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ATP-Induced Inflammation Drives Tissue-Resident Th17 Cells in Metabolically Unhealthy Obesity

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Obesity-induced inflammation is conducted by a metabolic pathway, which eventually causes activation of specialized immune cells and leads to an unresolved inflammatory response within the tissue. For this reason, it is critically important to determine how hypertrophic fat tissue alters T cell balance to drive inflammation. In this study, we identify the purinergic signaling as a novel mechanism driving the adaptive Th17 response in human visceral adipose tissue (VAT) of metabolically unhealthy obese patients. We demonstrate that ATP acting via the P2X7 receptor pathway promotes a Th17 polarizing microenvironment with high levels of IL-1β, IL-6, and IL-17 in VAT explants from lean donors. Moreover, in vitro blockade of the P2X7 receptor abrogates the levels of these cytokines. These findings are consistent with a greater frequency of Th17 cells in tissue from metabolically unhealthy obese donors, revealed not only by the presence of a baseline Th17-promoting milieu, but also by the higher expression of steadily recognized Th17 markers, such as RORC, IL-17 cytokine, and IL-23R, in comparison with metabolically healthy obese and lean donors. In addition, we demonstrate that CD39 expression on $CD4^+$ effector T cells represents a novel Th17 marker in the inflamed VAT, which also confers protection against ATP-induced cell death. The manipulation of the purinergic signaling might represent a new therapeutic target to shift the $CD4^+$ T cell balance under inflammatory conditions. ***The Journal of Immunology*****, 2016, 196: 000–000.**

Obesity is a severe health problem worldwide that leads to multiple comorbidities, including type 2 diabetes mellitus and cardiovascular diseases. It also increases the risk for autoimmunity and cancer (1–3). However, a proportion of obese individuals might not be at an increased risk for metabolic complications; therefore, their phenotype can be referred to as "metabolically healthy obese" (MHO). Conversely, those with metabolic abnormalities are referred to as "metabolically unhealthy obese" (MUO) (4).

Visceral adipose tissue (VAT), liver, muscle, and pancreas are themselves sites of inflammation in obesity (5–10). It has been recently accepted that T cells resident in nonlymphoid tissues regulate local inflammation by modulating immunological and nonimmunological processes (11–13). Both regulatory T cells (Tregs) and $CD4^+$ effector T cells (Teff) are found in VAT and influence the recruitment and function of other inflammatory cells into the tissue, thereby contributing to metabolic changes in obesity (14).

Among Teff subsets, Th17 cells are defined by their ability to produce IL-17. Retinoic acid–related orphan receptor γ thymus (RORγt; RORC in humans) was identified as their master transcription factor (15, 16). Th17 cells are mostly induced in the periphery from naive T cells upon Ag priming (17, 18). It has been well established that inflammatory cytokines such as IL-1β and IL-6 are involved in the induction of the Th17 polarization program (19–21). By contrast, IL-23 was demonstrated to participate in the stabilization and expansion of Th17 polarized cells (22). Interestingly, mice overexpressing IL-23 develop autoimmunity (23). Moreover, the IL-23R is expressed at higher levels on T cells mediating experimental autoimmune encephalitis, highlighting the importance of this cytokine in regulating this lineage activity (24).

Th17 cells contribute to the host defense against fungi and extracellular bacteria (25), and are involved in the pathogenesis of many inflammatory and autoimmune diseases (26–28). However, their pathogenic role in obesity metabolic disorders and the factors within tissue microenvironment that modulate a Th17 response in the enlarged VAT remain to be elucidated (29–32).

Under pathological conditions, ATP is released from intracellular stores to the extracellular space where it acts as a stressful signal by binding to the purinergic receptors (33). The P2X7 receptor (P2X7R), an extracellular ATP-gated channel, is implicated in the secretion of proinflammatory cytokines, cell death, and autophagy (34). Moreover, extracellular ATP acting via the P2X7R was shown to drive Th17 responses during inflammation (35, 36). The CD39 ectonucleotidase mediates the first step in the conversion of ATP into ADP, AMP, and adenosine. By diminishing extracellular ATP concentration, CD39 activity prevents proinflammatory and proapoptotic effects induced by this nucleotide (37–39). Although CD39 expression on $CD4^+$ T cells is a well-known Treg marker, it was recently described to identify Teff subsets, such as Th17 cells (40–42).

We have recently defined the purinergic signaling as a novel mechanism involved in chronic inflammation in VAT of MUO patients (43). In this article, we demonstrate that signaling through purinergic receptors, particularly P2X7R, drives adaptive Th17 responses in human VAT.

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Abbreviations used in this article: BMI, body mass index; BP, blood pressure; BzATP, 2′(3′)-O-(4-benzoylbenzoyl) ATP; MHO, metabolically healthy obese; MUO, metabolically unhealthy obese; P2X7R, P2X7 receptor; RA, retinoic acid; SVC, stromal vascular cell; Teff, $CD4^+$ effector T cell; Treg, regulatory T cell; VAT, visceral adipose tissue.

Materials and Methods

Subjects and tissue sampling

This study recruited 50 surgical patients who were included in three groups as defined by World Health Organization criteria for obesity and by the definition for metabolic syndrome (44, 45). We studied 30 obese patients [body mass index (BMI) >30 kg/m^2; 20 MUO and 10 MHO] undergoing laparoscopic bariatric surgery and 20 lean donors (BMI <24.9 kg/m^2) undergoing laparoscopic abdominal surgery (e.g., fundoplication for reflux disease, cholecystectomy for gallstone disease, appendectomy, or endoscopic repair of hernias). Participants with three or more of the following criteria were considered to be MUO: 1) waist circumference ≥102 cm in men and ≥88 cm in women; 2) triacylglycerol level ≥150 mg/dl; 3) high density lipoprotein–cholesterol level <40 mg/dl in men and <50 mg/dl in women; 4) systolic blood pressure (BP) ≥130 mm Hg and/or diastolic BP ≥85 mm Hg; and 5) fasting glucose level ≥100 mg/dl.

VAT samples were collected from patients after surgical resection at Hospital de Clínicas José de San Martín. The diagnosis from each participant was obtained at the same center. Patients with inflammatory and malignant diseases or those taking oral glucose-lowering drugs were excluded. Lean donors were age- and sex-matched with obese patients. The clinical characteristics of the donors are summarized in Table I. This study was approved by the Ethics Committee at the Hospital de Clínicas José de San Martín.

Explant preparation and stromal vascular cell isolation

Using a mini-knife, we prepared small pieces (200 mg) of tissue (explants) composed of adipocytes and stromal vascular cells (SVC). The tissue was minced and digested with collagenase type II (Life Technologies) for 1 h to separate SVC from adipocytes. The digested material was filtered through a 100-μm nylon cell strainer, and SVC were isolated through a Ficoll-Hypaque density gradient (Amersham Biosciences). The cells were washed and suspended in PBS.

VAT culture

Explants (200 mg fat tissue) from lean donors were injected with 100 μl of PBS (nontreated), 2′(3′)-O-(4-benzoylbenzoyl) ATP (BzATP, 350 μM, P2X7R agonist; Sigma Aldrich), BzATP plus KN-62 (1 μM; P2X7R antagonist; Sigma Aldrich), or KN-62. Doses for BzATP and KN-62 were selected based on titration curves. Explants were then floated on 0.5 ml serum-free RPMI 1640 medium in a 48 multiwell plate (GBO) and incubated at 37°C. After 24 h, the supernatants were collected for analysis of cytokine production.

In other experiments, explants from lean, MHO, and MUO were nontreated or treated with KN-62 and floated on serum-free RPMI 1640 medium for 24 h. Afterward, the supernatants were collected.

To test the baseline cytokine production, explants from the three groups of donors were only floated on serum-free RPMI 1640 medium for 24 h, and then the supernatants were collected.

Cytokine quantification

The release of cytokines by explants was analyzed in culture supernatants using commercially available IL-1β and IL-6 ELISA kit (BD OptEIA Set; BD Biosciences). Human IL-23 levels were analyzed by using Human IL-23 DuoSet ELISA (R&D Systems). Human IL-17 levels were analyzed by using Human IL-17A ELISA Ready-SET-Go! (eBioscience). A standard curve was plotted and a regression analysis was applied. Assays were performed in duplicates, and the results were expressed as mean ± SEM.

Real-time quantitative RT-PCR

Total RNA was extracted using TRIzol and subjected to reverse transcription using Improm-II Reverse Transcriptase (Promega, Madison, WI). A PCR analysis for RORC was performed with a real-time PCR detection system (Mx3000P; Stratagene) using SYBR Green as a fluorescent DNA-binding dye. The primer sets used for amplification were as follows: RORC-F 5′-TTTTCCGAGGATGAGATTGC-3′ and RORC-R 5′-CTTTCCACATGC-TGGCTACA-3′; GAPDH-F 5′-CGACCACTTTGTCAAGCTCA-3′ and GAPDH-R 5′-TTACTCCTTGGAGGCCATGT-3′. Primer sets yielded a single product of the correct size. The relative mRNA expression level was calculated using $2^{-\Delta Ct}$ (46), and the data were normalized according to GAPDH mRNA levels. The results are presented as a value relative to the control value.

Flow cytometry

SVC (1×10^5) were stained with anti-CD45 allophycocyanin-Cy7 (clone 2D1; Biolegend), anti-CD4 PerCP (clone SK3) or FITC (clone RPA-T4), anti-CD39 allophycocyanin (clone TU66), anti-CD25 allophycocyanin-Cy7 (clone M-A251; all from BD Biosciences), and anti–IL-23R PE (clone 218213; R&D Systems) Abs. Intracellular detection of Foxp3 with anti-Foxp3 PE or Alexa Fluor 488 (clone 259D/C7; BD Biosciences) Abs was performed on fixed and permeabilized cells following the manufacturer's instructions. Isotype-matched mAbs were used as control.

For detection of intracellular cytokine production, SVC were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of Monensin (Golgi-Stop; BD Biosciences) for 5 h and then stained with anti-CD45 allophycocyanin-Cy7, anti-CD4 FITC, anti-CD39 allophycocyanin, and anti–IL-17 PE (clone SCPL1362; BD Biosciences) after fixation and permeabilization (BD Biosciences). Data were acquired using a FACSAria II (Becton Dickinson) and were analyzed with FlowJo software. Statistical analyses were based on at least 100,000 events gated on the population of interest.

Apoptosis assay

In brief, 5×10^5 SVC were cultured in complete culture medium for 24 h in a 48 multiwell plate in the absence or presence of different concentrations of BzATP (3.5, 35, and 350 μM) in a final volume of 500 μl. SVC were labeled with anti-CD45 allophycocyanin-Cy7, anti-CD4 PerCP, anti-CD25 PE-Cy7, and anti-CD39 allophycocyanin Abs. Because expression of CD25 correlates positively with Foxp3 expression on Tregs (47), we used this surface marker to discriminate between unfixed Foxp3$^+$ and Foxp3$^-$ T CD4$^+$ cells. Percentage of apoptosis was determined by using FITC–Annexin V (BD Biosciences). SVC were exposed to KN-62 (1 μM; P2X7 antagonist; Sigma Aldrich) or nontreated for 1 h and then BzATP (350 μM) was added to the culture, to investigate whether the apoptotic effect of BzATP was mediated via the P2X7R pathway. Data were analyzed by flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. Data normality was evaluated by Shapiro–Wilk test. For comparisons between groups, Wilcoxon signed rank test, Kruskal–Wallis test, Friedman test, or one-way ANOVA test was used, as appropriate. Correlations were assessed using Spearman correlation test. A p value <0.05 was considered statistically significant.

Results

Signaling via P2X7R promotes a Th17 polarizing environment in VAT

Because the purinergic signaling activation is known to drive Th17 cell differentiation in intestinal lamina propria and skin (35, 36), we explored the ability of extracellular ATP to promote a Th17 response in fat tissue.

Using a well-established model of human VAT explants (48–51), we first cultured tissue from lean donors (n = 6) treated with BzATP, BzATP plus KN-62, KN-62, or nontreated for 24 h. As shown in Fig. 1A, in comparison with nontreated explants, BzATP induced an increased secretion of IL-1β (15 ± 8 versus 94 ± 24 pg/ml; $p < 0.01$) and IL-6 (3982 ± 1360 versus 24478 ± 6234 pg/ml; $p < 0.01$). Moreover, when KN-62 was added to BzATP, we quantified a significant decrease in both cytokines production (15 ± 8 and 7886 ± 3220 pg/ml, $p < 0.05$, for IL-1β and IL-6, respectively), suggesting that this effect was mediated via the P2X7R signaling. By contrast, BzATP did not increase IL-23 levels secreted by explants from lean donors. Interestingly, we also detected higher levels of IL-17 in the supernatants of BzATP-treated explants from lean donors in comparison with nontreated explants (66 ± 29 versus 17.5 ± 10 pg/ml; $p < 0.05$; Fig. 1B). As expected, this effect was abrogated by the addition of KN-62 to BzATP (17 ± 7.5 pg/ml; $p < 0.05$). Of note, KN-62 alone did not modulate the levels of any cytokine in this group of patients.

Because we have recently demonstrated that the purinergic signaling was upregulated in the MUO group (43), we addressed the question whether the in vitro findings described earlier could mimic what happens in tissue in the obese state. Thus, we compared the baseline levels of IL-1β, IL-6, and IL-23 secreted by explants from lean, MHO, and MUO donors (n = 6 in each group).

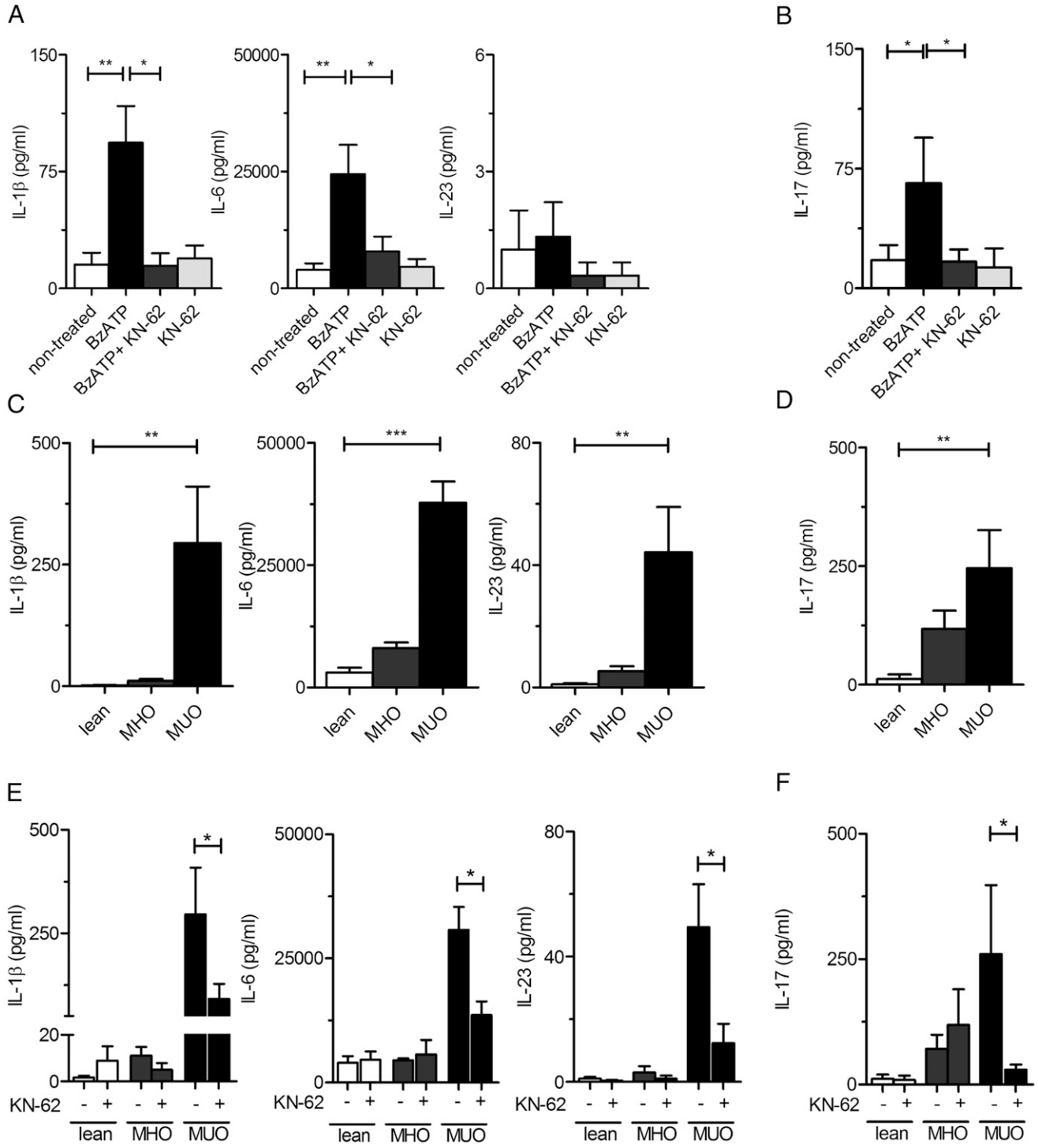

FIGURE 1. BzATP induces the release of Th17-biasing cytokines by VAT. (**A** and **B**) Explants (200 mg fat tissue) from lean donors ($n = 6$) were injected with BzATP (350 μM), KN-62 (1 μM), BzATP + KN-62, or PBS (nontreated) and cultured during 24 h. Then the supernatant cultures were collected and the levels of IL-1β, IL-6, and IL-23 (A) or IL-17 (B) were determined by ELISA. (**C** and **D**) Explants from lean, MHO, and MUO patients ($n = 6$ in each group) were floated in serum-free medium for 24 h. Then the supernatant cultures were collected and the baseline levels of IL-1β, IL-6, and IL-23 (C) or IL-17 (D) were determined by ELISA. (**E** and **F**) Explants from lean, MHO, and MUO donors ($n = 5$ in each group) were injected with KN-62 (1 μM) or PBS (nontreated) and cultured during 24 h. Then the supernatant cultures were collected and the levels of IL-1β, IL-6, and IL-23 (E) or IL-17 (F) were determined by ELISA. Data represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

The clinical characteristics of donors are shown in Table I. As depicted in Fig. 1C, MUO explants secreted significantly higher levels of IL-1β (294 ± 116 pg/ml) compared with MHO (11 ± 4 pg/ml; $p < 0.05$) and lean donors (2 ± 1 pg/ml; $p < 0.01$). In addition, MUO explants also secreted higher levels of IL-6 (37801 ± 4309 pg/ml) compared with MHO (8018 ± 1179 pg/ml) and lean donors (3046 ± 1021 pg/ml; $p < 0.001$). Moreover, whereas IL-23 levels were almost undetectable in lean (1 ± 0.4 pg/ml) and MHO (5 ± 2 pg/ml) explant cultures, the highest level of this cytokine was readily detected in MUO patients (44 ± 15 pg/ml; $p < 0.05$). Finally, we also found that MUO explants secreted significantly greater IL-17 baseline levels (246 ± 80 pg/ml) compared

Table I. Clinical characteristics of patients

	Lean	MHO	MUO
n	20	10	20
Age, y	40 ± 3.4	40 ± 3.3	44 ± 2.5
Sex, male/female	5/15	3/7	5/15
BMI, kg/m^2	22 ± 0.4	37 ± 3*	38 ± 1.8*
Metabolic score[a]			
0	20 (100)[b]	0 (0)	0 (0)
1	0 (0)	2 (20)	0 (0)
2	0 (0)	8 (80)	0 (0)
3	0 (0)	0 (0)	7 (35)
4	0 (0)	0 (0)	7 (35)
5	0 (0)	0 (0)	6 (30)

[a]Metabolic score: 0–5; in numbers by patient. Waist circumference: ≥102 cm in men and ≥88 cm in women; elevated triacylglycerol level: ≥150 mg/dl or reduced high density lipoprotein–cholesterol level: <40 mg/dl in men and <50mg/dl in women; elevated BP: systolic BP ≥130 mm Hg and/or diastolic BP: ≥85 mm Hg; and elevated fasting glucose level: ≥100 mg/dl.
[b]Metabolic score shown as number of individuals (%).
*$p < 0.001$ compared with lean.

with MHO (118 ± 38 pg/ml) or lean donors (12 ± 10 pg/ml; $p < 0.01$; Fig. 1D).

To confirm the involvement of the P2X7R in the tissue inflammation observed in the MUO group, we next cultured explants from the three group of donors treated with KN-62 (1 μM) or nontreated for 24 h ($n = 5$ in each group). As illustrated in Fig. 1E, the analysis revealed that KN-62 abrogated the levels of IL-1β (295 ± 113 versus 91 ± 37 pg/ml; $p < 0.05$), IL-6 (30780 ± 4610 versus 13590 ± 2766 pg/ml; $p < 0.05$), and IL-23 (49.5 ± 14 versus 12 ± 6 pg/ml, $p < 0.05$, for nontreated and KN-62, respectively) secreted by explants from the MUO group. This trend was not observed in MHO and lean donors. Importantly, we also detected a reduction of the IL-17 levels secreted by MUO explants in the presence of this ATP antagonist (260 ± 138 versus 29 ± 11 pg/ml; $p < 0.05$).

These findings suggested that, at the tissue level, an increase of activity of the purinergic signaling pathway could be involved in the inflammatory milieu, promoting the development of a Th17 polarizing microenvironment in MUO VAT.

Higher Th17 response in VAT of MUO donors

To investigate whether ATP-induced inflammation was related to the development of a Th17 response, we next analyzed the expression of Th17 markers in tissue from the three groups of donors. As depicted in Fig. 2A, we found that RORC was significantly upregulated in MUO SVC (2.6 ± 0.5; $n = 10$) compared with MHO (0.5 ± 0.2; $p < 0.001$; $n = 6$) and lean donors (1 ± 0.12; $p < 0.05$; $n = 10$). The strategy of gating by flow cytometry of CD4$^+$ T cells resident in tissue is shown in Fig. 2B. As depicted in Fig. 2C, we found that CD4$^+$ T cells from the MUO group secreted increased levels of IL-17 (1.6 ± 0.2%; $n = 6$) in comparison with MHO (1 ± 0.1%) and lean donors (0.7 ± 0.1%; $p < 0.01$). Interestingly, according to the higher levels of IL-23 secreted by explants from MUO donors, we also demonstrated a significant increase of the IL-23R expression on CD4$^+$ T cells from MUO (6.4 ± 1%) compared with MHO (3.3 ± 0.7%) and lean donors (2 ± 0.5%; $p < 0.05$; Fig. 2D).

Because we previously demonstrated that CD39 expression was increased in SVC from MUO donors (43), we next explored the possibility that this molecule could represent a Th17 marker in the inflamed VAT. As depicted in Fig. 2E, we found that CD4$^+$ T cells resident in tissue from MUO patients expressed higher levels of CD39 (8.3 ± 0.7%) in comparison with MHO (6.6 ± 0.75%) and lean donors (5.1 ± 0.4%; $p < 0.01$).

To assess the role of CD39 in tissue, we then explored the dual expression of this molecule with the Th17 markers previously described. Representative dot plots of CD39 and IL-17 production or IL-23R expression on CD4$^+$ T cells in VAT are shown in Fig 2F. As depicted in Fig. 2G, we observed that CD39$^+$ T cells contributed to the production of a quarter of the IL-17 secreted by CD4$^+$ T cells in all groups of donors (28.5 ± 2 versus 26.3 ± 3.5 versus 22.3 ± 3.4%, for MUO, MHO, and lean donors, respectively; p = NS). Moreover, we found a fit coexpression of CD39 and IL-23R in the MUO group (58.25 ± 4.4%) in comparison with MHO (42.8 ± 2.5%) and lean donors (17.8 ± 4.6%; $p < 0.01$).

Overall, our data demonstrated a higher frequency of Th17 cells in VAT from MUO donors. We also confirmed a link between CD39 expression and a Th17 response in the inflamed tissue.

CD39$^+$ Foxp3$^-$ T cells are increased in the inflamed VAT of MUO donors

Although in physiological conditions CD39 expression is restricted mostly to Tregs, we showed earlier that in the inflammatory site it could also represent a Th17 marker. To confirm that the higher expression of CD39$^+$ in MUO VAT denoted an effector function, we next assessed the coexpression of CD39 and Foxp3, which is the master regulator of Treg lineage, as defined previously (40).

Representative dot plots of CD39$^+$ Foxp3$^-$, CD39$^+$ Foxp3$^+$, and CD39$^-$ Foxp3$^+$ frequency in CD4$^+$ T cells in VAT are illustrated in Fig. 3A.

At tissue level, we found a significant increase of CD39$^+$ Foxp3$^-$ T cells in the MUO group (7 ± 0.7%; $n = 12$) in comparison with MHO (4.7 ± 0.7%; $n = 8$) and lean donors (3.4 ± 0.4%; $n = 12$; $p < 0.001$). By contrast, no statistical difference was found in the frequency of CD39$^+$ Foxp3$^+$ T cells among groups (1.4 ± 0.2 versus 1.9 ± 0.4 versus 2.1 ± 0.4%, for MUO, MHO, and lean donors, respectively; p = NS). Finally, we found that CD39$^-$ Foxp3$^+$ T cells were decreased in MUO SVC (0.9 ± 1.5%) compared with MHO (2.5 ± 0.8%) and lean donors (3.1 ± 0.6%; $p < 0.05$; Fig. 3B).

To clarify the role of CD39$^+$ Foxp3$^-$ T cells as a potential Th17 subset involved in the metabolic changes related to obesity, we further evaluated the potential association of RORC, IL-23R, and this cell subset frequency with the degree of metabolic disease of donors. We found a strong, positive association between the parameters evaluated and the metabolic score of donors ($r = 0.56$, $p < 0.01$; $r = 0.76$, $p < 0.001$; and $r = 0.73$, $p < 0.001$, for RORC, IL-23R, and CD39$^+$ Foxp3$^-$ cells, respectively).

Our data clearly demonstrated an unbalance between effector and regulatory forces on resident CD4$^+$ T cells from the MUO group. Moreover, CD39$^+$ Foxp3$^-$ T cells, which seem to include Th17 cells in tissue, may actively contribute to the metabolic changes in obesity.

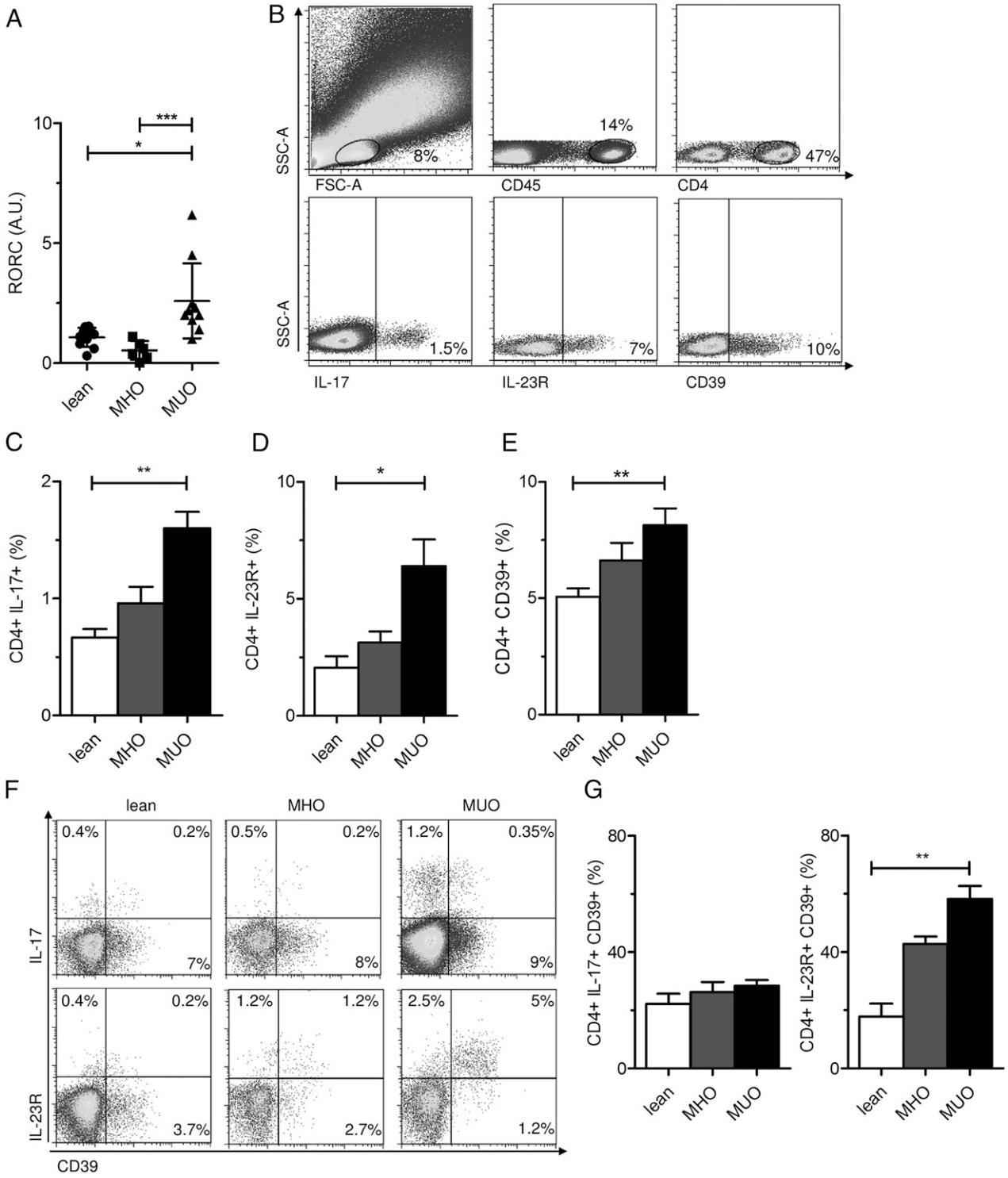

FIGURE 2. Higher expression of Th17 markers in VAT of MUO. (**A**) Basal expression of RORC in lean ($n = 10$), MHO ($n = 6$), and MUO ($n = 10$) SVC was quantified by real-time quantitative RT-PCR. (**B**) Representative FACS profile showing the gating strategy of $CD4^+$ T cells in VAT of MUO. (**C**) Frequency of IL-17^+CD4^+ T cells on SVC of lean, MHO, and MUO donors ($n = 6$ in each group). (**D**) Frequency of IL-$23R^+$ $CD4^+$ T cells on SVC of lean, MHO, and MUO donors ($n = 6$ in each group). (**E**) Frequency of $CD39^+$ $CD4^+$ T cells on SVC of lean, MHO, and MUO donors ($n = 6$ in each group). (**F**) Representative dot plots showing CD39 and IL-17 or IL-23R expression on gated $CD4^+$ T cells SVC from a lean, MHO, and MUO donor. (**G**) Frequency of IL-17^+ $CD39^+$ and IL-$23R^+$ $CD39^+$ $CD4^+$ T cells on SVC of lean, MHO, and MUO donors ($n = 6$ in each group). Data represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

CD39 gives a differential susceptibility to ATP-induced apoptosis via the P2X7R

CD39 has been implicated in resisting ATP-induced apoptosis (38, 39). Because we detected an increased frequency of $CD39^+$ $Foxp3^-$ in MUO VAT, we next investigated how in vitro exposure to ATP affects the viability of the $CD4^+$ T cell subsets resident in VAT.

In brief, 1×10^5 SVC obtained from lean donors were treated during 24 h with increasing doses of BzATP (3.5, 35, and 350 μM) or nontreated and then stained with Annexin V. The strategy of gating in VAT is shown in Fig. 4A. Representative histograms showing the percentage of cell death detected on $CD39^+$ $Foxp3^-$, $CD39^+$ $Foxp3^+$, and $CD39^-$ $Foxp3^+$ are shown in Fig. 4B.

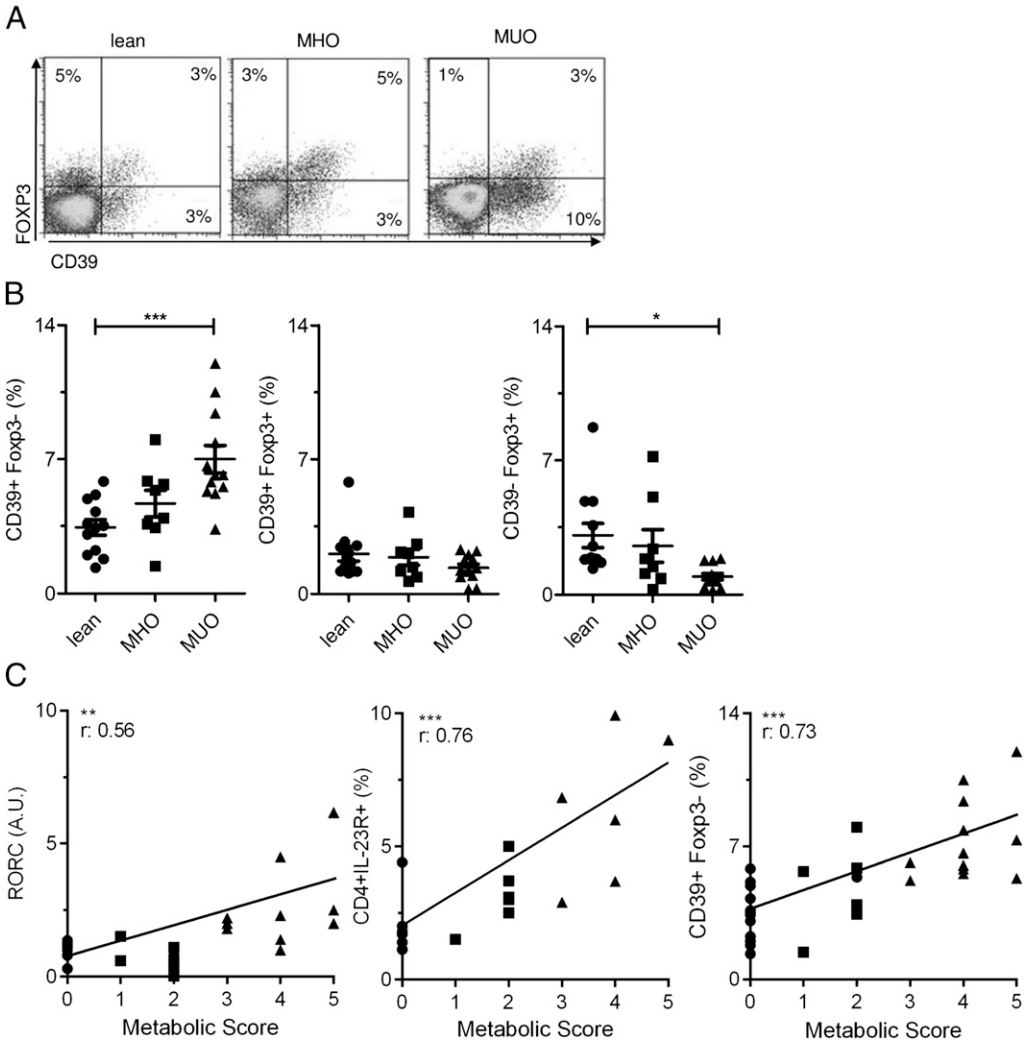

FIGURE 3. VAT from MUO exhibit an increased frequency of $CD39^+$ $Foxp3^-$ T cells. (**A**) Representative FACS profile showing the expression of Foxp3 and CD39 on $CD4^+$ T cells in VAT of lean, MHO, and MUO donors. (**B**) Frequencies of $CD39^+$ $Foxp3^-$, $CD39^+$ $Foxp3^+$, and $CD39^-$ $Foxp3^+$ T cells on SVC of lean ($n = 12$), MHO ($n = 8$), and MUO ($n = 12$) patients. Data are expressed as percentage of all $CD4^+$ T cells. Results show the mean ± SEM. (**C**) Graphs showing correlations between the RORC expression ($n = 26$), $IL23R^+$ $CD4^+$ ($n = 18$), and $CD39^+$ $Foxp3^-$ T cell frequency ($n = 32$) and the metabolic score of lean (circles), MHO (squares), and MUO donors (triangles). Correlations were evaluated by using Spearman rank correlation coefficient test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Six independent experiments showed a dose-dependent increased apoptosis on all $CD4^+$ T cell subsets exposed to BzATP. We found that, in the presence of the highest dose of BzATP (350 μM), $CD39^+$ $Foxp3^-$ and $CD39^+$ $Foxp3^+$ were less susceptible to cell death (12 ± 4.2 and 20 ± 6%, for $CD39^+$ $Foxp3^-$ and $CD39^+$ $Foxp3^+$, respectively) in comparison with $CD39^-$ $Foxp3^+$ (34 ± 7%) as depicted in Fig. 4C. Interestingly, this difference was only statistically significant between the $CD39^+$ $Foxp3^-$ and $CD39^-$ $Foxp3^+$ ($p < 0.01$). A similar trend was observed with the intermediate dose of BzATP (35 μM).

To evaluate whether this effect was mediated via the P2X7R signaling, we cultured SVC for 24 h in the presence of BzATP, KN-62, BzATP plus KN-62, or nontreated ($n = 6$).

As depicted in Fig. 4D, KN-62 was able to inhibit apoptosis in all subsets, reaching statistical significance in $CD39^+$ $Foxp3^+$ (30 ± 6.1 versus 11 ± 1.9%; $p < 0.05$) and $CD39^-$ $Foxp3^+$ subsets (56 ± 7.8 versus 19 ± 2%, $p < 0.05$, for BzATP and BzATP plus KN-62, respectively). Of note, the addition of KN-62 did not affect cell viability in the absence of BzATP.

These results demonstrated that BzATP acting via the P2X7R has a proapoptotic effect directly on $CD4^+$ T cells resident in tissue. Moreover, the differential susceptibility of $CD4^+$ T cell subsets in tissue could partially explain the higher frequency of $CD39^+$ $Foxp3^-$ and the diminished frequency of $CD39^-$ $Foxp3^+$ observed in MUO VAT.

Discussion

The major research focus in obesity-related inflammation has been on adipose tissue innate immune response. As obesity develops, enlarging adipocytes secrete chemokines that attract immune cells. Macrophages are among the earliest immune cells to infiltrate adipose tissue, because their numbers increase after 1 wk of high-fat diet (52–55). Within this local immune response other actors, like resident dendritic cells, mast cells, and eosinophil, are also involved (56–60). Concerning adaptive immunity in obesity, it was recently demonstrated that T cell regulation provides a link between inflammation and insulin resistance (11–13). In this study, we identified the purinergic signaling as a novel mechanism involved in driving the adaptive Th17 response in VAT of obese patients with metabolic changes.

Tissue microenvironment plays a critical role in controlling the differentiation of $CD4^+$ T cells into distinct subsets, including

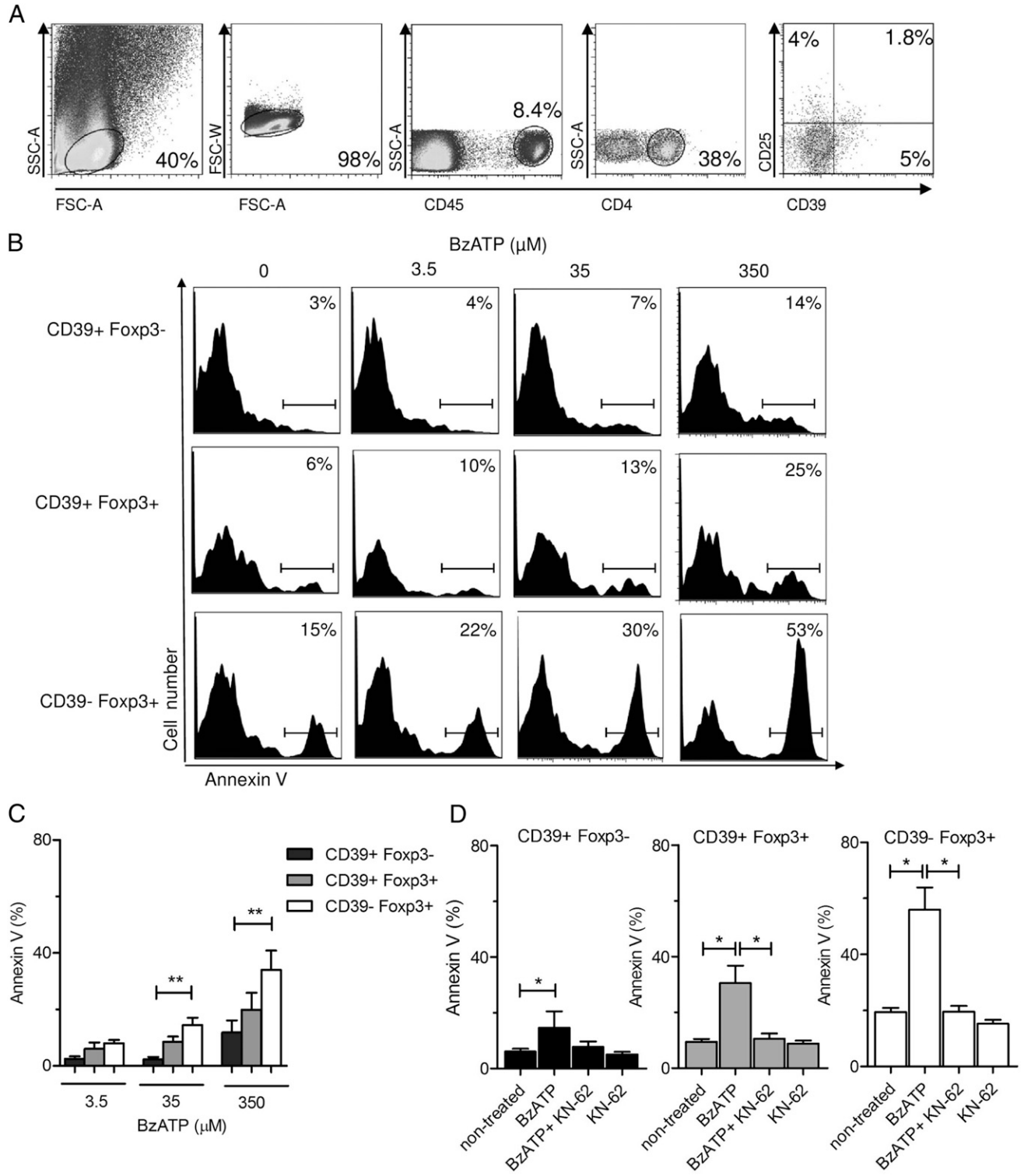

FIGURE 4. CD39 protects against ATP-induced apoptosis. SVC from lean donors were incubated with BzATP (0.35, 35, or 350 μM) or nontreated for 24 h. The assay discriminates between live and apoptotic cells labeled with anti-CD45, anti-CD4, anti-CD39, anti-CD25 Abs, and Annexin V. $CD45^+$ $CD4^+$ T cells were gated as $CD39^+$ $CD25^-$ ($CD39^+$ $Foxp3^-$), $CD25^{high}$ $CD39^+$ ($CD39^+$ $Foxp3^+$), and $CD25^{high}$ $CD39^-$ ($CD39^-$ $Foxp3^+$). (**A**) Representative FACS profile showing the gating strategy of $CD4^+$ T subsets. (**B**) Representative histograms showing Annexin V staining on $CD39^+$ $Foxp3^-$, $CD39^+$ $Foxp3^+$, and $CD39^-$ $Foxp3^+$ T cells. (**C**) Percentage of cell death induced by BzATP in $CD4^+$ T subsets after the subtraction of the corresponding controls (cells cultured in medium only). Data represent the mean ± SEM ($n = 6$). (**D**) SVC were incubated with BzATP (350 μM), KN-62 (1 μM), BzATP plus KN-62, or nontreated for 24 h. Percentage of cell death in $CD39^+$ $Foxp3^-$, $CD39^+$ $Foxp3^+$, and $CD39^-$ $Foxp3^+$ T cells was analyzed as described earlier ($n = 6$). Data represent the mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$.

Th17 cells (61–63). In humans, both IL-1β and IL-6 are essential for the differentiation of Th17 cells (20, 64). Furthermore, a failure in the IL-23/IL-23R axis determined a Th17 development delayed at the early stage and fewer Th17 cells in the lymph nodes and tissues (65). Thus, IL-23 is not required for the initial Th17 polarization but instead stabilizes and expands Th17 polarized

cells, especially in pathological settings (66–68). In addition, the production of these cytokines was shown to be pathologically dysregulated in the obese state, contributing to insulin resistance (9, 43, 69–72).

Regarding the signaling through purinergic receptors, the P2X7R was shown to play a role in the development of both innate and adaptive Th17 immune responses in skin and intestinal lamina propria (35, 36, 73). We previously showed that the purinergic signaling pathway was upregulated in VAT of MUO patients, and that it could chronically maintain the inflammasome activity and IL-1β production in tissue (43).

This study clearly demonstrates that, after P2X7R stimulation, IL-1β, IL-6, and IL-17 were significantly increased in explant cultures from lean donors. No changes on IL-23 levels were found in the in vitro cultures. In line with the upregulation of the purinergic signaling in MUO VAT (43), we also observed that, in comparison with MHO and lean, MUO explants secreted significantly higher baseline levels not only of IL-1β, IL-6, and IL-23 but also of IL-17 cytokine. Thus, our data suggest that the presence of a P2X7R agonist, such as ATP, induces a polarizing Th17 milieu located in VAT that mimics that observed in obese patients with metabolic changes. Importantly, this study also confirms the involvement of the P2X7R in tissue inflammation related to metabolic unhealthy obesity, because the treatment of MUO explants with KN-62 alone decreased the levels of these Th17 cytokines. Of note, this trend was not observed in lean or MHO donors.

We keep in mind that other factors may contribute to the Th17 cell differentiation in tissue. The effect of ATP in modulating VAT inflammation is more likely to be not directly on $CD4^+$ T cells but on other resident VAT cells like macrophages that secrete the proinflammatory cytokines IL-1β and IL-6. In this sense, the downstream signaling pathway mediated by the P2 receptors activation, which is involved in the generation of the local Th17 polarizing milieu, is not completely elucidated and will require further studies.

Although the pathogenicity of IL-17–producing T cells has been recognized in various autoimmune diseases (17, 74–77), only a few reports have explored their role in obesity. In this context, a higher frequency of Th17 cells was observed in peripheral blood and/or s.c. fat of obese subjects (30, 31, 78). However, the identification of Th17 cells in patients has remained problematic, which is due, in part, to the lack of reliable surface markers (22, 42).

In this study, we demonstrate for the first time, to our knowledge, a greater adaptive Th17 response located in VAT from MUO patients. This group of donors showed a higher RORC expression in SVC and an increased $IL\text{-}17^+$ and $IL\text{-}23R^+$ $CD4^+$ T cell frequency compared with MHO and lean donors. Because CD39 was proposed as a marker of the Th17 lineage (42), we also explored its expression on this subset in VAT. Surprisingly, the MUO group showed not only a higher frequency of CD39, but also a simultaneous expression of this enzyme with the other Th17 markers on $CD4^+$ T cells. These results supported the notion that CD39 could be a reliable Th17 marker in the inflamed fat. Furthermore, by analyzing the dual expression of CD39 and Foxp3, we observed that the MUO group had an increased frequency of $CD39^+$ $Foxp3^-$ T cells. Our findings also suggested both that the higher CD39 expression on $CD4^+$ T cells of MUO denoted an effector response and that $CD39^+$ $Foxp3^-$ T cells could encompass Th17 cells in tissue. The fact that RORC, IL-23R, and CD39 were strongly associated with the metabolic score of patients confirmed the link between a higher Th17 response and the metabolic changes in obesity. However, although IL-17 is traditionally thought to be secreted by Th17 cells, other cellular sources of IL-17 should be considered, as well as the cell subsets involved in their differentiation and the molecular mechanisms implied in the human model.

Although the tandem action of CD39 and CD73 on Tregs contributes to their suppressive function by catalyzing ATP/ADP into adenosine, it is not clear why Teff cells express CD39. Our data showed that BzATP had a proapoptotic effect on T cells through the P2X7R signaling and that CD39 expression conferred protection against ATP-induced cell death in $CD4^+$ T cells. In this context, it is possible that CD39 may offer a survival advantage to T cells, especially during ATP influx into the milieu caused by local inflammation. Finally, our findings could partially explain the increased frequency of $CD39^+$ $Foxp3^-$ T cells and the decreased frequency of $CD39^-$ $Foxp3^+$ T cells observed in MUO VAT.

In summary, we have demonstrated that signaling through purinergic receptors, particularly P2X7R, drives an adaptive Th17 response in VAT underlying the metabolic changes in obesity. Finally, CD39 expression on T cells represents a novel Th17 marker in the inflamed VAT giving protection against ATP-induced apoptosis. The manipulation of the purinergic signaling might represent a new therapeutic target to shift the $CD4^+$ T cell balance.

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Disclosures

The authors have no financial conflicts of interest.

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