Adenosine suppresses TNF-α production by FFA-activated caMϕ (1). We studied the effect of AR stimulation on TNF-α production, as FFA-induced TNF-α is a major mediator of insulin resistance in the adipose tissue (2). We stimulated cells with palmitate, which is the most abundant FFA in the adipose tissue. We found that AR stimulation with NECA suppressed TNF-α release by palmitate-stimulated A_{2A}AR deficient (1) and WT (Figure 1) but not A_{2B}AR deficient (data not shown) macrophages. We conclude that A_{2B}ARs mediate the adenosine suppression of TNF-α production by FFA-stimulated macrophages.

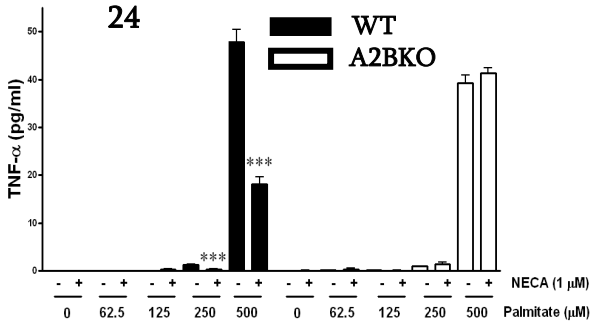

Figure 1. Bone marrow-derived macrophages from WT or A_{2B}AR deficient mice were treated with NECA 30 min before palmitate and TNF-α was quantitated in the supernatant taken 24 h after palmitate treatment. To prepare 20 mM palmitate solution, 5.14 mg palmitic acid (SIGMA) was dissolved in 888 μl of 10 mM NaOH at 70°C for 30, and then 111 ul of 5% FA-free BSA (SIGMA) was added drop by drop to the solution. This stock solution was further diluted with serum free DMEM for the treatment of macrophages. Vehicle solutions were prepared in the same way without palmitic acid but in the presence of NaOH and FA-free BSA.. ***p<0.001 vs. vehicle for NECA. Data shown are mean and s.e of n=3-4 wells. 1 of 3 experiments with similar results is shown.

A_{2B}ARs maintain insulin sensitivity and lipid homeostasis in mice (1). To begin to examine the role of A_{2B}ARs in metabolism, we studied the effect of A_{2B}AR

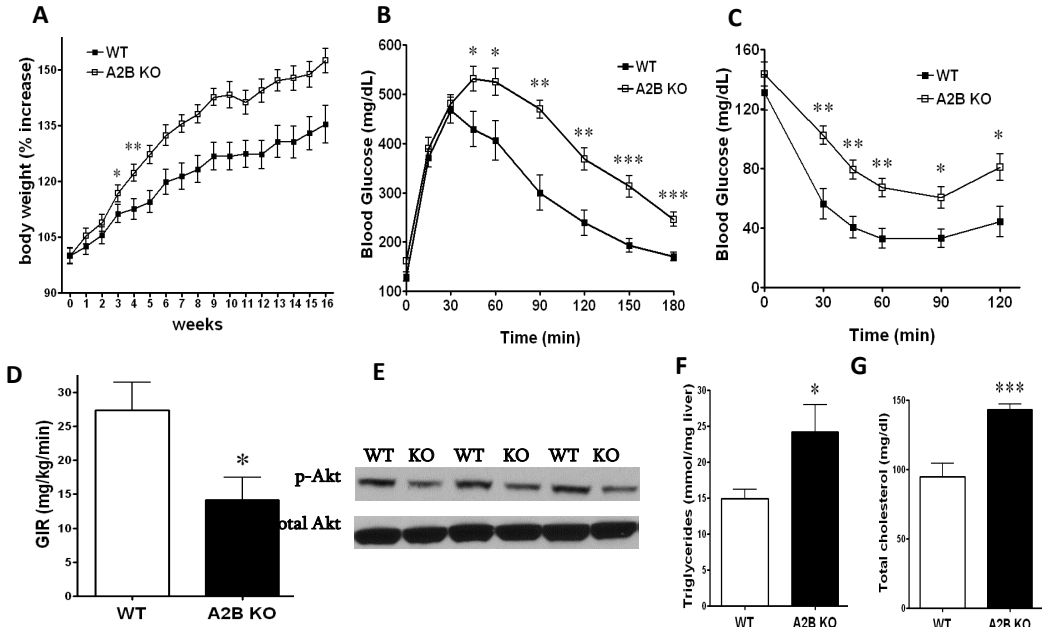

Figure 2. Male C57BL/6J WT or A_{2B}AR KO mice were maintained on regular rodent diet until 8-10 weeks of age, after which the mice were started on CD for 16 additional weeks. (A) Body weight during a 16-week-long CD. (B) ipGTT, (C) ipITT, (D) GIR, (E) Akt phosphorylation, (F) liver triglyceride, and (G) plasma cholesterol of WT and A_{2B}AR KO mice were measured after a 16-week-long CD. For ipGTT, mice were fasted overnight, and glucose (1g g/kg body weight) was injected intraperitoneally. Blood glucose was measured before and after injection at the indicated time points using Accu-Chek Active glucose monitoring system (Roche Diagnostic Co., Indianapolis, IN). ipITT was conducted by injecting 0.75 U insulin/kg body weight intraperitoneally and measuring glucose levels before and after the injection. Hyperinsulinemic–euglycemic clamp studies and GIR measurement were conducted by the Vanderbilt-NIH Mouse Metabolic Phenotyping Center in Nashville. For assessing insulin-induced Akt phosphorylation (Ser^{473}) in skeletal muscle, mice were injected with insulin (1U/kg) intraperitoneally, and 8 min after the insulin injection, gastrocnemius muscle was harvested for western analysis. TGs and cholesterol were quantitated with commercial kits. Except for GIR, where results are from 1 experiment, results are representative of 3 experiments; *n* = 7-10 mice/group in each experiment. Data are presented as mean ± s.e. *p < 0.05; **p < 0.01 vs. WT, ***p < 0.001 vs. WT.

inactivation on weight accumulation and insulin homeostasis of chow diet (CD)- (10% of calories comes from fat) and high fat diet (HFD)-fed mice (60% of calories comes from fat). A_{2B}AR KO ($A_{2B}AR^{-/-}$) mice gained more weight (Figure 2A) and had increased fat depots (1) than WT controls during a 16-week CD and the weight of A_{2B}AR KO mice and WT littermates on HFD was comparable (1). Intraperitoneal (i.p.) glucose tolerance test (ipGTT) showed that glucose tolerance was impaired in CD-fed A_{2B}AR KO vs. WT animals (Figure 2B). Insulin tolerance test (ipITT) showed that A_{2B}ARs are important in maintaining insulin sensitivity in CD-fed mice, as insulin tolerance was decreased in A_{2B}AR KO mice when compared to WT littermates (Figure 2C). ipGTT and ipITT were comparable in HFD-fed A_{2B}AR KO and WT mice (data not shown). Since most metabolic parameters were comparable between WT and KO mice on HFD (1), we conducted all further studies on mice on CD. We found decreased glucose infusion rate (GIR) in A_{2B}AR KO mice during hyperinsulinemic-euglycemic clamp studies (Figure 2D) and decreased Akt phosphorylation in muscle of A_{2B}AR KO mice (Figure 2E) following insulin injection, confirming decreased insulin sensitivity in KO vs. WT mice. In addition, CD-fed A_{2B}AR KO animals displayed elevated liver triglyceride (3) and plasma cholesterol levels (Figure 2 F,G) when compared to WT mice. Taken together, these findings indicate that A_{2B}ARs are required for maintaining glucose and lipid homeostasis in CD-fed mice.

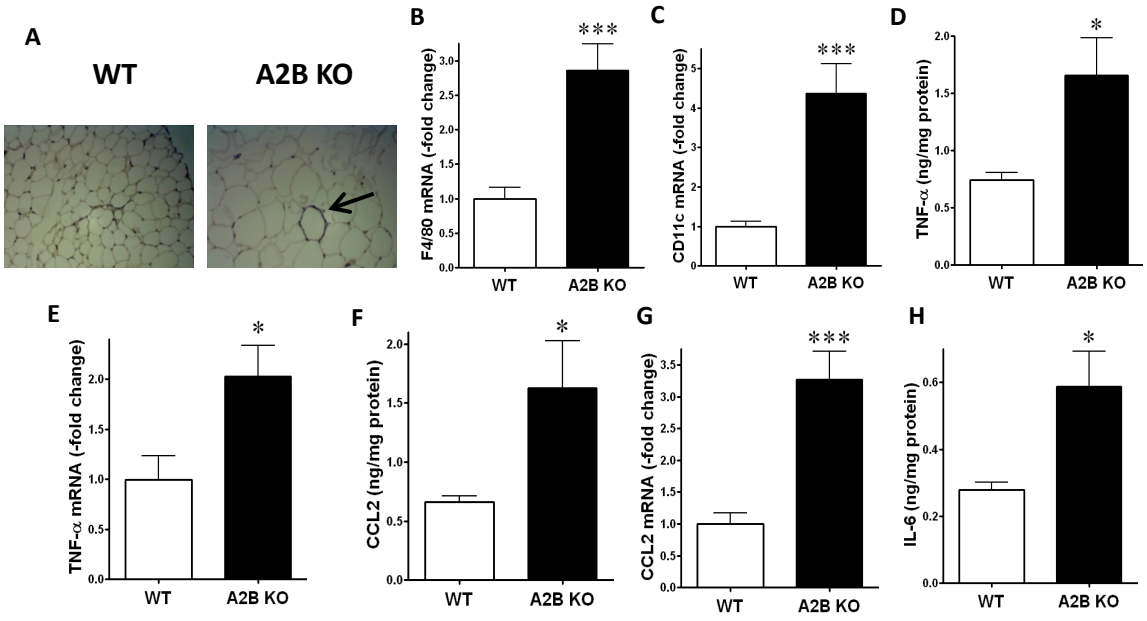

Figure 3. A_{2B}AR deletion exacerbates adipose tissue inflammation in adipose tissue of CD-fed mice. Representative images from immunohistochemistry for F4/80 in epididymal adipose tissue obtained from A_{2B}AR KO and WT animals after a 16-week CD. Epididymal adipose tissues were harvested, tissue sections prepared, and F4/80-stained tissue samples were subjected to histological examination. mRNA of F4/80 (B) and CD11c (C). Protein (D) and mRNA (E) levels of TNF-α, Protein (F) and mRNA (G) of CCL2 (B) in the epididymal adipose tissue of A_{2B}AR KO and WT animals that were kept on CD for 16 weeks. Results are representative of 3 experiments; *n* = 7-10 mice/group. Data are mean ± s.e. *p < 0.05 and ***p < 0.001 vs. WT.

Insulin resistance and increased adiposity are associated with accumulation of inflammatory macrophages that are both F4/80$^+$ and CD11c$^+$. Since, we found insulin resistance in CD-fed A_{2B}AR KO mice in comparison with their WT controls, we examined whether deletion of the A_{2B}AR has any impact on adipose tissue macrophages. In A_{2B}AR KO mice, "crown-like" structures, which are dead adipocytes surrounded by macrophages and are typically associated with obesity, adipose

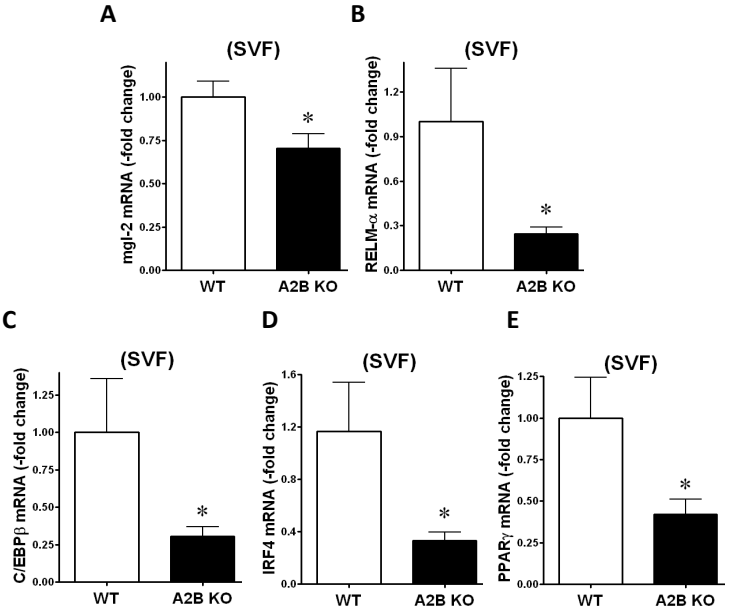

Figure 4. A_{2B}AR ablation downregulates the expression of aaMϕ-specific genes in SVF of CD-fed mice. mRNA expression of mgl-2 (A), RELMα (B), C/EBPβ (C), IRF4 (D), and PPARγ (E) in the SVF of epididymal adipose tissue obtained from A_{2B}AR KO and WT mice that were kept on CD for 16 weeks. For SVF isolation, epididymal tissues were collected, minced, and then digested with 0.2% collagenase type 2 for 30 min at 37°C. Cell suspensions from the digestion were passed through a 70-μm nylon mesh filter, and then the cells were centrifuged at 600 *g* for 10 min. Cells pellets after centrifugation were used for further analysis as SVF. Results are representative of 3 experiments; *n* = 7-10 mice/group. Data are presented as mean ± s.e. *p < 0.05 vs. WT

tissue dysfunction and chronic inflammation, were readily visible, whereas no such structures were present in WT mice (Figure 3A). In addition, mRNA levels of F4/80$^+$ and CD11c were increased

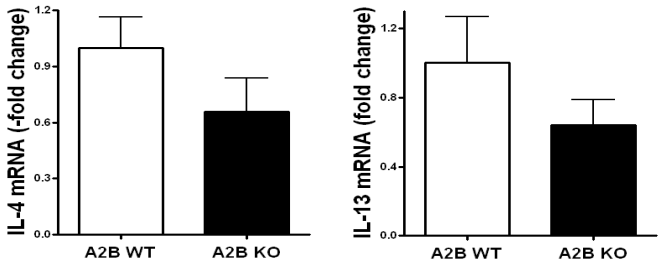

Figure 5. A_{2B}AR ablation fails to influence the expression of IL-4 and IL-13 in adipose tissue of CD-fed mice. mRNA expression of IL-4 (left) and IL-13 (right) in epididymal adipose tissue obtained from A_{2B}AR KO and WT mice that were kept on CD for 16 weeks. Results are *n* = 7-10 mice/group. Data are presented as mean ± s.e. *p < 0.05 vs. WT

in CD-fed A_{2B}AR KO animals compared to WT mice (Figure 3B and C), confirming the increased inflammation in A_{2B}AR KO mice. We also found increased classical macrophage activation in CD-fed A_{2B}AR KO vs. WT mice, as we detected elevated levels of TNF-α (Figure 3D and E), CCL2 (Figure 3F and G) and IL-6 (Figure 3H) in the epididymal fat tissue.

We then isolated stromal vascular fraction (SVF), which contains most macrophages, from epididymal fat tissue. In SVF of A_{2B}AR KO mice, we found downregulated expression of aaMϕ markers, including mgl-2 and resistin-like molecule (RELM)-α (Figure 4A and B) and diminished levels of several transcription factors that drive aaMϕ responses, which included C/EBPβ, IRF4, and PPARγ (Figure 4C-E).Taken together, our results indicate that A_{2B}ARs are required for maintaining insulin sensitivity, preventing adipose tissue inflammation and preserving aaMϕs in CD-fed mice (1).

To investigate whether or not A_{2B}ARs increased aaMϕ responses by increasing IL-4 or IL-13 levels, we measured the expression of these cytokines in the adipose tissue. We detected comparable levels of IL-4 and IL-13 between WT and A_{2B}AR KO adipose tissue (Figure 5), suggesting that A_{2B}ARs primarily affect macrophage function and not upstream regulators.

1. Csóka B., Koscsó B., Törő G., Kókai E., Virág L., Németh Z.H., Pacher P., Bai P., and **Haskó G**. A2B adenosine receptors prevent adipose tissue inflammation and insulin resistance by maintaining alternative macrophage activation. Diabetes 63, 850-866, 2014.

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