Interleukin-33 augments Treg cell levels: a flaw mechanism in atherosclerosis

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ABSTRACT: **Background:** Atherosclerosis is a well-established inflammatory disease in which T helper 1 (Th1) cells play a key role. Regulatory T (Treg) cells drive a shift from Th1 to Th2 response and were shown to be reduced in atherosclerosis. ST2/interleukin (IL)-33 signal was found to promote Th2 response, attenuating atherosclerotic plaque progression.

Objectives: To evaluate the effect of IL-33 on Treg cell number.

Methods: We employed flow cytometry to determine Treg cell number, as well as ST2 levels, among splenocytes of C57BL/6J vs ApoE-/- mice. Soluble ST2 (sST2) levels were detected by enzyme-linked immunosorbent assay.

Results: IL-33 contributed to an increase in Treg cells, but this association was attenuated in ApoE knockout (ApoE-/-) atherosclerotic mice. As a possible mechanism we demonstrated a reduction in the levels of CD4+ST2+ cells by flow cytometry, which is cotemporary to the previously described decrease in Treg cells in ApoE-/- mice. Additionally, the serum level of the soluble ST2 (sST2) decoy receptor was higher in ApoE-/- mice than in control animals.

Conclusions: Our results suggest that a repressed ST2/IL-33 signaling may contribute to the decrease in Treg cells observed in atherosclerosis.

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KEY WORDS: ST2, interleukin-33 (IL-33), Treg cells, apolipoprotein E (ApoE), inflammation

Atherosclerosis is an inflammatory disease of the arterial wall, in which lymphocytes play a major role [1]. T helper 1 cells and Th2 cells are key regulators of inflammation atherogenesis [2]. Cytokines involved in Th1 action, such as interferon-gamma and interleukins 12 and 18, have been shown to promote atherosclerosis [3,4]. On the other hand, Th2-related cytokines such as IL-10, IL-5 and IL-4 were shown to have anti-atherogenic effects [5,6]. T regulatory cells act as important regulators of the Th1/2 balance by forming a specific subgroup of T cells with suppressive effects on the immune system. We recently suggested a protective role for Treg cells in a mice model of atherosclerosis [7] as well as reduced levels and suppressive properties of these cells in the setting of acute coronary syndrome [8].

The ST2 receptor is a member of the IL-1 receptor family that takes part in the inflammatory process, mainly by modulating Th2 response, and its expression stands in direct correlation with the levels of inflammatory cytokines such as IL-1, IL-5, IL-6 and tumor necrosis factor-alpha [9]. ST2 has both a trans-membranal form, ST2 ligand, and a soluble form. Both ST2L and sST2 are induced by cardiomyocytes and fibroblasts in response to mechanical stress and are related to cardiovascular disease and atherosclerosis [10-12]. IL-33, which is the ligand of ST2, seems to have a protective effect in atherosclerosis and has been associated with IL-5 secretion and the production of oxidized low density lipoprotein antibodies [13]. In addition, IL-33 was shown to reduce the number of macrophage foam cells inside the atherosclerotic plaque [14]. sST2 serves as a decoy receptor to IL-33, and its presence prevents IL-33 binding to ST2L. Administration of sST2 was shown to block the beneficial effect of IL-33 in animal models of atherosclerosis [13,15].

Eventually, both ST2/IL-33 signal and Treg cells regulate the shifting of Th1 to Th2 inflammatory response, and both were shown to have beneficial effects on atherosclerosis progression. However, the interaction between the ST2/IL-33 signal and Treg cells is still unknown and stands at the center of this study.

MATERIALS AND METHODS

ANIMALS AND CELLS

Animal experiments were performed with the permission of the local ethics committee. Eight (adult) and five (young) month old male C57BL/6J and ApoE-/- mice were obtained

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Th = T helper
IL = interleukin

Treg = T regulatory
ST2L = ST2 ligand
ST2 = soluble ST2

from Harlan Laboratories (Israel). Mice were consistently kept under normal conditions with free access to water and a high fat diet. The pathological lipid profile and advanced atherosclerotic lesions of ApoE-/- mice are routinely assessed in our lab as previously described [7,16]. Mouse spleens were explanted, mechanically minced through 70 µm nylon mesh, and red cells were lysed using 155 mM ammonium chloride solution. Mouse red blood cell-depleted splenocytes were cultured in complete RPMI medium supplemented with 10% fetal bovine serum, 5% CO_2 at 37°C. Soluble anti-CD3 mA (3 µ/ml) and anti- CD28 (2.5 µg/ml) were added to the cultures to stimulate ST2 expression as previously described [9]. Alternately, recombinant IL-33 (ProSpec-Tany TechnoGene Ltd, Israel) at concentrations of 0, 10 and 100 ng/ml were added to cultures.

FLOW CYTOMETRY

Splenocytes were suspended in phosphate-buffered saline (10^6/100 µl) and stained with anti-mouse T1/ST2 monoclonal antibody (MD Bioproducts, Israel), and the percentage of CD4+ST2+ cells out of the total number of CD4+ cells was assessed. For assessing the number of Treg cells, splenocytes were stained with anti-mouse CD4-FITC and anti-mouse CD25-PE (Miltenyi, Germany) for 30 min at 4°C, washed and resuspended in 0.5 ml fixation buffer for 1 hour at 4°C and then washed twice with permeabilization buffer (E-Bioscience, USA). For CD4+CD25+Foxp3+ triple staining, cells were also stained with anti-mouse Foxp3-APC antibody (Miltenyi, Germany). Next, 8 x10^4 cells were acquired by flow cytometry and the percentage of Treg cells out of the total number of CD4+ cells was calculated.

ELISA

Levels of sST2 were measured by enzyme-linked immunosorbent assay (MD Bioproducts). Briefly, blood was drawn from the inferior vena cava of adult ApoE-/- and control mice (n=5/group) and sera were analyzed according to the manufacturer's instructions.

STATISTICAL ANALYSIS

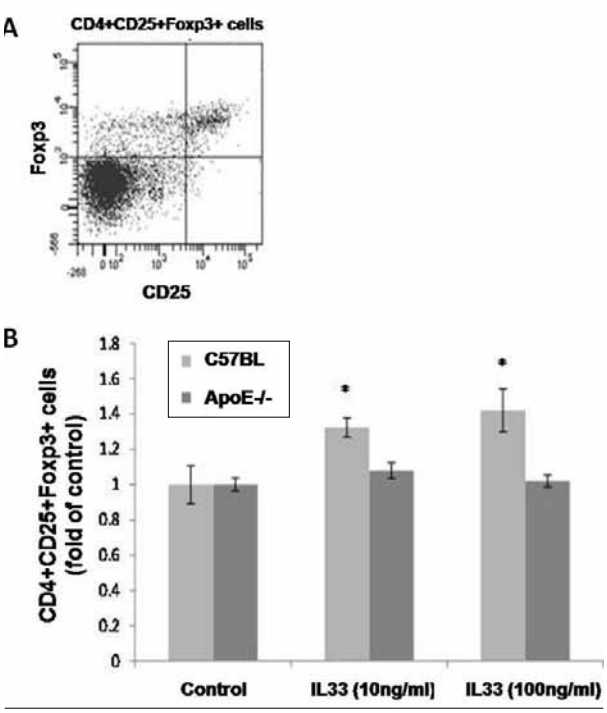
Comparisons between the two groups were performed using Student's *t*-test under the assumption that the two samples were of unequal variance with a one-tail distribution. Comparisons between multiple groups were performed using a one-way ANOVA test. $P < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

THE EFFECT OF IL-33 STIMULATION ON TREG CELL LEVEL

Spleen derived-lymphocytes harvested from both ApoE-/- and C57BL control mice were incubated with growing concentrations of recombinant IL-33. Following 48 hours of incubation, we assessed by flow cytometry the level of the CD4+CD25+Foxp3+ cells [Figure 1]. In control mice, IL-33 induced a dose-dependent elevation in CD4+CD25+Foxp3+ cells (6.57% ± 0.5, 8.7% ± 0.13 and 9.3% ± 0.65 following treatment with 0, 10 and 100 ng/ml of recombinant IL-33, respectively, $P < 0.005$).

Figure 1. Effect of IL-33 stimulation on the expression of Treg cells. **[A]** Representative flow cytometry analysis of CD4+CD25+Foxp3+ cells. **[B]** Comparison of Treg cell levels following stimulation of splenocytes derived from ApoE-/- mice (dark grey) vs. control mice (light grey) using 0, 10 and 100 ng/ml of recombinant IL-33.

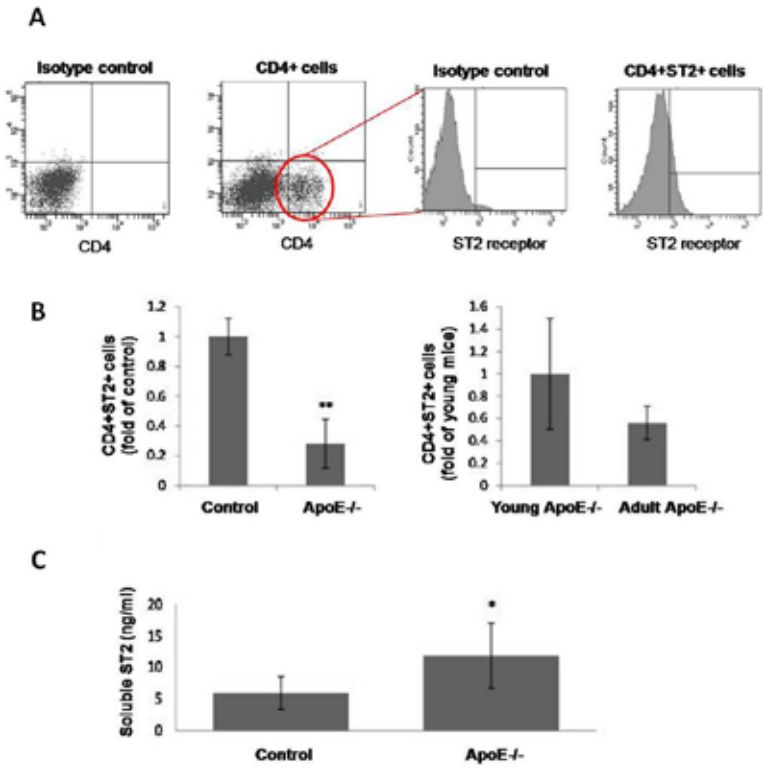


Interestingly, while applying a similar IL-33 stimulation on splenocytes derived from atherosclerotic ApoE-/- mice, no significant increase in the level of CD4+CD25+Foxp3+ cells was noticed ($P > 0.1$). Treg cell number and suppressive activity were previously shown to be defective in atherosclerosis, leading to skewing of the immune response from the more protective TH2-mediated response to TH1 [7]. However, the nature of the defect in Treg cell function is still unknown. Here, we suggest that a flawed response to IL-33 potentially contributes to Treg cell dysfunction in atherosclerosis.

CD4+ST2+ CELLS LEVELS ARE REDUCED IN ATHEROSCLEROTIC MICE

As a possible mechanism of reduced response to IL-33 signal, we assessed ST2 receptor levels by flow cytometry. As described previously, we did not detect the expression of ST2 receptor on Treg cells [17]. Since CD4+ cells are the precursor population of Treg cells [18], we measured the percentage of

Figure 2. Potential mechanisms. **[A]** CD4+ST2+ are reduced in atherosclerosis: the percentage of CD4+ST2+ cells out of the total number of CD4+ splenocytes was assessed by flow cytometry (representative analysis). **[B]** Expression of CD4+ST2+ cells, compared to control mice in ApoE-/- mice (left panel) and in young vs. adult ApoE-/- mice (right) (absolute levels appear in the text). **[C]** High expression of serum soluble ST2 in atherosclerosis: serum levels of soluble ST2 (ng/ml) in ApoE-/- vs. control mice as measured by ELISA. * $P < 0.05$, ** $P < 0.01$.



CD4+ST2+ cells out of the total number of CD4+ lymphocytes in ApoE-/- vs control mice (n=5/group) [Figure 2A]. Decreased levels of ST2 expression were found in ApoE-/- mice compared to C57BL mice (0.46% ± 0.2 vs. 1.67% ± 0.2, $P < 0.001$). A similar trend, although not statistically significant, was detected in young ApoE-/- mice, where the level of CD4+ST2+ cells was 1.77 fold higher than in older ApoE-/- mice.

SERUM SOLUBLE ST2 LEVELS IS INCREASED IN ATHEROSCLEROTIC MICE

Soluble ST2 is a decoy receptor of IL-33 and acts by blocking IL-33 action on the ST2 receptor. We measured the serum level of soluble ST2 by ELISA and found the level to be markedly elevated in ApoE-/- mice compared to controls (11.8 ± 5.16 ng/ml vs. 5.94 ± 2.59 ng/ml, respectively, $P < 0.05$) [Figure 2B]. This finding suggests an additional mechanism by which an elevated level of soluble ST2 in atherosclerosis is responsible for an attenuated response of CD4+ST2+ cells to IL-33, which subsequently affects Treg cell number.

ELISA = enzyme-linked immunosorbent assay

CONCLUSIONS

Both the ST2/IL33 signal system and Treg cell activities were shown to attenuate TH1 type systemic inflammation, provoking beneficial effects on the progression of atherosclerosis. In the present study we show that IL-33 stimulation of regular lymphocytes, but not of lymphocytes derived from atherosclerotic mice, induce an increase in Treg cells. As a possible explanation for this flawed response, we demonstrated a decrease in the level of ST2 receptor in CD4+ cells in atherosclerotic mice. This phenomenon may indicate a flawed ST2/IL-33 interaction in the setting of atherosclerosis/systemic inflammation. Finally, we demonstrated high serum sST2 levels in atherosclerotic mice, suggesting an additional mechanism for an impaired ST2/IL-33 interaction, that is possibly responsible for the reduced level of Treg cells in atherosclerosis.

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Capsule

A voltage-gated sodium channel is essential for the positive selection of CD4+ T cells

The sustained entry of Ca2+ into CD4+CD8+ double-positive thymocytes is required for positive selection. Lo and co-workers identified a voltage-gated Na+ channel (VGSC) that was essential for positive selection of CD4+ T cells. Pharmacological inhibition of VGSC activity inhibited the sustained Ca2+ influx induced by positively selecting ligands and the in vitro positive selection of CD4+ but not CD8+ T cells. In vivo short hairpin RNA (shRNA)-mediated knockdown of the gene encoding a regulatory β-subunit of a VGSC specifically inhibited the positive selection of CD4+ T cells. Ectopic expression of VGSC in peripheral and CD4+ T cells bestowed the ability to respond to a positively selecting ligand, which directly demonstrated that VGSC expression was responsible for the enhanced sensitivity. Thus, active VGSCs in thymocytes provide a mechanism by which a weak positive selection signal can induce the sustained Ca2+ signals required for CD4+ T cell development.

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Eitan Israeli

Capsule

Chromatin organization is a major influence on regional mutation rates in human cancer cells

Cancer genome sequencing provides the first direct information on how mutation rates vary across the human genome in somatic cells. Testing diverse genetic and epigenetic features, Schuster-Böckler et al. show that mutation rates in cancer genomes are strikingly related to chromatin organization. Indeed, at the megabase scale, a single feature – levels of the heterochromatin-associated histone modification H3K9me3 – can account for more than 40% of mutation-rate variation, and a combination of features can account for more than 55%. The strong association between mutation rates and chromatin organization is upheld in samples from different tissues and for different mutation types. This suggests that the arrangement of the genome into heterochromatin- and euchromatin-like domains is a dominant influence on regional mutation-rate variation in human somatic cells.

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Eitan Israeli

The tragedy in the lives of most of us is that we go through life walking down a high-walled land with people of our own kind, the same economic situation, the same national background and education and religious outlook. And beyond those walls, all humanity lies, unknown and unseen, and untouched by our restricted and impoverished lives

Florence Luscomb (1887-1985), American architect and women's suffrage activist

In the cellars of the night, when the mind starts moving around old trunks of bad times, the pain of this and the shame of that, the memory of a small boldness is a hand to hold

John Leonard (1939-2008), American literary critic