MATERIALS AND METHODS

Mice

All mice used were bred in the pathogen-free facility at the New Research Building (Harvard Medical School, Boston, MA), in accordance with the guidelines of the Committee of Animal Research at the Harvard Medical School and the National Institutes of Health Animal Research Guidelines. Sulfatrim (Sulfamethoxazole/Trimethoprim) was administered to mice starting at birth and continuing for the duration of the experiment.

The *ldlr*$^{-/-}$*rag1*$^{-/-}$ mice were generated by breeding *ldlr*$^{-/-}$ and *rag1*$^{-/-}$ C57BL/6 mice, both purchased from Jackson Laboratories (Bar Harbor, ME). Age matched groups of female *ldlr*$^{-/-}$ *rag1*$^{-/-}$ and *ldlr*$^{-/-}$ mice were fed high-fat diet (HFD) containing 1.25% cholesterol (Cat. No. D12108C, Research Diets Inc.) [1] for seven weeks starting at eight weeks of age. In the main cohort, mice were treated with isotype control IgG, anti-CD90.2 or IL-2 complexes for the last five weeks of the experiment. In a follow up study mice were fed HFD for eight weeks and injected with PBS or IL-2 complexes for the last four weeks of the experiment.
Mice were randomly assigned to groups.

Innate lymphoid cell depletion and expansion

Mice were injected intraperitoneally twice a week with 0.25 mg of cell depleting rat anti-mouse anti-CD90.2 mAb (clone 30H12; BioXCell) or an IgG2b isotype control antibody (clone LTF2; BioXCell). In order to expand CD25^{+} ILCs, mice were injected i.p. twice a week with 6 □g IL-2/JES6-1-complexes that were prepared by mixing IL-2 (R&D) with anti-IL-2 (clone:JES6-1A12, BioXCell) [2]

IL-5 blockade

Mice were fed HFD for seven weeks and injected with IL-2/JES6-1 co-administered with either anti-IL-5 (15 mg/injection; clone: TRFK5, BioXCell) or an isotype control (15 mg/injection; clone: HRPN) for the last four weeks before sacrifice.

Tissue digestion and cell sorting

Atherosclerotic artery from the ascending aorta to iliac bifurcation was to enzymatic digestion. In some experiments, perivascular adipose tissue (PVAT) was removed from aortas before digestion. Aortas were minced and subsequently digested with 450 U/ml collagenase I-S (C1639, Sigma), 125 U/ml collagenase XI (C7657, Sigma), 60 U/ml hyaluronidase (H3506, Sigma) and 60 U/ml DNase1 (D5025, Sigma) for 1 hour. Cells were washed with PBS twice and then stained with fluorochrome-conjugated antibodies and viability stain. Cells sorted by FACS (FACS Aria, BD Biosciences) were resuspended in RPMI containing 10% fetal bovine serum with PMA (1ug/ml) and ionomycin (20 ng/ml) and cultured for 24h at 37 °C before collection of supernatant.

Liver was dissected and passed through a 70 μm cell strainer. Non-parenchymal cells from the liver were separated from parenchymal cells by centrifugation at 50 RCF. The non-parenchymal cells in the supernatant were isolated and put on a Lympholyte gradient (Cedarlane, Ontario, Canada). Cells were centrifuged at 1250 RCF and the lymphocyte fraction was isolated. Cells were washed and resuspended in PBS.

Epididymal visceral adipose tissue was minced and incubated at 37 °C for 40 minutes with 4 mg/ml collagenase type II (Worthington) supplemented with 0.5% bovine serum albumin (BSA). Digested tissue was passed through 70 μm cell strainer and washed with PBS (+0.5% BSA) and centrifuged at 500 RCF where after cells were resuspended in PBS.

Flow cytometric analyses and cell sorting
Splenocytes, mesenteric lymph nodes and aortic digests were stained using the following antibodies (from Biolegend unless indicated): CD90.2 (53-2.1), CD127 (A7R34), Siglec-F (E50-2440, BD Biosciences), CD25 (PC61), CD11b (M1/70), Gr-1 (RB6-8C5), B220 (RA3-6B2), NKp46 (29A1.4), NK1.1 (PK136) CD45.2 (104), ST2 (DIH9). Live/Dead Viability staining (Life Technologies) or 7-AAD (BD Pharmingen) was used for exclusion of non-viable cells. Samples were run on a DXP12 flow cytometer (Cytek) and analyzed using FlowJo software (Treestar).

Multiplexed Cytokine assays
Serum cytokines were analyzed using a Luminex bead-based multiplex assays specific for IL-2, 4, 5, 6, 10, 12p40, 12p70, IL17a, IFNγ, TNFα, and MCP-1. Recombinant cytokine standards (Bio-Rad, Hercules, CA) were used to calculate cytokine concentrations and data were analyzed using StarStation 2.3 software (Applied Cytometry, Sheffield UK). Cytokines in supernatants from PMA/ionomycin-treated aortic cells were measured by multiplex analysis (Eve Biotechnology, Calgary, Alberta, Canada).

Serum lipid analysis
Mouse blood cholesterol and triglycerides were quantified on the c501 module of the Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN), and lipoprotein profiles were further analyzed by high-performance liquid chromatography (HPLC; Liposearch, Tokyo, Japan).

Immunohistochemical staining of aortic lesions and liver
Frozen sections (8 μm) were stained with antibodies specific for Mac-3 (M3/84, BD Pharmingen) to detect macrophages or with Van Gieson staining kit (Sigma) for collagen assessment. Sections were stained with Oil Red O to measure neutral lipids. Lesions area was presented as average± SEM. Liver sections (formalin fixed and parafin-embedded) were stained with H&E and Masson's trichrome.

Quantitative RT-PCR (qRT-PCR) analysis
Total RNA was extracted from cultured cells by Rneasy kit (QIAGEN Inc., Valencia, California, USA) and reverse-transcribed using the ThermoScript RT-PCR system and random hexamer primers according to the manufacturer's instructions (Invitrogen). cDNA was amplified by real-time PCR with SYBR Green PCR mix (Applied Biosystems) and Step-One Detection System (Applied Biosystem) according the manufacturer's instructions. Levels of specific gene expression in the samples were normalized to expression of *hprt* and values were expressed as relative to control IgG treated mice.

Serum markers of liver function
Alanine aminotransferase, aspartate aminotransferase, and total bilirubin were measured in serum using the ALTL, ASTL and BILTS assays (all Roche Diagnostics) and the c501 analyzer.

Statistical analysis
All statistical analyses were performed using Prism software. Differences between two groups of mice were analyzed by Student's *t* test or by the Mann-Whitney U tests (for nonparametric data) and expressed as mean ± SEM. One-way ANOVA with Tukey's Multiple Comparison post test or Kruskal-Wallis with Dunn's multiple comparison tests for three or more group experiments was used depending on normal distribution. A value of $p<0.05$ was considered to be significant. For experiments with n≤3 no statistical test was performed.

References

1. Lichtman AH, Clinton SK, Iiyama K, Connelly PW, Libby P, Cybulsky MI. Hyperlipidemia and atherosclerotic lesion development in ldl receptor-deficient mice fed defined semipurified diets with and without cholate. *Arteriosclerosis, thrombosis, and vascular biology*. 1999;19:1938-1944
2. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, Grey ST, Sprent J. In vivo expansion of t reg cells with il-2–mab complexes: Induction of resistance to eae and long-term acceptance of islet allografts without immunosuppression. *The Journal of Experimental Medicine*. 2009;206:751-760