], which binds both, membrane bound and soluble TNF-α with high affinity and specificity [13]. We decided to utilize infliximab because a recent study by Grounds *et al.* demonstrated that TNF-α blockade using infliximab was biologically active in a murine model of

Duchenne muscular dystrophy, protecting dystrophic skeletal muscle from necrosis [14].

Material and methods

Abdominal aortic transplantation and morphometric analysis

CBA.J mice ($H2^k$) were used as recipients and syngeneic donors and C57Bl/6 ($H2^b$) as fully allogeneic donors of aortic allografts. Abdominal aortic transplantation was performed as described earlier [15]. For morphometric analysis, aortic grafts were removed on day 30 after transplantation and evaluated for intimal proliferation using Elastin–van Gieson staining as described earlier [15]. Results were given as the mean per group ± standard deviation. All mice used in this study were aged between 6 and 12 weeks and had an average body weight of 25 g at the time of experimental use. All mice were treated in accordance with institutional and state guidelines. All experiments were approved by the state review board.

Treatment protocols

The chimeric anti-TNFa antibody infliximab and the human immunoglobulin G (IgG) control (Intratect®, Biotest, Germany) were obtained from the local hospital pharmacy and injected intraperitoneally in a final volume of 500 µl of PBS. The first group of recipients received 250 µg infliximab, corresponding to the dosing used by Grounds *et al.* with 10 µg per gram body weight [14]. In our study, the mice in group 1 received the antibody on days 0, 3, 7, 14 and 21 (cumulative dose 1.25 mg, $n = 4$). The second group was treated with 500 µg, equivalent to 20 µg per gram body weight on days 0, 2–12, 16 and 22 (cumulative dose 4.5 mg, $n = 4$) following allogeneic aortic transplantation. As infliximab is composed of human constant regions, we had to exclude an unspecific IgG-mediated effect on the development of transplant arteriosclerosis. Therefore, an additional control group ($n = 4$) received 250 µg human IgG (Intratect®) according to the protocol of the first treatment group.

Analysis of intragraft mRNA expression

Intragraft mRNA expression for ICAM-1, PDGF-B and iNOS was determined on day 14 following aortic transplantation using real-time PCR. Aortic grafts ($n = 4$ for each respective group) were removed on day 14, which was previously established as the time point of the highest cytokine expression [16]. Total mRNA was isolated from the entire aortic graft and reverse transcript according to standard protocols. Oligonucleotide sequences for GAPDH and iNOS were described elsewhere [17]. For ICAM-1 and PDGF-B the following oligonucleotides were used: forward 5′-atgggaatgtcaccaggaatg-3′, reverse 5′-tcacgaggcccaccatga-3′, probe 5′-tgacagtactgtaccactct-3′ and PDGF-B forward 5′-cctcggcctgtggactagaagtc-3′, reverse 5′-ttaccagtgggctcga actc-3′, probe 5′-agcgagccaagacg-3′. A cloned PCR product of the respective amplified gene was run in parallel as internal standard. Expression of ICAM-1, PDGF-B and iNOS was normalized against GAPDH as house keeping gene. All amplifications were run in duplicate. Results were given as relative expression units, representing the mean per group ± standard deviation.

Flow cytometry

Murine splenocytes and human peripheral blood mononuclear cells, used as positive control, were stimulated *in vitro* using phorbol myristate acetate (PMA, 5 ng/ml) and ionomycin (1 µg/ml). After incubating the cells with biotinylated infliximab or biotinylated human IgG as isotype control, the cells were stained using fluorescein-labelled streptavidin. The flow cytometry was run on a FACS Calibur (BD Biosciences, Heidelberg, Germany).

Statistical analysis

Results are given as the mean per group ± standard deviation (SD). Data between treatment group and untreated controls were compared using an unpaired, two-tailed Student's *t*-test. A P-value < 0.05 was considered as significant.

Results

Effect of TNF blockade on intima proliferation

Fully mismatched aortic grafts from untreated recipients developed 77 ± 5% luminal occlusion, whereas syngeneic controls showed no sign of transplant arterisosclerosis (Fig. 1). Treatment with the lower dose of infliximab (group 1, cumulative dose 1.25 mg) resulted in a significant reduction of luminal occlusion to 52 ± 6% (Fig. 1). Increasing the amount of administered antibody by threefold (group 2, cumulative dose 4.5 mg) had no additional beneficial effect. As infliximab as a chimeric antibody is composed of a human constant region, we had to consider that normal human immunoglobulin G (IgG) has unspecific anti-inflammatory properties. Therefore, an additional control group received human IgG (Intratect®) according to the infliximab treatment protocol to exclude an unspecific IgG-mediated effect on the reduction of transplant arteriosclerosis. Grafts from recipients that had received the human IgG control antibody showed a similar degree of luminal occlusion with 76 ± 9% as grafts from untreated recipients.

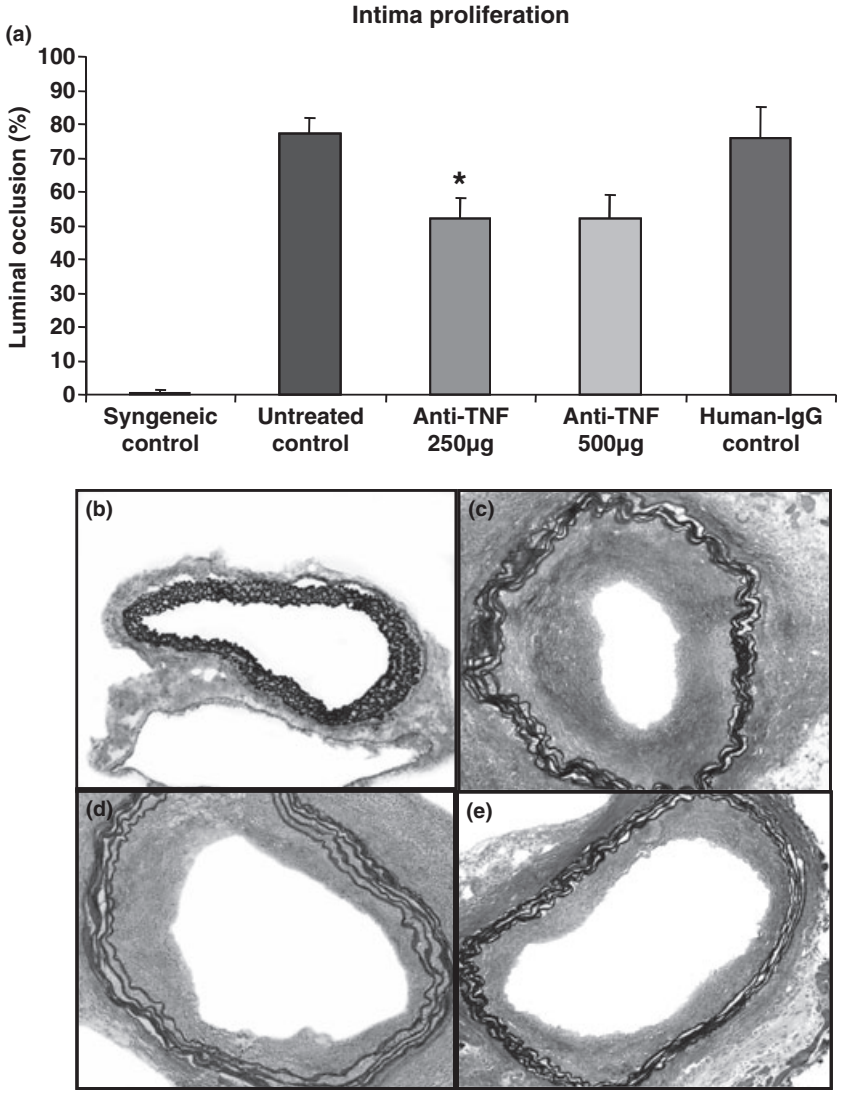

Figure 1 Intimal proliferation was analysed on day 30 after abdominal transplantation of C57Bl/6 ($H2^b$) aortic grafts in CBA.J ($H2^k$) recipients (a). Whereas syngeneic controls (n = 3) were free from transplant arteriosclerosis (b), grafts from untreated recipients (n = 5) developed 77 ± 5% of luminal occlusion (c). Application of 250 µg infliximab (d) resulted in a significant reduction of luminal occlusion to 52 ± 6% compared with untreated controls (*, $P < 0.05$). This effect could not further be improved in the 500-µg infliximab group (e). Application of human IgG, as appropriate control antibody for the chimeric antibody infliximab, had no unspecific effect on the development of transplant arteriosclerosis. Results are given as the mean per group ± standard deviation.

Effect of TNF blockade on intragraft cytokine expression

Next, we analysed the effect of TNF-α blockade on intragraft mRNA expression for intercellular adhesion molecule (ICAM)-1, platelet-derived growth factor (PDGF) and inducible nitric oxide synthetase (iNOS) on day 14 after transplantation, the time point of the highest cytokine expression in this model [16]. In this study, a strong expression of ICAM-1 was detected in grafts from untreated recipients (Fig. 2a). Treatment with infliximab significantly reduced ICAM-1 expression in a dose-dependent manner, twofold in the 250-µg group and 3.9-fold in the 500-µg group (Fig. 2a).

In our model, treatment with 250 µg infliximab strongly reduced the expression of PDGF-B by 5.5-fold compared with untreated controls (Fig. 2b). No additional effect on PDGF-B expression was observed in the high dose group. On analysis of iNOS, we found that TNF-α blockade resulted in a dose-dependent increase in iNOS expression by 1.6-fold in the group receiving 250 µg and by threefold in the group receiving 500 µg infliximab, compared with untreated controls (Fig. 2c).

Binding of infliximab to membrane-bound murine TNF-α

Although the biological effect of infliximab was demonstrated previously in a murine disease model and in an *in vivo* bioassay [14,18], binding of the therapeutic antibody *in vitro* to murine TNF-α could not be demonstrated so far. Despite the use of several experimental approaches including sandwich solid phase assays to analyse binding of infliximab to soluble murine TNF-α, none of these tests gave convincing results (data not shown). Next, we used

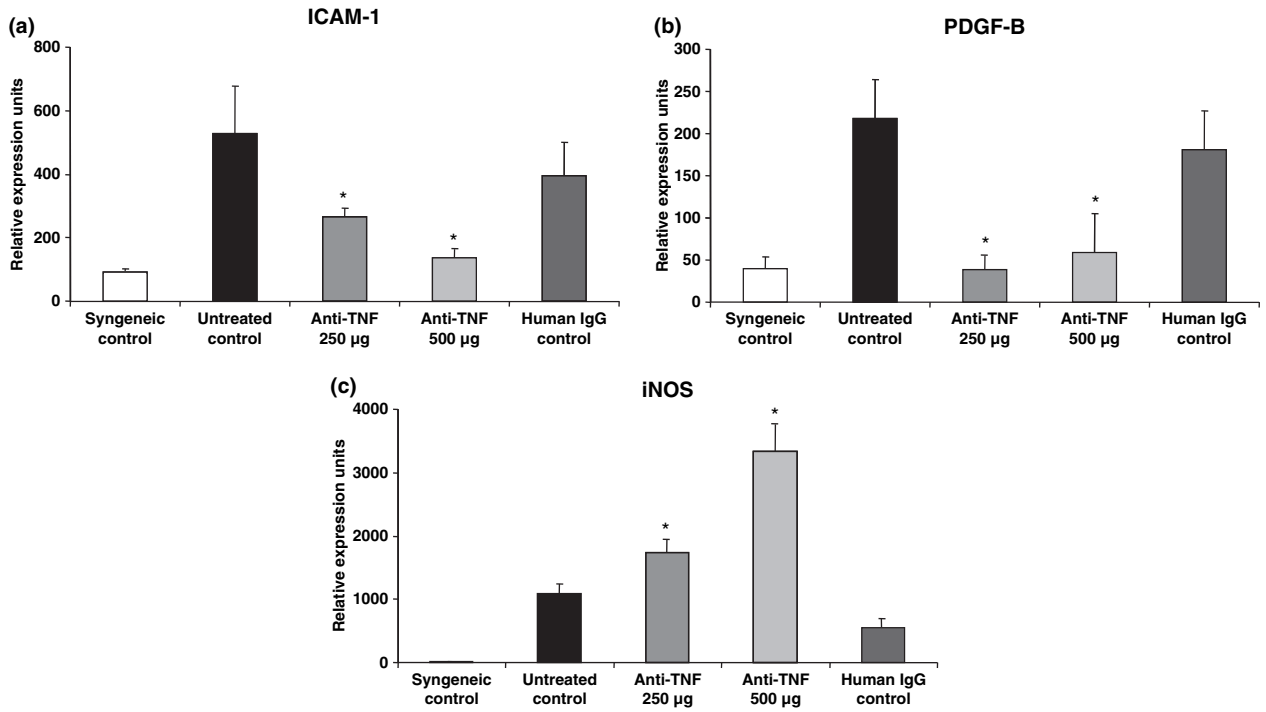

Figure 2 Intragraft mRNA expression for ICAM-1 (a), PDGF-B (b) and iNOS (c) was determined on day 14 following aortic transplantation using real-time PCR. Expression of the gene of interest was normalized against GAPDH as house keeping gene and four grafts per group were analysed. Results are shown as relative expression units, representing the mean per group ± standard deviation. (*$P < 0.05$ compared to untreated controls).

biotinylated infliximab to investigate its binding of membrane TNF-α. In this assay, the stimulated mouse splenocytes were incubated with biotinylated infliximab, stained with streptavidin and analysed by flow cytometry using stimulated human peripheral blood mononuclear cells as control (Fig. 3). Here, we could show for the first time that infliximab binds membrane-bound murine TNF-α.

Discussion

In this study, the blockade of TNF-α using chimeric antibody infliximab reduced the formation of transplant arteriosclerosis in a murine aortic allograft model. This finding is in contrast to a previous study that could not demonstrate a beneficial effect of TNF-α blockade on arteriosclerotic lesion formation despite attenuation of myocardial remodelling in a rat cardiac allograft model [19]. Besides differences in the transplant models, another possible explanation are variations in the biological activity of the construct or antibody used for TNF-α blockade. This problem, which also makes it difficult to compare study outcomes, could be addressed by the comparative biological assays as suggested by Grounds *et al.* [18].

Infliximab is a chimeric monoclonal antibody composed of a murine variable and human constant region of the IgG1 subclass [12]. When choosing the appropriate control, we had to consider that normal human immunoglobulin G (IgG) therapy has anti-inflammatory and immuno-regulatory properties [20] that are successfully used in clinical transplantation [21]. Furthermore, normal human immunoglobulin G (IgG) has been shown to prevent TNF-α-mediated endothelial cell activation [22].

Recent interesting data suggest that the anti-inflammatory property of an Ig preparation is dependent on the sialylation of the Fc part of the immunoglobulin rather than the IgG subclass [23]. We have therefore decided to use a human Ig preparation that is also used clinically as a control for unspecific anti-inflammatory Ig activity instead of an arbitrary monoclonal human IgG1 antibody. The standard clinical human immunoglobulin preparation used in this study is composed of nearly 60% IgG1 and contains the other IgG subclasses according to their average serum concentration. In this study, grafts from recipients that had received the human Ig preparation as control showed a similar degree of luminal occlusion with 76 ± 9% as grafts from untreated recipients (Fig. 1). This indicated that the reduction of transplant arteriosclerosis following infliximab treatment was a specific effect from the therapeutic antibody and not an unspecific Ig-mediated outcome.

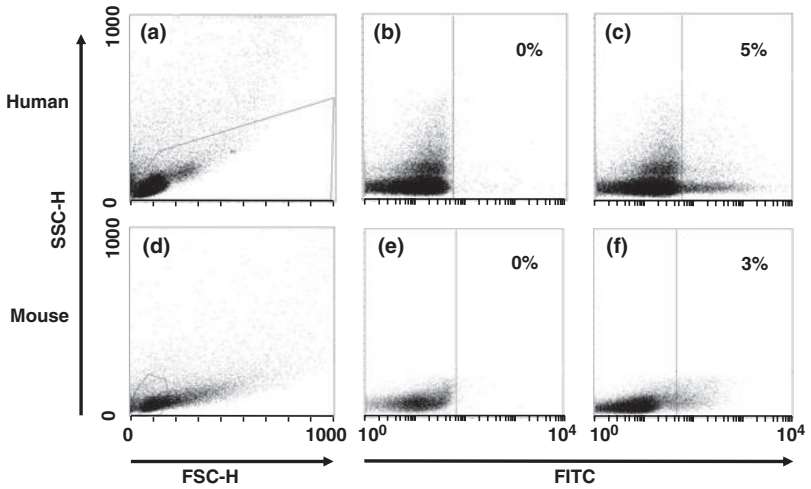

Figure 3 Flow cytometry analysis of infliximab binding to membrane TNF-α. Human peripheral blood mononuclear cells (a–c, upper panel) and murine splenocytes (d–f, lower panel) were stimulated using PMA/ionomycin and plate bound anti-CD3 antibody. After incubating the cells with biotinylated infliximab or biotinylated human IgG, the cells were stained using fluorescein-labelled streptavidin. The flow cytometry was run on a FACS Calibur (BD Biosciences, Germany). Figures a and d show the analysed cell populations. The binding of biotin-conjugated infliximab is displayed in Figures c and f compared with the isotype control (b and d). The percentage of cells binding infliximab is shown on each histogram. In total, 3% of stimulated murine splenocytes stained positive compared with 5% of human peripheral blood mononuclear cells.

The dosage of 250 or 500 μg per mouse used in this study corresponded to 10 and 20 mg/kg respectively. The dosing in the lower treatment group corresponded to the one used by Grounds *et al.* in their initial report of the beneficial effect of inflixiamb in a murine model of Duchenne muscular dystrophy [14]. The dosage used in this study was higher than the standard clinical dose of infliximab with 5 mg/kg per single application. As the antibody was given repeatedly, the cumulative dosage per mouse exceeded the usual clinical dosing, where the antibody application is generally repeated after 2 and 6 weeks, by approximately factor 5 and 18 respectively. It has to be taken into account that there are no data available, showing the avidity of infliximab for murine TNF-α, albeit we were able to demonstrate that infliximab binds to membrane TNF-α (Fig. 3). However, the higher dosage might have been a reason for the biological effect observed in this study.

The alloimmune response plays a pivotal role in the development of transplant arteriosclerosis, the hallmark feature of chronic rejection [24], although coronary artery disease can develop early after heart transplantation [25]. In this study, we used a murine aortic allograft model, which allows accurate quantification of intimal proliferation. In a previous study using a combined cardiac and aortic transplant model, we could already show that the aortic allograft model as a single vessel graft is a representative model for the transplant arteriosclerosis seen in fully vascularized cardiac allografts [15]. Although it has been shown that the graft itself may modify the donor-specific immune response [26], the development of transplant arteriosclerosis in the aortic allograft was not influenced by the presence or absence of an additional solid organ transplant in the combined cardiac and aortic transplant model [15]. Furthermore, the murine aorta contains smooth muscle cells that are important for neointima formation. However, because of its small size, the aortic allograft model is limited with respect to graft-associated immunological analysis, as isolation of graft infiltrating cells is not possible in contrast to heart allografts. Analysis of cytokine expression was performed using mRNA isolated from whole aortic grafts on day 14 after transplantation.

It has been shown that TNF-α mediates activation of endothelial cells and induces upregulation of adhesion molecules including ICAM-1, which plays a critical role in the development of transplant arteriosclerosis [10]. Deletion of the ICAM-1 gene in aortic graft recipients or blocking ICAM-1 in conjunction with lymphocyte function-associated antigen-1 strongly reduced neointimal proliferation [27,28]. Likewise, TNF-α promotes the expression of platelet-derived growth factor (PDGF) [29], which has been involved in cardiovascular disease including transplant arteriosclerosis [30]. Suppression of PDGF inhibited the development of transplant arteriosclerosis by regulation of cell migration and proliferation [31,32]. In addition, inhibiting the signal-regulated kinase pathway attenuates PDGF-mediated transplant vasculopathy [33]. In this study, treatment with infliximab reduced the intragraft expression of both ICAM-1 and PDGF, which

may contribute to the observed reduction in transplant arteriosclerosis.

The major source of intragraft expression of inducible nitric oxide synthase (iNOS) are vascular smooth muscle cells and macrophages. In contrast to the endothelial form, an upregulation of inducible NOS contributes to the development of transplant arteriosclerosis [34,35]. Upregulation of iNOS leads to nitrosative stress, which in turn triggers the activation of the nuclear enzyme poly (ADP-ribose) polymerase, contributing to cardiac dysfunction during cardiac allograft rejection [36]. TNF-α has been shown to induce iNOS expression [37,38]. We had therefore hypothesized that TNF blocked might reduce iNOS expression. However, TNF-α blockade resulted in a dose-dependent increase in iNOS expression within the aortic grafts. The reason for this unexpected finding is currently unclear. A recent study has shown that TNF blockade had little effect on iNOS expression in human arteries transplanted in immunodeficient murine hosts reconstituted with human peripheral blood mononuclear cells [39]. It might be that species specific differences of the transplant model or variations of the therapeutic antibody contribute to the increased iNOS expression following TNF blockade in this study. All data on cytokine expression were derived on mRNA level, and the biological role of these findings will have to be confirmed in further experimental studies.

When TNF-α blockade is discussed as a potential post-transplant treatment, it has to be taken into account that recent clinical trials using TNF-α blockade in chronic heart failure were disappointing and in some cases even resulted in increased adverse events [40,41], contradicting earlier beneficial observations [42,43]. However, in organ transplantation, TNF-α is produced in response to the allogeneic stimuli, and therefore TNF-α antagonism may have different effects compared with the failing heart.

The hypothesis that the blockade of TNF-α may be beneficial for cardiac allografts is substantiated further by the recent data from the EFECT (Effect of Etanercept on Cardiac Transplantation) trial. The results of this placebo-controlled study demonstrated that TNF-α blockade using etanercept, a recombinant human soluble TNF-α receptor fusion protein, was safe and reduced extracellular matrix deposition and hypertrophy in the heart transplants, albeit not yet reaching significance at 6 months post-transplant [44].

In summary, experimental and clinical data indicate that the TNF-α blockade is a potentially valuable therapeutic tool to reduce the post-transplant alloresponse. Further studies addressing mechanisms and safety issues seem warranted.

Authorship

MW, SA, HB: performed research. MW, JRK: discussed results and provided reagents. SME, BMS: designed the study, analysed the data and wrote the manuscript.

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