Chapter 5

The CXCR3 Antagonist NBI-74330 Attenuates Atherosclerotic Plaque Formation In LDL Receptor Deficient Mice

EJA van Wanrooij, SCA de Jager, T van Es, P de Vos, HL Birch, DA Owen, RJ Watson, EAL Biessen, GA Chapman, TJC van Berkel, J Kuiper

Abstract

The chemokine receptor CXCR3 is implicated in migration of leukocytes to sites of inflammation. Antagonizing CXCR3 could therefore be a potential strategy to inhibit inflammation induced leukocyte migration, and subsequently reduce atherosclerotic lesion formation. In this study we used the CXCR3 specific antagonist NBI-74330 to block CXCR3 mediated signaling in thioglycolate induced peritonitis and diet induced atherosclerosis. Antagonizing CXCR3 with NBI-74330 resulted in a significant reduction in CD4+ T cell and macrophage migration to the peritoneal cavity. Ex-vivo migration studies with cells isolated from the peritoneal cavity after NBI-74330 treatment showed that this reduction in migration was totally CXCR3 dependent. Atherosclerotic lesion formation in both the aortic valve leaflet area as well as the total aorta was significantly inhibited in NBI-74330 treated mice. Lymph nodes draining from the aortic arch were significantly smaller in treated mice and showed enrichment in regulatory-and less activated T cells. This study shows for the first time that treatment with a CXCR3 antagonist results in attenuating atherosclerotic lesion formation, not only by blocking direct migration of effector cells from the circulation to the atherosclerotic plaque, but also by beneficially modulating the inflammatory response in lymph nodes draining from the atherosclerotic lesion.
Introduction

Atherosclerosis is a progressive multi-factorial disease of the larger arteries characterized by cholesterol deposition, leukocyte influx, cell death and fibrosis. In recent years, it has become increasingly clear that next to a lipid storage disorder, atherosclerosis can be considered as an ongoing inflammatory process within the vasculature. Migration of leukocytes into the vessel wall is an essential step in atherosclerotic lesion formation and progression and chemokines are defined as key regulators of this process.

Chemokine receptors are trans-membrane spanning, G-protein-coupled receptors which are classified by the position of the N-terminal cysteins (CC, CXC, C, CXXXC). They play an important role in the recruitment, migration and trafficking of immune cells to sites of inflammation. An increasing amount of evidence underscores the relevance of chemokines in the pathogenesis of atherosclerosis.

The chemokine receptor CXCR3 is expressed on different types of leukocytes, including T cells, B cells, natural killer (T) cells and monocytes. Its expression is highly induced upon CD4 T cell activation and is preferentially expressed on activated auto-reactive T cells. Antibody-mediated blockade of CXCR3 results in a decreased recruitment of Th1 cells to sites of inflammation.

CXCR3 has 3 known ligands; MIG (monokine induced by IFN-γ (CXCL9)), IP-10 (IFN-γ-inducible protein, (CXCL10)) and ITAC (IFN-γ-inducible T-cell chemoattractant (CXCL11)). The expression of these ligands is highly inducible by interferon-γ (IFN-γ) and associated with several inflammatory disorders.

Recent publications point towards a prominent role for CXCR3 mediated migration of inflammatory cells in atherosclerosis. Human atherosclerotic lesions express high amounts of all three CXCR3 ligands. Targeted deletion of CXCR3 in ApoE deficient (ApoE−/−) mice resulted in decreased lesion formation in the abdominal aorta. Furthermore, deletion of the CXCR3 ligand CXCL10 in ApoE−/− mice resulted in decreased lesion formation by reducing the migration of CD4 effector T cells to the atherosclerotic plaque.

Blockade of CXCR3 mediated migration could therefore provide a potential strategy to reduce leukocyte migration to sites of inflammation and in this way attenuate atherosclerotic lesion formation. In this study, we describe the highly specific CXCR3 antagonist NBI-74330 and its inhibitory effects on cell migration and diet induced atherosclerosis in LDL receptor deficient (LDLr−/−) mice.
Material and methods

Determination of appropriate in vivo dosage of NBI-74330
The quinazolinone-derived CXCR3 antagonist NBI-74330 was synthesized as described by Medina et al (WO02083143, 2002 oct 24). Mice were treated with NBI-74330 in 0.1% Na Docusate in 0.5% 400Cp Methylcellulose and serum concentration at indicated time points were determined using LC-MS-MS. Serum was subjected to protein precipitation prior to analysis. HPLC mobile phase consisted of H2O with 0.1% (v/v) formic acid and Acetonitrile with 0.1% (v/v) formic acid using a gradient profile.

Peritonitis induced migration and mobility assay
LDLr⁻/⁻ mice were treated with a subcutaneous injection of 100mg/kg CXCR3 antagonist NBI-74330 in 100 μl 0.1% Na Doc in 0.5% 400Cp Methylcellulose (n=6) or vehicle (n=5) for 6 days. At day 2, all mice were injected intraperitoneally with sterile 3% (w/v) Brewers thioglycolate solution. Peritoneal cells were isolated by peritoneal cavity lavage with PBS and counted and phenotyped by flow cytometry at day 6. Migration capacity of the isolated peritoneal cells in response to CXCL10 (100ng/ml), and the chemotactic peptide FMLP (1µM) was quantified using a chemokinesis assay19.

Atherosclerosis experiments
Female LDLr⁻/⁻ mice, 10 weeks old (n=8-12 per group), were fed a Western-type diet containing 0.25 % cholesterol and 15% cocoa butter two weeks before collar placement20. Mice were treated with a subcutaneous injection of 100mg/kg CXCR3 antagonist NBI-74330 in 100 μl 0.1% Na Doc in 0.5% 400Cp Methylcellulose every day during the entire experiment. After 8 weeks of Western-type diet and treatment, the mice were sacrificed and organs were harvested for histology, FACS and RNA isolation. Blood samples were collected by tail bleeding from non-fasted animals and concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures.

Histological analysis
Cryostat sections of the aortic root (10 µm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:50). Goat anti-rat IgG-AP (dilution 1:100) was used as secondary antibody and NBT-BCIP as enzyme substrates. Masson trichrome staining (Sigma Diagnostics) was used to visualize collagen (blue staining). TGFβ was stained with a polyclonal rabbit antibody (Santa Cruz, USA) and biotinylated goat anti rabbit (Dako cytomatics, The Netherlands) was used
as a secondary antibody with Nova Red as enzyme substrate (Vector Laboratories).

Real time PCR assays
Total RNA was isolated from aortic arch and collar induced atherosclerotic plaques and was DNase treated. Quantitative gene expression analysis was performed on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Online table I) were designed using Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Flow cytometry
Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada). Cell suspensions from spleen, blood, lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.2 μg Ab/300,000 cells) and subsequently subjected to flow cytometric analysis (FACS). Antibodies were purchased from Immunoscource (Belgium). All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

Statistical analysis
Values are expressed as mean ± SEM unless indicated otherwise. Two-tailed student’s T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to compare not normally distributed data. A probability value of P<0.05 was considered to be significant for both tests.

Figure 1: Daily s.c. injections of 100 mg/kg NBI-74330 result in serum levels sufficient to fully antagonize CXCR3 in vivo. A formulation of NBI-74330 was constructed using 1% Na Doc in 0.5% 400Cp Methylcellulose and the mice (n=3) were treated with 100 mg/kg compound every day for 5 days. Serum levels were determined at indicated time points using LC-MS-MS. Dotted line indicates ~Kᵢ.
Antagonizing CXCR3 Decreases Atherosclerosis

Figure 2: Macrophage and T cell migration to thioglycollate induced peritonitis is inhibited by NBI-74330 treatment. **A**: Female LDLr-/- mice were treated with a subcutaneous injection of 100mg/kg CXCR3 antagonist NBI-74330 in 100 µl 1% Na Doc in 0.5% 400ºCp Methylcellulose (n=6) or vehicle (n=5) for 6 days. At day 2, all mice were injected intra-peritoneally with sterile 3% (w/v) Brewers thioglycollate solution. Peritoneal cells were isolated by peritoneal cavity lavage with PBS and counted and phenotyped by FACS at day 6. **A**: Recruitment of cells to the peritoneal cavity was reduced by 56% after NBI-74330 treatment. The percentage of CD4+ T cells and macrophages (F4/80+, Gr-1 low) present in the peritoneal cavity was significantly decreased in mice treated with NBI-74330 compared to control-treated mice. (Error bars represent SEM, *: p<0.05, **: p<0.01)

**B**: 5000 isolated peritoneal cells (triplicate per mouse/situation) were seeded on fibrinogen coated 96-wells and left to adhere at a nearly upright position. Migration in response to CXCL10 and FMLP (positive control) was then assessed at a 15º tilted position by counting the number of cells that migrated across a defined threshold. (Error bars represent SD, ***: p<0.001 compared to cells from control mice cultured with X100ng/ml CXCL10, ###: p<0.001 compared to cells from control mice cultured with 100 ng/ml CXCL10, $$: p<0.01, $$$p<0.001 FMLP versus control stimulated cells).

Results

**In vivo use of NBI-74330**

NBI-74330 is a small molecular high affinity CXCR3 antagonist that is a potent inhibitor of CXCR3 ligand binding with a Kᵢ in the low nanomolar range (~8nM). *In vitro* data have shown that it inhibits CXCL10 and ITAC induced calcium mobilization at concentrations below 10 nM\(^2\). A formulation of NBI-74330 was constructed using 1% Na Docusate in 0.5% 400ºCp Methylcellulose.
We tested the optimal dosing of this formulation in vivo and found that daily dosage of 100 mg/kg via subcutaneous injections resulted in serum concentrations of approximately 1 μM (figure 1). This concentration is sufficient to fully block the CXCR3 receptor in vivo.

The CXCR3 antagonist NBI-74330 inhibits CD4 T cell and macrophage migration during thioglycolate induced peritonitis

To show the in vivo capacity of NBI-74330 to antagonize CXCR3 mediated cell migration, we used a peritonitis model. In this model, leukocytes migrate to the peritoneal cavity in response to a single intraperitoneal thioglycolate challenge. Female LDLr⁻/⁻ mice were treated with NBI-74330 or control for 6 days. At day 2, all mice were injected intra-peritoneally with sterile 3% (w/v) Brewers thioglycolate solution and 5 days later the number of cells present in the peritoneum was quantified. A significant 56% reduction in leukocyte recruitment could be observed after 5 days in NBI-74330 treated mice compared to control treated mice (Fig. 2A, p=0.01). FACS analysis of the isolated peritoneal cells showed that this reduction was mainly due to reduced migration of CD4 T cells and macrophages (Fig 2A). To investigate the capacity of the isolated peritoneal cells from control and NBI-74330 treated mice to migrate in response to the CXCR3 ligand CXCL10 we performed an ex vivo mobility study. Isolated peritoneal cells from control and treated mice were allowed to accumulate at the lower end of a 96-well culture plate and migration in response to 100 ng/ml CXCL10 and FMLP (positive control) was then assessed. Results are shown in figure 2B.

Peritoneal cells isolated from mice treated with vehicle for 6 days had a clear migratory response when exposed to 100 ng/ml CXCL10, and this effect could be completely reversed by the in vitro addition of the CXCR3 antagonist NBI-74330 to the culture medium. Cells isolated from mice treated with CXCR3 antagonist in vivo during the thioglycolate challenge were not able to respond to CXCL10. Their capability to migrate while exposed to the general chemotactic peptide FMLP was not different compared to control treated mice.
These findings clearly show that the reduced migration towards thioglycollate induced peritonitis by NBI-74330 treatment is the result of an effective in vivo blockade of CXCR3.

**Figure 4: Atherosclerotic lesion formation is significantly inhibited in mice treated with NBI-74330**

Representative photomicrographs of oil red O stained cross sections of the aortic root of control treated mice (A) and NBI-74330 treated mice (B) are shown. A significant reduction in plaque size was found as compared to control (p=0.03, n=8 per group) (C). Relative plaque area was quantified in en face pinned aortas. Representative pictures are shown for control (D) and NBI-74330 treated mice (E). Lesion formation is significantly inhibited by NBI-74330 treatment by 53% (p=0.01).

**Atherosclerotic lesion formation in LDLr⁻/⁻ mice is attenuated by antagonizing CXCR3**

We use LDL receptor deficient mice on a Western type diet as a model for atherosclerosis and the mRNA expression of CXCR3 during lesion formation was monitored in the aortic arch at different stages of western type diet feeding. A significant increase was observed in CXCR3 mRNA expression after 9 weeks of diet, indicating initial influx of CXCR3 expressing leukocytes (Figure 3). This showed that CXCR3 could be involved in the atherosclerotic process in our model. We then assessed the effect of NBI-74330 treatment on atherosclerotic lesion formation.

Female LDLr⁻/⁻ mice were fed a Western-type diet and received daily subcutaneous injections of 100mg/kg NBI-74330 or vehicle. No difference was observed in serum cholesterol and triglyceride levels between control and treated animals (data not shown). Figure 4 shows representative sections of control treated (figure 4A) and NBI-74330 treated (figure 4B) mice.
Atherosclerotic lesion formation in the aortic valve leaflet area was significantly inhibited in mice treated with NBI-74330 (536*10^3 μm² vs. 391*10^3 μm², p<0.05). Relative macrophage staining (MOMA-2) was comparable in plaques from control and treated animals, as well as the relative collagen content as determined by Masson trichrome staining (Figure 5).

Next to lesion formation in the aortic valve leaflets, we quantified the percentage of lesion area in the aorta of control and NBI-74330 treated mice by en face pinned out aortas stained with oil red O. Representative pictures of control (4D) and NBI-74330 treated aortas (4E) are shown. NBI-74330 treatment resulted in a 53% reduction in lesion formation compared to control treated mice (18 ± 2% vs. 8 ± 2%, p<0.01).

**Figure 5: NBI-74330 treatment has no effect on relative macrophage and collagen content of atherosclerotic plaques.** Plaque composition of NBI-74330 treated and control treated mice was determined using a macrophage specific antibody (MOMA-2) (figure 4A-C), and Masson Trichrome staining to visualize collagen (figure 4D-F). Representative sections are shown for each group. No significant effects could be observed between control and treated animals (n=8 per group).

**CXCR3 antagonist NBI-74330 treated mice have smaller lymph nodes draining from the aortic arch and a beneficial regulatory/effector T cell balance during atherogenesis**

Recent publications suggest a role for CXCR3 in the migration of effector cells towards the site of inflammation\(^{18,22,23}\). We isolated the lymph nodes draining from the aortic arch of LDLr\(^{-/-}\) mice after 8 weeks of Western type diet and subsequent treatment with either control or the CXCR3 antagonist NBI-74330. The size of the isolated lymph nodes was assessed by quantifying the number of cells. Treatment with NBI-74330 resulted in a 64% reduction of cell numbers in lymph nodes draining from the aortic arch (Fig. 6). Characterization of the isolated cell population was performed using FACS.
A significant increase was observed in the percentage CD4^+CD25^+ regulatory T cells in NBI-74330 treated mice (p<0.05). This increase in regulatory T cells was accompanied by an increase in the expression of CD62L on CD4 cells (p<0.001), suggesting a reduction in the activation state of effector T cells. These effects were restricted to the lymph nodes draining from the aortic arch, as no such effects were observed in spleen or the circulating white blood cell population.

Plaques from mice treated with NBI-74330 express more genes associated with regulatory T cells

To determine whether the local reduction in effector cells and the increase in regulatory T cell phenotype resulted in a resembling cytokine profile inside the atherosclerotic plaque we stained for the regulatory T cell cytokine TGFβ. Representative slides are shown for control (7A) and NBI-74330 treated (7B) mice. A significant increase was observed in the relative expression of TGFβ in the plaque of mice treated with NBI-74330 compared to control.

We performed QPCR on atherosclerotic plaques isolated from the carotid artery after collar induced atherosclerosis from both control and treated mice and determined the expression of the regulatory T cell associated genes FOXP3, CD25 and CTLA-4. The expression of FOXP3 was 2.5 fold upregulated (p<0.05), and the expression of CTLA-4 was 6 fold upregulated (p<0.05) compared to expression in control treated mice. The expression of CD25 showed a trend towards an increased expression, but this was not significant.

![Figure 6: Draining lymph nodes from the aortic arch of NBI-74330 treated mice are smaller in size and contain less activated and more regulatory T cells](image)

Draining lymph nodes were isolated from control and NBI-74330 treated mice after 8 weeks of western type diet feeding. Single cell suspensions were prepared and total cell number was quantified. NBI-74330 treatment resulted in a 64% decrease in cell number compared to control. (Error bars represent SEM, *: p< 0.05). FACS was used to assess the relative amount of CD4^+CD25^+ regulatory cells, and the % CD4^+CD62L^hi effector cells. (Error bars represent SEM, n=6 per group, *:p<0.05, ***:p<0.001)
Figure 7: Atherosclerotic plaques from NBI-74330 treated mice express increased levels of regulatory T cell associated molecules
A: Sections of the aortic leaflet area were stained for TGFβ using anti TGFβ antibodies. We then quantified the TGFβ positive area relative to total plaque area. Treatment with NBI-74330 resulted in a significant increase in relative TGFβ positive area (red staining).
B: mRNA expression of different genes isolated from collar induced atherosclerotic plaques (8 weeks) is expressed relative to 36B4 and HPRT, and subsequently related to the expression in control mice. White bars represent control mice; black bars represent NBI-74330 treated mice. An unpaired Student t test was applied to test if mRNA levels were significantly different from the mRNA levels in chow fed animals (n=5-10 per group) (*P<0.05).

Discussion

Evidence is building that CXCR3 mediated cell migration plays an important role in several (auto-) immune diseases.24-27. CXCR3 positive cells enter the site of inflammation followed by a local upregulation of CXCR3 expression. These CXCR3 positive cells are attracted by the 3 known ligands, CXCL9, 10 and 11, and it was shown that these ligands are highly expressed in atherosclerotic lesions.17.

In this study we used the highly specific CXCR3 antagonist NBI-74330 to block CXCR3 mediated signalling and migration. Firstly, we showed the in vivo capacity of this compound to reduce cell migration to a site of inflammation using a thioglycolate induced peritonitis model. Total leukocyte migration to the peritoneal cavity was reduced by 56% upon daily treatment with NBI-74330. The reduction in total cell migration was the consequence of a reduction in the number of CD4+ T cells and macrophages to the peritoneal cavity. The observed decrease in migration was the result of selective blockage of CXCR3, as shown by a subsequent ex vivo mobility assay. Peritoneal cells isolated from control mice were able to migrate in response to both CXCL10 and FMLP. Cells isolated
from mice that received NBI-74330 during the induction of peritonitis were not responsive to CXCL10, but were still capable of migrating in response to an FMLP stimulus.

After we established the in vivo efficacy of the compound, we used LDL receptor deficient mice to test the effect of antagonizing CXCR3 on atherosclerotic lesion formation.

We show that the expression of CXCR3 is upregulated in the aortic arch of LDLr<sup>−/−</sup> mice fed a western type diet for 9 weeks. This indicates that CXCR3 positive leukocytes migrate to the developing atherosclerotic plaque. It was shown that CXCR3 deficient mice on an ApoE background show reduced lesion burden compared to control ApoE mice. In addition, mice lacking CXCL10, the main ligand for CXCR3, show decreased atherogenesis compared to control ApoE mice. These are interesting observations, clearly suggesting that CXCR3 is involved in the disease initiation and progression of atherosclerosis in ApoE deficient mice. However, these mice lack the expression of these proteins throughout their development and compensating mechanisms to overcome this deficiency may have taken place.

In this study, we use the compound NBI-74330, a highly specific low molecular weight CXCR3 antagonist, to investigate the effect of antagonizing CXCR3 in a diet induced model for atherosclerosis. Treatment with NBI-74330 resulted in a significant decrease in atherosclerotic lesion formation at the aortic valve leaflet area as well as the total aorta. We observed a more pronounced decrease in lesion size in the aorta compared to the valve leaflet area. In their study with CXCR3/apoE double deficient animals, Veillard et al. also showed a difference in lesion formation between these two sites, since they observed a significant decrease in the degree of lesion formation in the descending aorta was observed, but no effect in the aortic sinus. They suggest a more prominent role for CXCR3 in the initial stages of lesion formation. Our findings strengthen this idea as we had quite large and advanced lesions in the valves, while the lesion burden in the aorta was more moderate.

As shown in literature, CXCR3 mediates the migration of effector cells to the site of inflammation. Absence of CXCR3 or its main ligand CXCL10 in mice on an ApoE background resulted in an induction of regulatory T cells markers within the atherosclerotic plaque but not in lymph nodes or in the circulation. Our experiments however clearly show that administration of a pharmacologically active CXCR3 antagonist results in smaller lymph nodes draining from the aortic arch compared to control treated mice. Next to this, the activation state of T cells is decreased and regulatory phenotype is enhanced in these lymph nodes of NBI-74330 treated mice.
When LDLr−/− mice are fed a Western type diet, the lymph nodes draining from the aortic arch increase in size due to locally induced inflammatory signals. Administering a CXCR3 antagonist clearly reduces the migration of leukocytes to the lymphatic sites draining to the site of inflammation. This indicates that not only direct migration of effector cells from the circulation to the atherosclerotic plaque is inhibited, but that also migration to the draining lymph system is beneficially modulated. Possibly CXCL9 and 11 are involved in this process, because no increase in regulatory T cells was observed in lymph cells isolated from CXCL10 deficient mice. The amount of TGFβ in plaques from mice treated with NBI-74330 is significantly increased. Several mechanisms could lead to this finding. We observed that NBI-74330 treated mice have an increased number of CD4+CD25+ positive regulatory T cells in the lymph nodes draining directly from the aortic arch28. Next to this treated mice have an increased expression of the regulatory T cell markers FOXP3, CTLA-4 and CD25 within the atherosclerotic plaque. This indicates that regulatory T cells are the likely source of the observed increase in (surface bound) TGFβ expression within the atherosclerotic plaque29-34.

In general, we hypothesize that the observed reduction in lesion formation and the accompanying induction in regulatory T cell phenotype is a result of a reduction in migration of effector cells both from the circulation to the atherosclerotic plaque and to the locally draining lymph nodes. This will in turn lead to a relative induction of anti-inflammatory and regulatory cells. Eventually, due to the continuing exposure to antigens such as oxLDL or heat shock proteins, the ongoing attraction of leukocytes via other pathways than CXCR3 will facilitate the process of lesion formation. The blockade of CXCR3 thus provides a "lag-time" in this response.

It is clear that selective blockade of CXCR3 migration in vivo using NBI-74330 provides an attractive way to beneficially balance the immune response in an auto-inflammatory situation such as atherosclerosis. A possible drawback of interfering with CXCR3 mediated migration could be a potentially hampered immune response against invading pathogens. For example, lung infection with B. Bronchiseptica results in strong upregulation of CXCR3 ligands and influx of CXCR3+ cells. However, CXCR3 deficient mice do not show increased mortality to this pathogen. Furthermore, Chackavarty et al. have shown that CXCR3 deficient mice are more resistant to Mycobacterium tuberculosis infection35. CXCR3 is also associated with cellular influx into CMV infected liver. Infection of the liver with CMV attracts CXCR3+ CD8+ cells that contribute to the protective response to the virus but these cells are not exclusive required for its clearance36. Based on these observations treatment with a selective CXCR3 antagonist is unlikely to result in severe infections with pathogens.

Small molecular antagonists are the preferential and most widely used drugs and have clear advantages over protein or antibody formulations. We conclude that
this study shows for the first time that a small molecular CXCR3 antagonist inhibits lesion formation in an animal model for atherosclerosis. This study therefore provides evidence that CXCR3 antagonists can be a possible new therapeutic strategy to counteract the development of atherosclerosis or other (auto)-immune diseases.
References

Antagonizing CXCR3 Decreases Atherosclerosis

atherogenesis by modulating the local balance of effector and regulatory T cells. Circulation. 2006;113:2301-2312.