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Chapter 7

Potential role of regulatory T cells in Aortic Aneurysms Formation

-Manuscript in preparation-

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Abstract

Regulatory T cells (Tregs) suppress autoimmune and inflammatory diseases by exerting suppressive effects on various immune cells such as effector CD4 T cells. We examined the importance and potential role of Tregs in a mouse model for abdominal aortic aneurysm formation. ApoE^{-/-} mice were fed a western type diet during 4 weeks after which AngII infusion was started by means of an osmotic pump that was placed subcutaneously. Tregs were either depleted by injection of anti-CD25 or induced by the injection of anti-CD3. We observed a 40% death rate due to aortic rupture in mice in which the Tregs were depleted, suggesting a protective role of Tregs in preventing AAA rupture. In addition, we performed microarray analysis on the aorta's after anti-CD25 treatment and observed a significant lower expression of genes involved in energy metabolism, more specifically the Krebs cycle, indicating a reduced energy production which could eventually lead to cell death. Treatment with anti-CD3 in ApoE^{-/-} infused with AngII did not result in increased numbers of Tregs and had consequently no effect on the number or type of aneurysm formation. In conclusion our data demonstrate a possible protective role of Tregs in aneurysm formation but further research is needed in which the induction of Tregs is optimized to definitively show the protective effect of these cells on aneurysm formation.

Introduction

An aneurysm of the abdominal aortic (AAA) is a focal balloon-like dilation of the aorta. AAA is a common disease, which mainly affects men aged 60 or older. Mostly, aneurysms remain undiscovered and they grow approximately 0.3 cm each year and will, if untreated, rupture. Rupture of aneurysm has a high mortality rate and is the 13th leading cause of death in the United States.^{1, 2} Development of AAA is associated with inflammation, tissue remodeling and the upregulation of matrix-degrading proteinases and it correlates with age, sex, pulmonary emphysema and high blood pressure.³⁻⁵ Treatment of AAA mainly focuses on controlling the risk factors and/or the surgical removal of the aneurysm by techniques such as stent graft repair or complete replacement of the diseased part. Suitable pharmacological treatment is currently unavailable, although inhibitors of Angiotensin Converting Enzyme (ACE) and statins have been shown to beneficially influence the progression or rupture of an aneurysm in patients.⁶

There is an increasing body of evidence supporting the role of the immune system in aneurysm formation where chronic inflammation of the aortic wall results in degradation of collagen and the elastic lamina. Inflammatory cells accumulate in AAA lesions with a predominance of CD4⁺ T cells and to a lesser extent B cells and macrophages.⁷⁻⁹ CD4⁺ T cell depletion protects against AAA formation in a calcium-chloride induced model for AAA in mice¹⁰ and CD28^{null} T cells play an important role in the pathology of AAA¹¹ and CAD patients.¹² Therefore, the concept of T cell mediated inflammation is favored in AAA formation. CD4⁺CD25⁺ regulatory T cells (Tregs) play a critical role in the maintenance of immune homeostasis and tolerance to self-antigens. Tregs potently suppress the proliferation and cytokine production of effector CD4⁺ and CD8⁺ T cells and deficiency of Tregs leads to autoimmunity in both murine experimental models as in humans.^{13, 14} In atherosclerosis, Tregs have been shown to play a protective role.¹⁵⁻¹⁷ However, the exact role of Tregs in AAA has not been elucidated yet. Therefore we determined the role of Tregs in a mouse model of AAA formation.

Materials and methods

Mice

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male ApoE^{-/-} mice on a C57Bl6 background were bred in-house. Mice were kept under standard laboratory conditions and were fed a normal chow diet or Western-type diet (WT-diet) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 6 months old at the start of the experiment. Diet and water were administered *ad libitum*.

Experimental protocol

Mice were put on WT-diet for 4 weeks prior to infusion with AngII and WT-diet feeding was continued during the rest of the experiment. Before placement of the osmotic pumps, mice were distributed into groups according to their cholesterol levels. 1.44 mg AngII/kg/day was infused using Alzet osmotic minipumps (Model 2004, Alzet, Charles River, The Netherlands) which were subcutaneously implanted in anesthetized mice. 24 hours later mice were *i.p.* injected with anti-CD25 or rat control IgG (100 µg/mouse). In the second experiment mice were *i.p.* treated with 50 µg/mouse anti-CD3 to induce Tregs prior to pump placement.

En face aorta

After perfusion with PBS and Zinc-Fix, heart and aorta were isolated and stored in zinc-Fix. Aorta's were prepared and photographed. Aneurysm formation was classified using the morphological grade of aneurysm (grade 0-IV) according to Daugherty *et al.*¹⁸

Micro-array analysis

Aorta's were crushed using liquid nitrogen and RNA was isolated using the GTC-method. aRNA was amplified using the NanoAmp RT-IVT Labeling Kit (Applied Biosystems, The Netherlands) and labeled with DIG-UTP. aRNA from 2 mice were pooled. The Applied Biosystems (AB) Mouse Genome Survey Micro-array was used which contains ~34,000 features including a set of ~1000 controls. Each micro-array uses 32,996 probes targeted to 32,381 curated genes representing 44,498 transcripts. Micro-array hybridization (using 10 µg of fragmented, DIG-labeled cRNA), processing, chemiluminescence detection, imaging, auto-gridding, and image analysis were performed according to AB protocols, using the 1700 Chemiluminescent Micro-array Analyzer Software v.1.0.3. For each group we performed 2 micro-array analyses. Panther Probe_ID annotations were updated to Mouse Micro-array V2 using the AB1700_MouseV2_MB_Updater. Transcriptome analysis based on raw intensity data was performed essentially as described¹⁹ using the limma package²⁰ and in-house scripts in R/Bioconductor.²¹ Intensities were log₂-transformed and then normalized using quantile normalization.²² Differential expression between the treatments of interest was assessed using a moderated t-test.²³ This test is similar to a standard t-test for each probe except that the standard errors are moderated across genes to ensure more stable inference for each gene. Genes were considered significant if the P values, adjusted for multiple

testing by using Benjamini and Hochberg's method were <0.05 . The false discovery rate was thereby controlled to be $<5\%$. Finally, we used Panther software for the analysis of enriched biological processes and molecular functions.²⁴

Validation of candidate genes with Real-Time PCR

Total RNA was converted into cDNA using the RevertAid M-MuLV reverse transcriptase according to manufacturer's protocol. Relative gene expression was performed on the ABI7300 using SYBR Green technology. PCR primers were designed using Primer Express software (Applied Biosystems). 36B4, 18s ribosomal RNA, HPRT and GAPDH were used as housekeeping genes.

Flow cytometry

After sacrificing the mice, blood, spleen and draining lymph nodes (para-aortic and mediastinal) were isolated ($n=6$ per group). Single cell suspensions were obtained by smashing the cells through a 70 μm cell strainer (Falcon, The Netherlands). Blood and spleen cells were lysed using 0.83% NH_4Cl in 0.01 M Tris/HCL pH 7.2. Subsequently 300.000 cells were stained with antibodies in the presence of 1 % mouse serum. FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software. All antibodies were obtained at Immunosource, Belgium.

Statistical analyses

Values are expressed as mean \pm SEM. Data were analyzed with a two-tailed Student's t-test or Mann-Withey U test. Statistical analyses were performed using the InStat3 software. Probability values of $P<0.05$ were considered significant.

Results

Depletion of Tregs using anti-CD25 induces aortic rupture

To study the role of regulatory T cells in aneurysm formation, we depleted Tregs with anti-CD25 treatment in our aneurysm model. Six months old ApoE^{-/-} mice were fed a WT-diet for 4 weeks, after which AngII (1.44 mg/kg/day) was infused using osmotic pumps and mice were kept on a WT-diet. Twenty-four hours after placement of osmotic pumps, mice were injected *i.p.* with a depleting anti-CD25 antibody (100 µg) or rat control IgG. Mice were sacrificed two days or two weeks after anti-CD25 treatment and the number of Tregs in the draining lymph nodes was determined by flow cytometry. The percentage of CD4⁺CD25^{high} cells was almost completely depleted two days after anti-CD25 treatment (Figure 1A). However, the percentage of CD4⁺CD25^{high} cells, although still significantly lowered in the anti-CD25 treated group, already started to recover two weeks after treatment (Figure 1B).

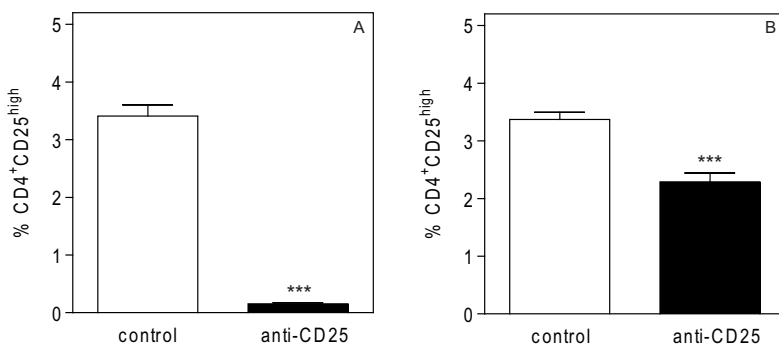


Figure 1: Depletion of Tregs after anti-CD25 treatment

ApoE^{-/-} mice were fed a western type diet for 4 weeks after which osmotic pumps were placed and AngII infusion was started (1.44 mg/kg/day). The next day mice received a single *i.p.* injection of either control IgG or anti-CD25 (100 µg/mouse). Two days (A) or two weeks later (B), mice were sacrificed and the percentage of Tregs in draining lymph nodes was determined by flow cytometry (***) $P < 0.001$).

With regard to aneurysm formation, we observed an extreme decreased survival rate due to rupture of the abdominal aorta in mice treated with anti-CD25 with a 60% survival rate after nine days after injection. In contrast, there were no deaths in the control group (Figure 2). The number of aneurysms were equally distributed between groups when taking into account the dead mice due to rupture (control IgG: 8 of 15 mice had AAA; anti-CD25: 8 of 15 mice had AAA of which 6 mice died due to rupture).

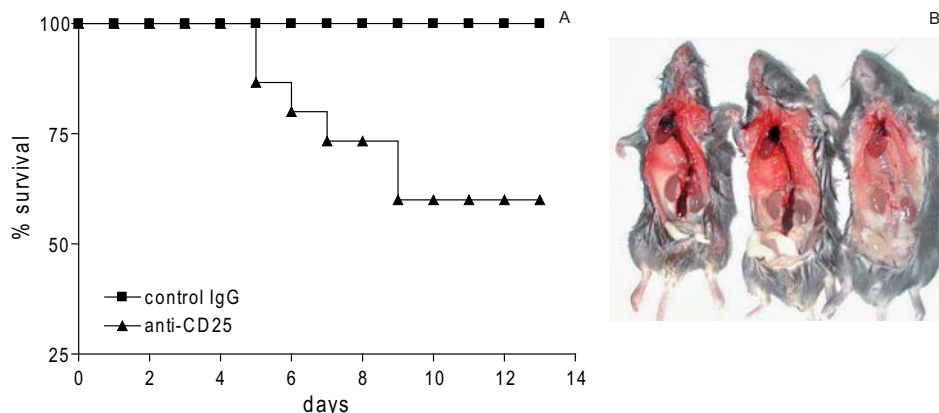


Figure 2: Kaplan-Meier survival curve of mice treated with control IgG or anti-CD25

Mice treated with anti-CD25 died due to rupture of the aorta. At day nine, the survival rate was reduced to 60% (A). Representative pictures of aortic rupture (B).

Micro-array analysis of dissected aorta’s

Micro-array analysis was performed on dissected aorta’s of mice treated with anti-CD25 or control IgG during AngII treatment. During analysis genes were rearranged according to disease pathology and we observed that genes involved in Aneurysm ($P=2.09E-07$) and Aortic Aneurysm ($P=3.16E-06$) were highly regulated in mice treated with anti-CD25. Interestingly, also Glucose Metabolism ($P=5.05E-07$) and Metabolic Disorders ($P=4.6E-06$) were in the top 5 of disease related genes (Table 1). Using a different approach to analyze the micro-array data we arranged the regulated genes according to the affected cellular processes and found that genes involved in the generation of intermediates in the biosynthetic pathway of glucose synthesis and energy metabolism were affected ($P=1.08E-09$). Further in depth analysis revealed that many of the genes involved in the Krebs cycle were significantly lowered in mice treated with anti-CD25 (Table 2). These results were confirmed with real-time PCR where we observed a reduction in the expression of pyruvate dehydrogenase beta (PDHB), pyruvate dehydrogenase alpha1 (PDHA1) and malate dehydrogenase 2, key enzymes involved in the Krebs cycle (Figure 3).

Table 1: Analysis of micro-array data of aorta’s from anti-CD25 treated animals: gene rearrangement according to disease pathology

Disease	Ratio		P-value
Aneurysm	13	93	2,09E-7
Glucose Metabolism Disorders	45	901	5,05E-7
Aortic Aneurysm, Thoracic	6	19	3,16E-6
Metabolic Diseases	58	1405	4,60E-6
Cardiovascular Diseases	63	1670	2,99E-5

Table 2: Analysis of micro-array data of aorta's from anti-CD25 treated animals: genes involved in the Krebs cycle were negatively regulated

Gene	Fold	Bayes P
Malec enzym, supernatant	-20,4	0,021
Pyruvate carboxylase	-16,8	0,03
Pyruvate dehydrogenase (lipoamide) beta	-9,8	0,019
Pyruvate dehydrogenase kinase, isoenzym 4	-5,82	0,011
Isocytate dehydrogenase	-5,53	0,021
Lactate dehydrogenase 2, B chain	-4,51	0,016

Flow cytometry analysis: diet-dependent effects of AngII treatment

AngII induces a wide range of inflammatory responses. To study the effects of AngII on the immune system in ApoE^{-/-} mice, we treated these mice with AngII for 4 consecutive days and used mice on a normal chow diet or mice fed a western-type diet for four weeks. In mice that were fed a chow diet, we observed an increased percentage of T cells and activated T cells in the blood and an increased percentage of CD4⁺CD28^{null} T cells in the draining lymph nodes. However, these effects were not observed in mice on a WT diet (Table 3). In contrast, infusion of AngII in mice on a WT diet induced an increased percentage of CD11c⁺ and CD11b⁺ in blood and a decreased percentage of CD4⁺CD28^{null} cells in lymph nodes.

Table 3: Flow cytometry data after subcutaneously AngII injections

Chow diet							
BLOOD	PBS	AngII		Lymph Nodes	PBS	AngII	
CD4	7,63 ± 1,02	12,68 ± 1,03	P=0.006	CD4	23,70 ± 1,13	27,04 ± 1,90	ns
CD8	6,89 ± 1,08	9,38 ± 0,79	ns	CD8	21,18 ± 1,97	21,62 ± 2,37	ns
CD4CD62L low	0,52 ± 0,05	1,24 ± 0,14	P=0.001	CD4CD62L low	4,62 ± 0,31	5,47 ± 0,40	ns
CD8CD62L low	1,05 ± 0,17	1,39 ± 0,18	ns	CD8CD62L low	5,86 ± 1,16	3,87 ± 0,54	ns
CD4CD25high	1,65 ± 0,20	2,15 ± 0,63	ns	CD4CD25high	4,73 ± 0,24	5,56 ± 0,25	ns
CD4CD28null	2,35 ± 0,50	3,31 ± 0,45	ns	CD4CD28null	8,45 ± 0,59	12,47 ± 1,29	P=0.018
CD8CD28null	5,17 ± 0,83	6,68 ± 0,65	ns	CD8CD28null	17,23 ± 1,72	18,08 ± 2,12	ns
CD11c	37,17 ± 6,30	31,35 ± 4,38	ns	CD11c	1,59 ± 0,30	1,50 ± 0,56	ns
CD11b	42,83 ± 0,42	37,26 ± 4,30	ns	CD11b	8,30 ± 1,11	6,41 ± 0,89	ns

Western type diet							
BLOOD	PBS	AngII		Lymph Nodes	PBS	AngII	
CD4	9,24 ± 0,57	7,220 ± 1,65	ns	CD4	22,58 ± 0,85	18,44 ± 1,58	0,044
CD8	7,82 ± 0,83	5,470 ± 1,55	ns	CD8	19,97 ± 1,03	13,53 ± 1,25	ns
CD4CD62L low	0,84 ± 0,11	0,610 ± 0,16	ns	CD4CD62L low	4,73 ± 0,38	4,06 ± 0,39	ns
CD8CD62L low	1,11 ± 0,18	0,700 ± 0,24	ns	CD8CD62L low	4,67 ± 0,52	3,08 ± 0,59	ns
CD4CD25high	1,71 ± 0,46	2,360 ± 0,54	ns	CD4CD25high	5,78 ± 0,27	5,01 ± 0,22	ns
CD4CD28null	2,67 ± 0,37	2,730 ± 0,56	ns	CD4CD28null	9,18 ± 1,35	8,69 ± 1,10	ns
CD8CD28null	5,52 ± 0,56	4,170 ± 1,26	ns	CD8CD28null	15,72 ± 1,17	10,97 ± 1,08	0,014
CD11c	31,26 ± 1,95	52,780 ± 7,46	P=0.019	CD11c	1,83 ± 0,32	1,90 ± 0,26	ns
CD11b	35,47 ± 2,15	60,520 ± 9,74	P=0.02	CD11b	9,60 ± 1,47	8,81 ± 1,02	ns

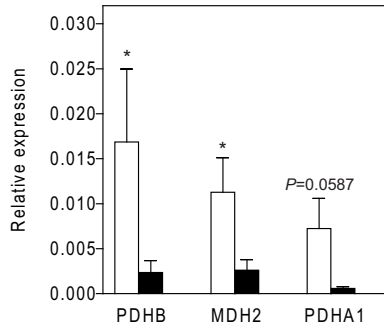


Figure 3: Expression of genes involved in Krebs cycle confirmed by real-time PCR
Gene expression of pyruvate dehydrogenase beta (PDHB), pyruvate dehydrogenase alpha1 (PDHA1) and malate dehydrogenase 2 (MDH2) were reduced in animals treated with anti-CD25 (* $P < 0.05$).

Effect of anti-CD3 treatment on aneurysm formation

To further elucidate the importance of Tregs in aneurysm formation, we aimed to increase the number of Tregs by anti-CD3 treatment prior to AngII infusion. The working mechanism of this strategy is depicted in Figure 4A.

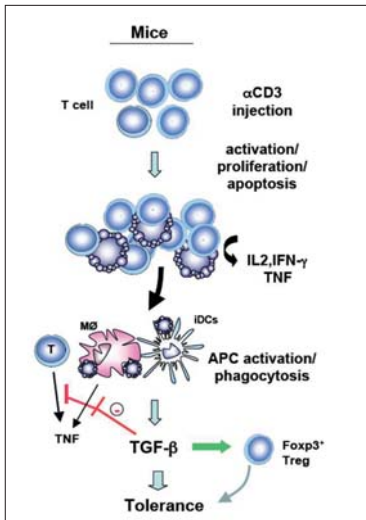


Figure 4: Mechanism of Treg induction by T cell induced apoptosis

Proposed model of T cell apoptosis induced tolerance. Anti-CD3 treatment results in T cell apoptosis. Macrophages and immature DCs digest apoptotic T cells and produce TGF- β . TGF- β down-regulated TNF- α induced immune responses but also induces Tregs. (Adapted from Perruche *et al.* 2008)

In short, injection of intact mitogenic CD3-specific antibody will result in apoptosis of T cells. Phagocytosis of these apoptotic cells by macrophages or dendritic cells will result in TGF- β production and a subsequent increase of Tregs. Treatment with anti-CD3 in our aneurysm model did not affect body weight (data not shown) and more importantly, mice did not die as a consequence of the induction of aneurysm formation. When we assessed the percentage of T cells in blood one week after anti-CD3 treatment, we observed a decreased percentage of CD3⁺, CD4⁺ and CD8⁺ T cells in comparison to control treated mice (Figure 5A, B and C, respectively).

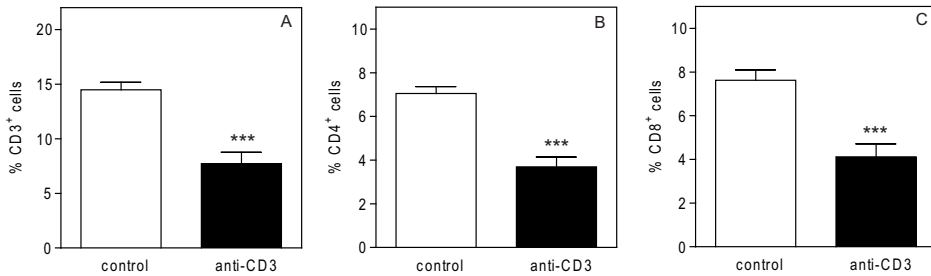


Figure 5: Flow cytometry analysis of blood samples one week after treatment

One week after anti-CD3 treatment blood was drawn by tail vein bleeding and the percentage of T cells was determined by flow cytometry. The percentage of CD3⁺ T cells (A), CD4⁺ T cells (B) and CD8⁺ T cells (C) was reduced in animals treated with anti-CD3 (***) ($P < 0.001$).

However, when we determined the percentage of Tregs in spleen, para-aortic lymph nodes and in mediastinal lymph nodes at four weeks of AngII treatment, we observed a non-significant reduction in the percentage of CD4⁺CD25^{high} cells (Figure 6). In addition, after isolation of the aorta's we observed that the number of different types of aneurysms was not affected. Both groups showed equal amounts of Type 0-IV AAA and TAA (Figure 7).

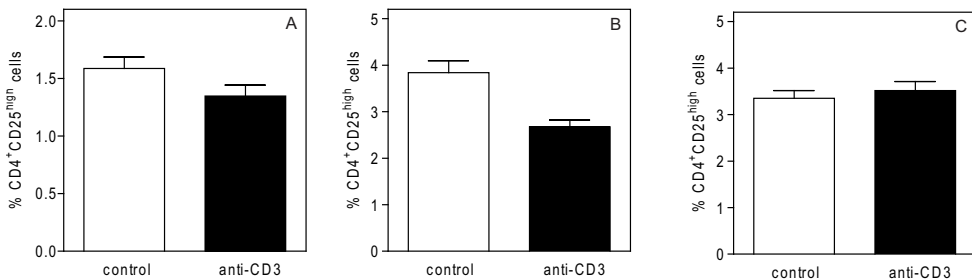


Figure 6: Treg number were not increased in mice treated with anti-CD3

Mice were treated with anti-CD3 or control IgG. AngII infusion was continued for 30 days after which mice were sacrificed. The percentage of Tregs was determined by flow cytometry in spleen, para-aortic lymph nodes and mediastinal lymph nodes (A, B and C, respectively).

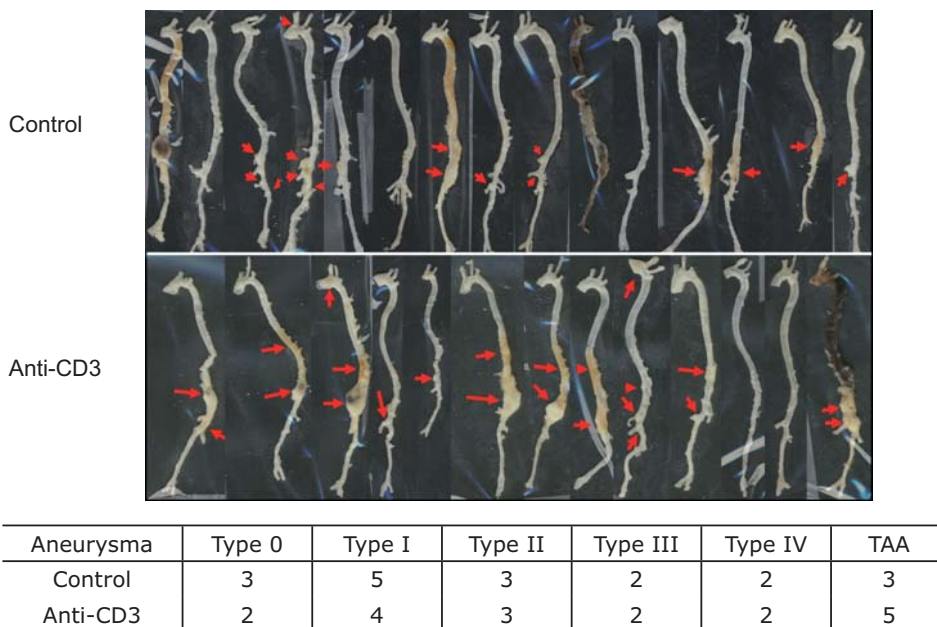


Figure 7: Quantity and severity of aneurysm was not affected by anti-CD3 treatment

After 30 days of AngII infusion, mice were sacrificed and the entire aorta was isolated and photographed. Aneurysm formation was classified (AAA: Type 0-IV and TAA).

Discussion

In the present study we determined the role of Tregs in the formation of AAA. Within AAA there is a prominent role for inflammation in the initiation and progression of disease. Various types of immune cells have been found in AAA and in contrast to atherosclerosis, which is specifically marked by a Th1-type immune response, both Th1 and Th2 responses play an important role in AAA.^{25, 26} It has been shown that AngII injections lead to inflammation and to AAA formation.²⁷ Therefore, we investigated the effect of AngII injection in mice on a normal chow diet compared to mice on a high-fat, high cholesterol, WT diet. We observed that in mice on chow, the number of CD4⁺ T cells and moreover, the activated CD4⁺ T cells are increased in circulation by infusion of AngII. Our findings are in line with prior studies, which demonstrated that AngII increases the number of circulating CD4⁺ lymphocytes and furthermore, that the number of T cells in the aortic adventitia, and particularly in the peri-adventitial fat, is increased.⁸ However, when comparing the two diets, we did not observe an increase in CD4 T lymphocytes in mice on the WT diet. In literature it is known that the number of circulating CD4⁺CD28^{null} and CD8⁺CD28^{null} cells are enriched in AAA patients compared to healthy controls.^{11, 12, 28} CD4⁺CD28^{null} T cells are a subgroup of inflammatory T cells which lack the costimulatory molecule CD28. Functionally, these T cells are capable of producing

large amounts of interferon- γ , perforin and granzyme B providing them with the capacity to lyse cells.²⁹ Also in the present study we observed an increase in CD4⁺CD28^{null} T cells in the draining lymph nodes in mice on chow. Again, we did not observe these changes in mice on the WT diet. On the other hand, mice on a WT diet had higher percentages of macrophage and dendritic cells markers, CD11b and CD11c, respectively. These molecules are also integrins and increased expression may facilitate the transmigration of macrophages or dendritic cells into the arterial wall.³⁰ Taken together, our data showed that AngII injection in mice on chow diet indeed induced T cell proliferation/activation and the induction of CD4⁺CD28^{null} T cells. Moreover we showed that WT diet has a possible compromising role in this activation. Consequently, we have to conduct more research on the role of AngII infusion and the WT diet in aneurysm formation.

To investigate the potential role of Tregs in aneurysm formation we performed several experiments in which we depleted or induced Tregs. CD4⁺CD25⁺ regulatory T cells are implicated in the maintenance of self-tolerance and play a protective role in atherosclerosis. Recently, the adaptive transfer of Tregs has been shown to be effective in quenching autoimmune and allergic diseases.^{31, 32} At present, we show that depletion of Tregs via anti-CD25 treatment resulted in increased death as a result of aortic rupture, suggestive of a protective role for Tregs in aneurysm rupture. It has recently been shown that ApoE^{-/-}IFN- γ ^{-/-} dKO mice experienced death with similar survival curves as the one observed after Treg depletion. Also in this experiment mice died due to rupture of the abdominal aorta.³³ Lack of IFN- γ is associated with reduced frequency and function of Treg and a subsequent heightened susceptibility to Collagen Induced Arthritis (CIA) and Experimental Autoimmune Encephalomyelitis (EAE).^{34, 35} Therefore the study of King *et al.* could provide us with a direct link between IFN- γ production, Tregs and rupture of AAA.³³ Furthermore, when we assessed gene expression in aorta's of anti-CD25 treated mice we observed a high regulation of genes involved in aneurysms but also in glucose metabolism. Especially expression of genes involved in the Krebs cycle was negatively influenced by anti-CD25 as confirmed by real-time PCR. As a result, we can hypothesize that the reduced production of these essential products can increase cell death which could eventually affect the integrity of the aortic wall and result in rupture.

To further support the results obtained by anti-CD25 treatment, we attempted to increase Treg numbers. Treatment with non-mitogenic anti-CD3 has been shown to induce Tregs by induction of T cell apoptosis.³⁶ Although we induced the necessary T cell death by anti-CD3 treatment, we observed only a trend towards a reduced percentage of Tregs in spleen and draining lymph nodes. In line with the observation that Tregs were not affected, AAA was also not affected by anti-CD3 treatment.

In conclusion, we have observed a possible protective effect of Tregs in AAA formation and rupture but could not confirm these data because of failed induction of Tregs. Therefore, procedures to induce Tregs *in vivo* should be further optimized. For example: adoptive transfer of Tregs or injection of IL-2 murine antibody complexes could provide us with an effective method to induce Tregs *in vivo*³⁷ and to elucidate the precise role of Tregs in aneurysm formation or rupture.

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