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Endogenous Oils Derived From Human Adipocytes Are Potent Adjuvants That Promote IL-1α–Dependent Inflammation

Obesity is characterized by chronic inflammation associated with neutrophil and M1 macrophage infiltration into white adipose tissue. However, the mechanisms underlying this process remain largely unknown. Based on the ability of oil-based adjuvants to induce immune responses, we hypothesized that endogenous oils derived from necrotic adipocytes may function as an immunological "danger signal." Here we show that endogenous oils of human origin are potent adjuvants, enhancing antibody responses to a level comparable to Freund's incomplete adjuvant. The endogenous oils were capable of promoting interleukin (IL)-1α–dependent recruitment of neutrophils and M1-like macrophages, while simultaneously diminishing M2-like macrophages. We found that endogenous oils from subcutaneous and omental adipocytes, and from healthy and unhealthy obese individuals, promoted comparable inflammatory responses. Furthermore, we also confirmed that white adipocytes in visceral fat of metabolically unhealthy obese (MUO) individuals are significantly larger than those in metabolically healthy obese individuals. Since adipocyte size is positively correlated with adipocyte death, we propose that endogenous oils have a higher propensity to be released from hypertrophied visceral fat in MUO individuals and that this is the key factor in driving inflammation. In summary, this study shows that adipocytes contain a potent oil adjuvant which drives IL-1α–dependent proinflammatory responses in vivo.

Obesity is characterized by chronic, low-grade inflammation resulting in insulin resistance. Understanding the mechanisms underlying this inflammation would provide valuable insights into the disease and potentially offer new therapeutic targets. Early studies showing that sodium salicylate can reverse the symptoms of type 2 diabetes (1) highlighted roles for inhibitor of κB kinase β (IKKβ) and Jun N-terminal kinase (JNK) in this process. JNK can directly phosphorylate Serine307 on insulin receptor substrate 1, thus impairing insulin signaling and mediating obesity-induced insulin resistance (2). IKKβ can also phosphorylate insulin receptor substrate 1 (3), and myeloid-specific deletion of IKKβ in obese mice protects against insulin resistance (4). In addition, IKKβ-mediated translocation of nuclear factor-κB into the nucleus

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results in transcription of a plethora of proinflammatory cytokines that further activate JNK and IKKβ in a positive feedback loop (5). Hotamisligil et al. (6) first demonstrated a role for proinflammatory cytokines in driving obesity-induced insulin resistance when reporting that neutralization of tumor necrosis factor (TNF)-α in genetically obese rats improved insulin sensitivity. Subsequently, it was shown that genetic or diet-induced obese mice deficient in TNF-α are protected from insulin resistance (7).

White adipose tissue (WAT) contains resident macrophages that, in nonobese individuals, have an anti-inflammatory profile with an "alternatively activated" phenotype and are referred to as M2 macrophages. The polarization of resident M2 macrophages is achieved under the influence of the cytokines interleukin (IL)-4 and IL-13, which are primarily secreted by T helper (Th) 2 cells and innate lymphoid cells (8,9). However, eosinophils and regulatory T cells are also important for alternative activation of M2 macrophages in WAT through production of IL-4 (10) and IL-10 (11), respectively. Crucially, obesity induces a phenotypic shift from M2 to M1 adipose tissue macrophages, which exhibit a proinflammatory profile and a "classically activated" phenotype (12). M1 macrophages originate from bone marrow precursors and subsequently infiltrate WAT (13). There is a significant increase in the number of proinflammatory M1 macrophages in obesity, particularly in visceral fat, which is more closely associated with pathology than subcutaneous fat (14,15). Th1 cells present in WAT promote the polarization of infiltrating macrophages toward an M1 phenotype by secreting the Th1 signature cytokine interferon (IFN)-γ (8). In addition, cytotoxic T cells (16), B cells (17), and mast cells (18) contribute to obesity-induced inflammation. Neutrophils also play a crucial role in the chronic inflammation associated with obesity and transiently infiltrate intra-abdominal tissue during the early stages of high-fat feeding (19,20).

Currently, the mechanisms responsible for neutrophil and M1 macrophage infiltration into WAT and the inflammatory response associated with obesity remain largely unknown. However, it has been reported that adipocytes in WAT of obese mice exhibit features of necrosis and subsequently release endogenous dietary oils into the extracellular milieu. Macrophages are recruited exclusively to sites of necrotic adipocyte death where they aggregate to form crown-like structures (21). Based on the established ability of oil-based vaccine adjuvants to induce potent immune responses (22–24), we hypothesized that endogenous oils derived from necrotic adipocytes may function as a potent immunological "danger signal," thus providing a crucial link between obesity and chronic immune stimulation.

RESEARCH DESIGN AND METHODS

Reagents
The reagents used were Freund's incomplete adjuvant (FIA; Sigma-Aldrich), alum (Alhydrogel, Brenntag Biosector), Pam3CSK4 (Pam3; InvivoGen), CpG (Oligos Etc. Inc.), and 10 μm polystyrene (PS) particles (Phosphorex Inc.).

Animals
C57BL/6, C3H/HeN, and C3H/HeJ mice were obtained from Harlan Olac (Bicester, U.K.), $Il1r1^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and $Nlrp3^{-/-}$ breeding pairs were provided by the late Jurg Tschopp (University of Lausanne, Switzerland). These mice were housed in the Bioresources Unit in Trinity College Dublin. Animals were maintained according to the regulations of the European Union and the Irish Department of Health. Animal studies were approved by the Trinity College Dublin Animal Research Ethics Committee (ethical approval number 091210) and were performed under license number B100/3321. $Il1\alpha/\beta^{-/-}$ and $Il1\alpha^{-/-}$ mice were provided by Stuart Allan (University of Manchester, U.K.), and experiments involving these mice were performed at the University of Manchester under the Animals (Scientific Procedures) Act 1986 (license number PPL 40/3617).

Defining Profile of Individuals
Obese individuals had a BMI of >30. The metabolic profile of obese individuals was determined as described previously (25) and as outlined in Supplementary Table 1, according to the International Diabetes Federation worldwide consensus definition of the metabolic syndrome of 2006. Healthy lean individuals were nonmalignant and noninflammatory and had a BMI of <25.

Human Adipose Tissue
With informed consent, omental and subcutaneous adipose tissue biopsies were obtained from obese patients undergoing bariatric surgery and healthy lean patients undergoing a laparoscopic colonoscopy for exploratory reasons at St. Vincent's University Hospital (Elm Park, Dublin, Ireland). The St. Vincent's University Hospital Ethics and Medical Research Committee approved the use of these patient samples for this study.

Isolation of Human Omental and Subcutaneous Oils from WAT
Adipocytes were isolated as described previously (25) and were centrifuged at 2,500g for 1 min to release endogenous oils. Where indicated, a lipid-extraction procedure was performed using the Bligh and Dyer method described previously (26). When testing for immunostimulatory activity in vivo, the individual fractions were further separated with reversed-phase high-performance liquid chromatography. When preparing samples for fatty acid compositional profiling, the organic layer containing the lipid fraction was transesterified using 14% boron trifluoride-methanol as described previously (27) and was analyzed by gas chromatography as described previously (28).

Histological Analysis of Human Adipose Tissue
Omental adipose tissue was immediately fixed in formalin, paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin. Adipocyte size was determined using Image Pro Plus 7 software.

Endotoxin-Free Ovalbumin

Ovalbumin (OVA; Sigma-Aldrich) was resuspended in sterile PBS (Biosera). This preparation was filter sterilized with a 0.22 μm syringe-driven filter (Millipore) and passed through three separate endotoxin removal columns (Pierce) three times each.

Heat-Killed *Escherichia coli*

BL21 strain *E. coli* were killed by heating at 70°C for 15 min.

Isolation and Culture of Peritoneal Exudate Cells

Mice were killed by cervical dislocation, and the peritoneal cavity was washed with 5 mL of ice-cold PBS. The wash fluid was collected, and peritoneal exudate cells (PECs) were pelleted by centrifugation. The supernatant was collected, and PECs were resuspended in RPMI 1640 medium (Biosera). PEC numbers were determined by light microscopy using a KOVA Glasstic cell counter slide with grids (Medical Supply Co. Ltd.) following trypan blue (Sigma-Aldrich) exclusion. PECs (1×10^6/mL) were incubated at 37°C in a humidified atmosphere (5% CO_2).

Flow Cytometry

PECs (1×10^6) were preincubated for 10 min with an anti-CD16/CD32 monoclonal antibody (0.25 μg; BD Pharmingen). PECs were then incubated for 30 min with AQUA fluorescent dye (0.5 μL; Invitrogen) and for an additional 30 min with antibodies specific for CD11b (0.005 μg; BD Pharmingen), Gr-1 (0.04 μg; BD Pharmingen), F4/80 (0.1 μg; eBioscience), and SiglecF (0.02 μg; BD Pharmingen). Samples were acquired with a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences), and the data were analyzed using FlowJo software (Treestar, Oregon).

Dot plots are shown in the figures as a single representative population from each treatment group.

Measurement of Cytokines by ELISA

The concentration of the cytokine IL-12p40 was measured by ELISA using antibodies obtained from BD Pharmingen. Concentrations of the cytokines TNF-α, IL-10, and IL-12p70 were measured with DuoSet ELISA Development Kits (R&D Systems).

Measurement of Antigen-Specific Serum Antibodies by ELISA

Titres of antigen-specific total serum IgG as well as the IgG subtypes IgG1, IgG2b, and IgG2c were determined using commercially available antibodies from Sigma-Aldrich (total IgG), BD Pharmingen (IgG1 and IgG2b), and AbD Serotec (IgG2c).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software. The means for three or more groups were compared by one-way ANOVA. Where significant differences were found, the Tukey–Kramer multiple comparisons test was used to identify differences between individual groups. The means for two groups were compared using an unpaired Student *t* test.

RESULTS

Endogenous Omental Oils Are Potent Adjuvants

Oil-based emulsions such as MF59 and FIA are potent vaccine adjuvants. Therefore, the first question addressed was whether endogenous oils derived from the human omentum of metabolically healthy obese (MHO) individuals (oils were derived from MHO individuals for each experiment, unless stated otherwise) could function as an endogenous adjuvant. Remarkably, mixing OVA with endogenous oils significantly enhanced antigen-specific IgG1, IgG2b, and IgG2c responses to a degree comparable to FIA (Fig. 1*A*).

In order to rule out the possibility that nonlipid components were responsible for the immunostimulatory activity of the preparation of endogenous oils, a lipid-extraction procedure was performed. The aqueous phase containing nonlipid components failed to enhance any of the OVA-specific IgG subtypes tested. However, the organic fraction containing nonpolar lipids potently enhanced OVA-specific antibody titres in the serum, including IgG1, IgG2b, and IgG2c (Fig. 1*B*). Thus it is likely that lipids, and not nonlipid components, are responsible for the immunostimulatory activity of the endogenous oils in vivo.

Endogenous Oils Promote Recruitment of Neutrophils and M1-Like Macrophages

Since oil-in-water adjuvants have been shown to act as powerful chemoattractants for macrophages and granulocytes (29), we investigated whether endogenous oils can promote inflammatory cell recruitment. The peritoneum was chosen as a relevant site to study oil-driven modulation of the innate immune system, as endogenous oils from necrotic adipocytes may leak into the abdomen of obese humans. Furthermore, the application of intraperitoneal injection to demonstrate the immunostimulatory activity of endogenous inducers of sterile inflammation, including monosodium urate crystals (30) and necrotic cells (31), is well established. Remarkably, the endogenous oils induced almost a threefold increase in PEC number after 24 h (Fig. 2*A*).

Neutrophil and M1 macrophage infiltration into WAT is a hallmark of obesity-induced inflammation (12,19). Given the significant increase in total PEC number following injection with endogenous oils, the capacity of the endogenous oils to promote neutrophil and M1 macrophage infiltration was determined. Importantly, "M1-like" and "M2-like" macrophages represent the peritoneal equivalent of M1 and M2 macrophages, respectively (32,33).

There was almost a complete absence of neutrophils (CD11b^{inter} F4/80$^-$ Gr-1^{high}) in the peritoneum of control mice. However, injection of the endogenous oils induced neutrophil recruitment after only 15 min, and the magnitude of this response increased with time, being highest after 24 h (Fig. 2*B*). The endogenous oils also promoted significant infiltration of M1-like macrophages (CD11b^{inter}
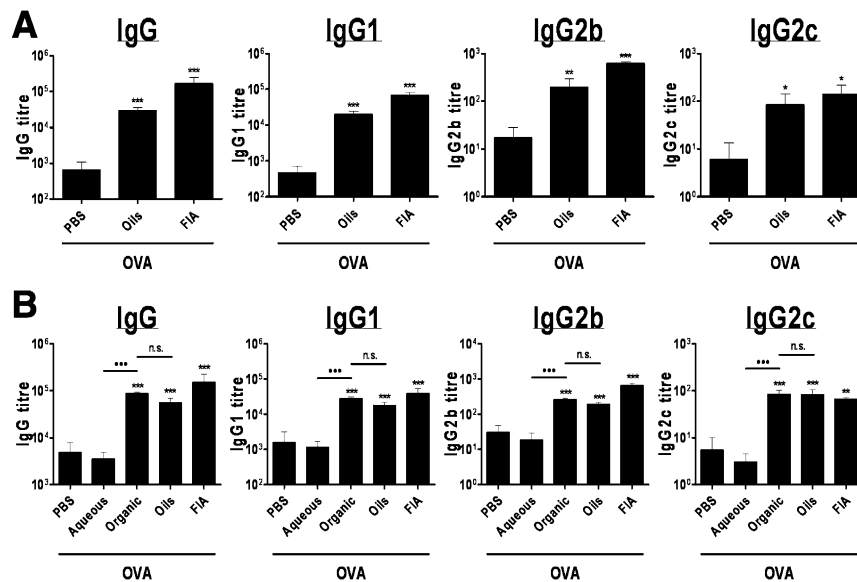
Figure 1—Endogenous oils have potent adjuvant properties. *A*: Female C57BL/6 mice were immunized intraperitoneally on day 0 with PBS or OVA (200 µg/mouse) either alone or mixed with an equal volume (100 µL) of endogenous oils derived from the human omentum. OVA was also mixed with an equal volume (100 µL) of FIA, and this solution was emulsified by sonication. The mice were boosted with these same treatments on day 14 and were killed on day 21. Blood samples were recovered and the serum was removed following centrifugation. OVA-specific antibody titres (IgG, IgG1, IgG2b, and IgG2c) in the serum were determined by ELISA. Results are mean titres (+SEM) for five mice per group. Versus PBS (OVA): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *B*: Female C57BL/6 mice were immunized intraperitoneally with PBS or OVA (200 µg/mouse) either alone or mixed with an equal volume (100 µL) of endogenous oils or an aqueous or organic fraction derived from this preparation following a lipid-extraction procedure. OVA was also mixed with an equal volume (100 µL) of FIA, and this solution was emulsified by sonication. The mice were boosted with these same treatments on day 14 and were killed on day 21. Blood samples were recovered and the serum was removed following centrifugation. OVA-specific antibody titres (IgG, IgG1, IgG2b,, and IgG2c) in the serum were determined by ELISA. Results are mean titres (+SEM) for four mice per group. Versus PBS (OVA), **, $P < 0.01$; ***, $P < 0.001$. Organic fraction (OVA) versus aqueous fraction (OVA): ●●●, $P < 0.001$. n.s., not significant.

F4/80^{inter} SSC^{low}) into the injection site. Recruitment of these cells was only detectable after 3 h but was again most prominent after 24 h (Fig. 2C). The endogenous oils concomitantly mediated the rapid depletion of M2-like macrophages (CD11b^{high} F4/80^{high}) (Fig. 2D). Furthermore, these effects were still observed when as little as 1 µL of endogenous oils was injected (Supplementary Fig. 1A and B).

The immunostimulatory activity of a number of adjuvants has been reported to be at least partially dependent on their ability to induce necrotic cell death at the injection site (34,35). However, we found that the endogenous oils failed to exhibit significant toxicity in contrast to alum (Supplementary Fig. 2A and B). Therefore, the immunostimulatory activity of the endogenous oils is not likely to be mediated by toxicity. In support of this finding, the oil-in-water emulsion FIA also failed to induce necrotic cell death at the injection site (Supplementary Fig. 2C).

Endogenous Oils Enhance the Production of Proinflammatory Cytokines, Including TNF-α, and Decrease the Production of IL-10

Under steady-state conditions, resident macrophages serve to dampen immune responses in WAT through secretion of anti-inflammatory cytokines such as IL-10 (12). In obesity, however, WAT recruits M1 macrophages that secrete

proinflammatory cytokines such as TNF-α (13) and IL-12 (36–38).

Importantly, PECs isolated from mice injected with endogenous oils exhibited a significant increase in secretion of TNF-α and IL-12p40 following restimulation with pattern recognition receptor (PRR) agonists, whereas IL-12p70 release was selectively augmented following restimulation with heat-killed (HK) *E. coli*. In contrast, PECs isolated from mice injected with endogenous oils secreted significantly less IL-10 in comparison with those isolated from mice injected with PBS (Fig. 2E). These effects were also apparent in PECs derived from mice injected with only 1 µL of endogenous oils (Supplementary Fig. 1C). Thus injection of endogenous oils recruits inflammatory cells into the peritoneum and primes PECs for secretion of proinflammatory cytokines, which parallels that seen in the inflamed WAT of obese individuals.

Endogenous Oils Derived From Subcutaneous Adipocytes Promote Innate Immune Responses Comparable to Those Derived From Omental Adipocytes

Accumulation of visceral fat is associated with adverse metabolic conditions such as type 2 diabetes and fatty liver and cardiovascular disease (39). Therefore, this study
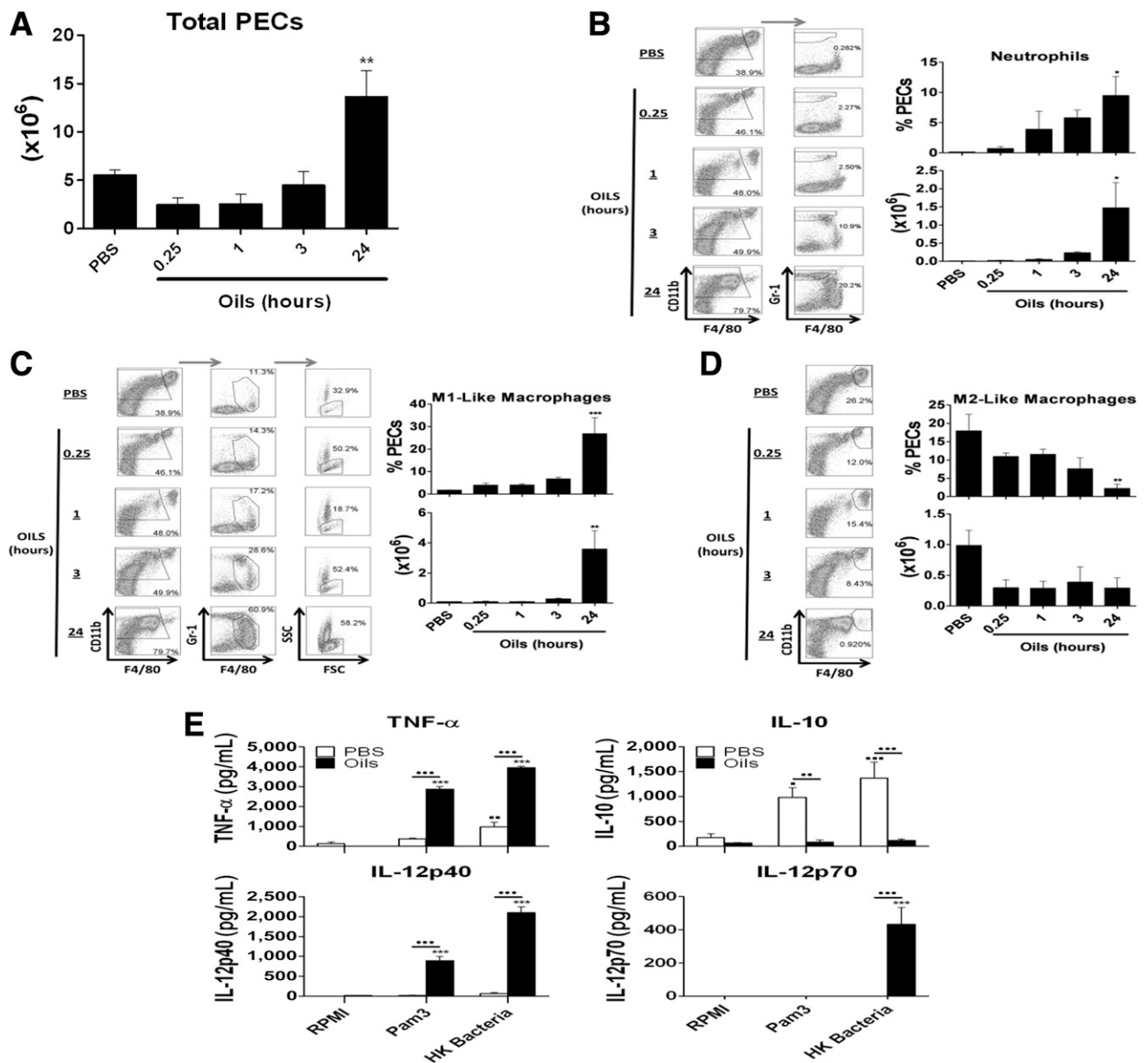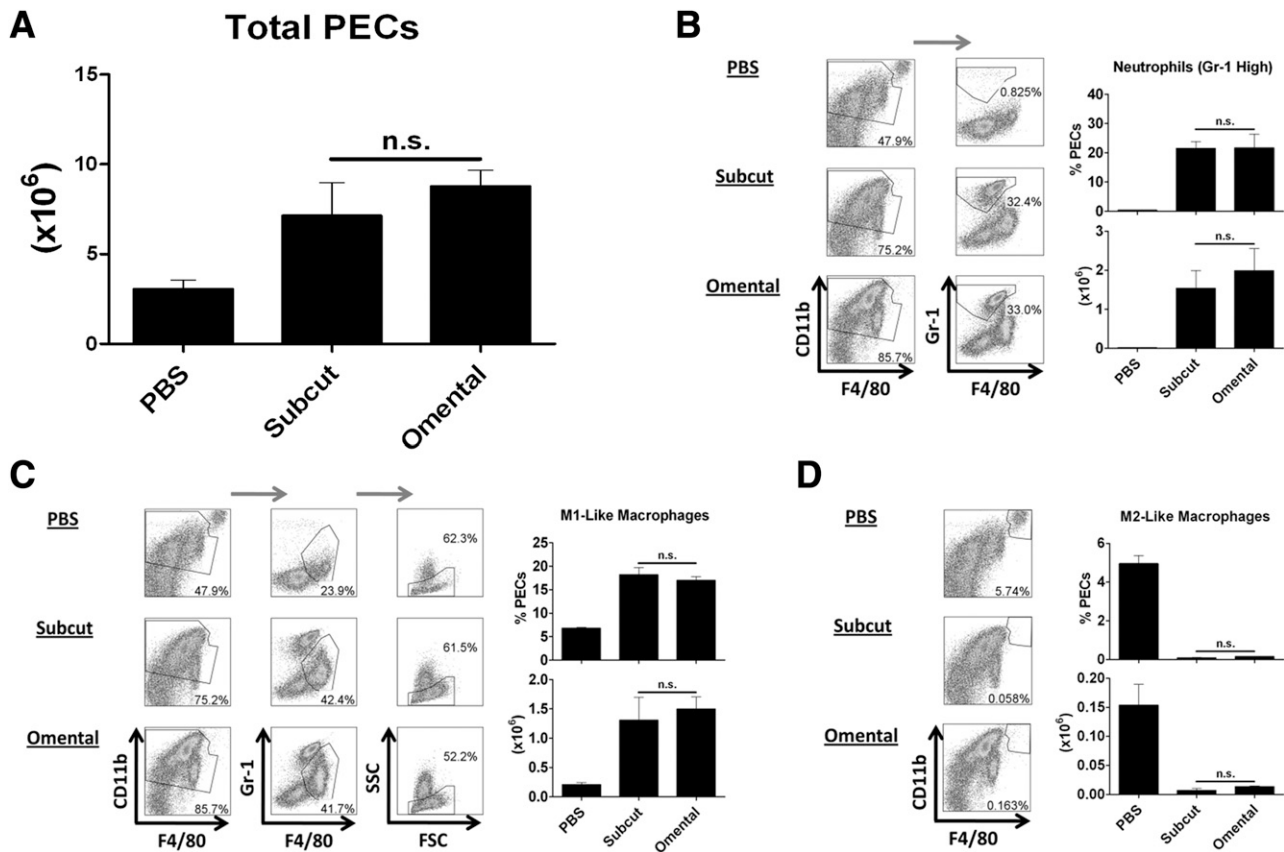
Figure 2—Endogenous oils promote the recruitment of inflammatory cells. *A*: Female C57BL/6 mice were immunized intraperitoneally with PBS or endogenous oils (10 μL/mouse). The mice were killed after the indicated number of hours (0.25 for PBS), and total PEC numbers were determined. *B*: Neutrophils (CD11b[inter] F4/80^{-} Gr-1^{high}), (*C*) M1-like macrophages (CD11b[inter] F4/80[inter] SSC^{low}), and (*D*) M2-like macrophages (CD11b^{high} F4/80^{high}) were identified by flow cytometry. Bar graphs represent mean (+SEM) cell numbers for three mice per group. Versus PBS: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *E*: Female C57BL/6 mice were immunized as described (*A*). The mice were killed 24 h later. PECs were restimulated with RPMI, Pam3 (5 μg/mL), or HK *E. coli* (10 *E. coli*: 1 PEC). After 24 h, supernatants were analyzed for TNF-α, IL-10, IL-12p40, and IL-12p70 by ELISA. Results are mean cytokine concentrations (+SEM) for four mice per group tested individually in triplicate. Versus PECs from oil-injected mice restimulated with RPMI: **, $P < 0.01$; ***, $P < 0.001$. Versus PECs from PBS-injected mice restimulated with RPMI: ■, $P < 0.05$; ■■, $P < 0.01$; ■■■, $P < 0.001$. PECs from oil-injected mice versus PECs from PBS-injected mice for corresponding stimulus: ●●, $P < 0.01$; ●●●, $P < 0.001$.

next sought to address the question of whether cell recruitment into the injection site was an immunostimulatory property specific for endogenous oils derived from omental adipocytes or was also characteristic of endogenous oils derived from the less metabolically active subcutaneous fat.

Endogenous oils derived from subcutaneous and omental adipocytes were comparable in their capacity to

recruit cells into the peritoneum (Fig. 3*A*). Both forms of endogenous oils also promoted similar recruitment of neutrophils and M1-like macrophages (Fig. 3*B* and *C*) while simultaneously depleting the M2-like macrophage population (Fig. 3*D*). Ultimately, there was no significant difference between endogenous oils derived from subcutaneous or omental adipocytes in their respective ability to promote inflammatory effects in the peritoneum.

Figure 3—Endogenous oils derived from subcutaneous adipocytes and omental adipocytes are equally immunogenic. (*A*) Female C57BL/6 mice were immunized intraperitoneally with PBS or endogenous oils derived from either subcutaneous or omental adipocytes (10 μL/mouse) from the same obese individual. The mice were killed 24 h later and total PEC numbers were determined. (*B*) Neutrophils (CD11b^{inter} F4/80^{-} Gr-1^{high}), (*C*) M1-like macrophages (CD11b^{inter} F4/80^{inter} SSC^{low}), and (*D*) M2-like macrophages (CD11b^{high} F4/80^{high}) were identified by flow cytometry. Bar graphs represent mean (+SEM) cell numbers for three mice per group. n.s., not significant.

Endogenous Oils Derived From Visceral Fat of MHO, Metabolically Unhealthy Obese, and Lean Individuals Are Comparable in Their Ability to Drive Inflammation

Since both the omental and subcutaneous oils elicited immunostimulatory effects in vivo, it was hypothesized that the ability of endogenous oils to promote obesity-induced inflammation was dependent on their release from ruptured adipocytes in WAT rather than their specific composition. Importantly, there is a positive correlation between adipocyte hypertrophy and adipocyte necrosis in visceral fat of obese mice and humans (21). In agreement with previous work (25), MHO individuals possessed an average adipocyte diameter of ~75 μm, but metabolically unhealthy obese (MUO) individuals had significantly larger adipocytes, which were ~85 μm in diameter, thus representing almost a 15% increase in adipocyte size (Fig. 4*A*).

Since adipocyte size is positively correlated with adipocyte death, we propose that endogenous oils have a higher propensity to be released from hypertrophied visceral fat in MUO individuals and that this is the key factor in driving inflammation. In support of this hypothesis, endogenous oils derived from MHO and MUO individuals both increased total PEC number, with no significant difference in their ability to promote this effect (Fig. 4*B*). Similar results were evident for recruitment of neutrophils and M1-like macrophages into the peritoneum (Fig. 4*C*), while both treatments effectively depleted the resident M2-like macrophage population (Fig. 4*D*). This comparable capacity of endogenous oils derived from MHO and MUO individuals to modulate the local PEC population was also reflected in terms of PEC cytokine secretion following ex vivo restimulation (Fig. 4*E*).

The immunostimulatory effects outlined above in response to endogenous oils derived from MHO and MUO individuals were also observed following injection with endogenous oils isolated from healthy lean individuals (Fig. 5*A*–*C*). These findings confirm that endogenous oils derived from adipocytes in WAT of MHO, MUO, and lean individuals are equally immunostimulatory. Analysis of the fatty acid profile of endogenous oil preparations derived from MHO, MUO, and lean individuals did not reveal significant differences in composition between the different groups. Furthermore, there was minimal variability between individuals, regardless of their BMI or metabolic state (Fig. 5*D*).
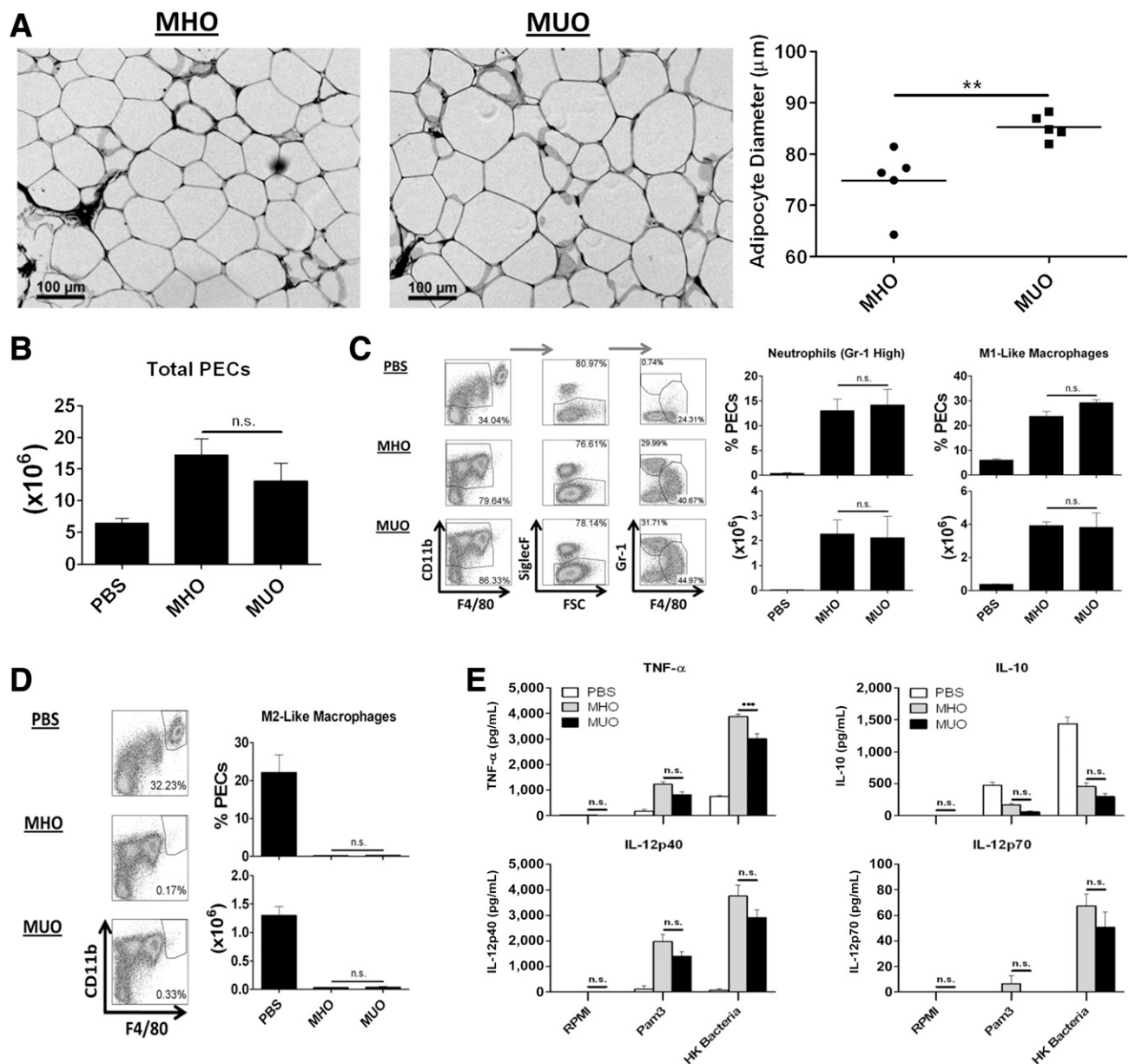
Figure 4—Endogenous oils are released from the hypertrophied visceral fat of MUO individuals and subsequently promote inflammation. *A*: Approximately 10–30 g of omental adipose tissue was obtained during bariatric surgery. A piece of this tissue was immediately fixed in formalin, prior to paraffin mounting and preparation of hematoxylin and eosin sections. Adipocyte size was determined using Image-Pro Plus 7 software. The mean diameter of a range of adipocytes from five MHO individuals and five MUO individuals was calculated from 10 separate photographs of randomized areas of the same section. Single representative image of a section containing WAT from an MHO individual and also a section containing WAT from an MUO individual. Scatter plot represents mean for five individuals per group. Versus MHO: **, $P < 0.01$. *B*: Female C57BL/6 mice were immunized intraperitoneally with PBS or endogenous oils derived from omental adipocytes of an MHO or MUO individual (10 μL/mouse). The mice were killed 24 h later, and total PEC numbers were determined. *C*: Neutrophils (CD11b^{inter} F4/80$^-$ Gr-1^{high}), M1-like macrophages (CD11b^{inter} F4/80^{inter} SiglecF$^-$), and (*D*) M2-like macrophages (CD11b^{high} F4/80^{high}) were identified by flow cytometry. Bar graphs represent mean (+SEM) cell numbers for four mice per group. *E*: Female C57BL/6 mice were immunized as described (*B*). The mice were killed 24 h later, and PECs were isolated. PECs were restimulated with RPMI, Pam3 (5 μg/mL), or HK *E. coli* (10 *E. coli*: 1 PEC). After 24 h, supernatants were analyzed for TNF-α, IL-10, IL-12p40, and IL-12p70 by ELISA. Results are mean cytokine concentrations (+SEM) for four mice per group tested individually in triplicate. MHO versus MUO for corresponding stimulus: •••, $P < 0.001$. n.s., not significant.

Toll-Like Receptor 4 Is Dispensable for Cell Recruitment Into the Peritoneum in Response to Endogenous Oils

Saturated fatty acids have previously been shown to promote cytokine production in macrophages in vitro in a Toll-like receptor 4 (TLR4)-dependent manner (40,41). Since the preparation of endogenous oils derived from the human omentum contains free fatty acids, this study next investigated whether TLR4 is required for inflammatory cell recruitment into the peritoneum in response to these oils.
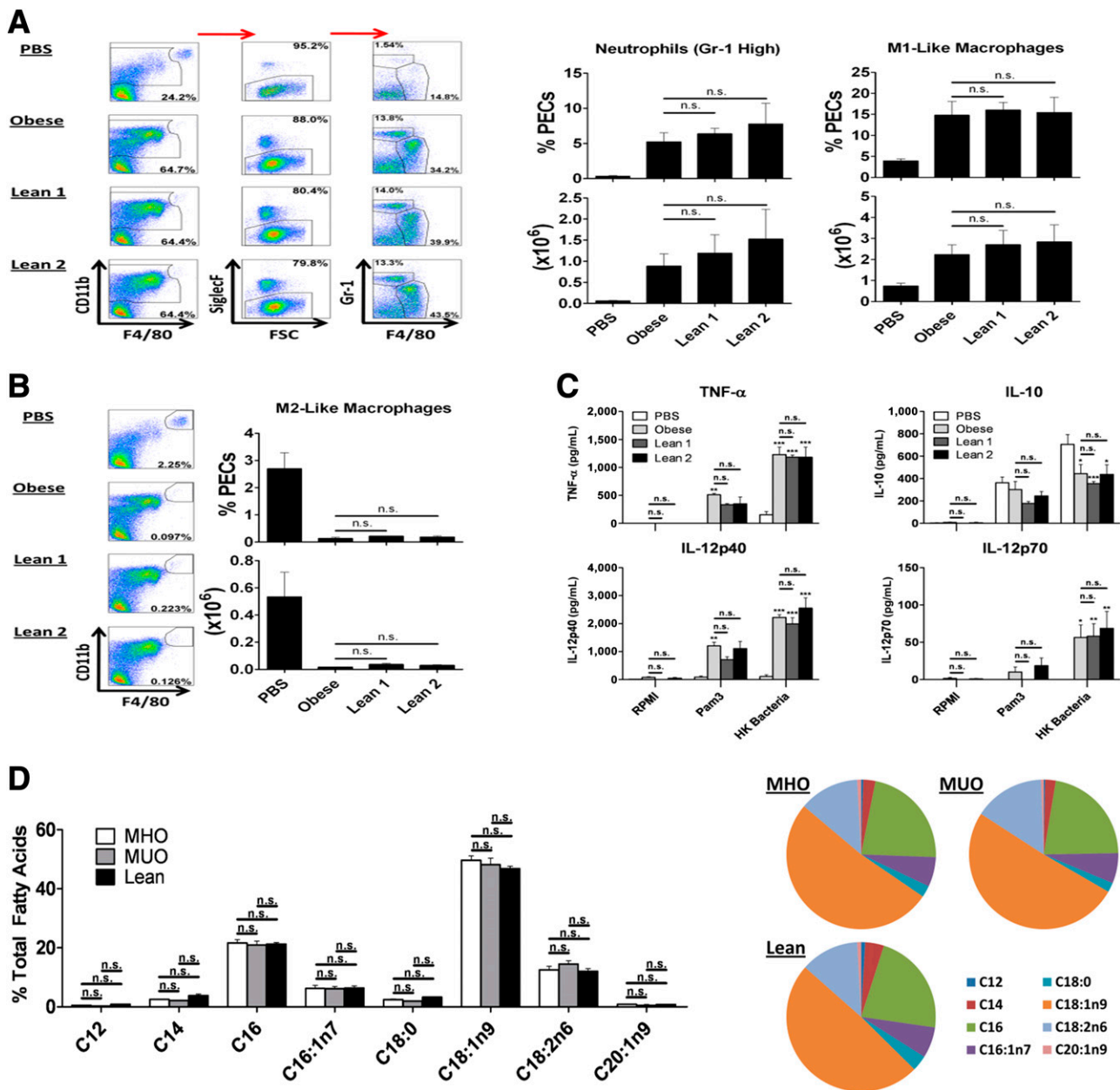
Figure 5—Endogenous oils derived from omental adipocytes of MHO, MUO, and lean individuals share an identical fatty acid profile. *A*: Female C57BL/6 mice were immunized intraperitoneally with PBS or endogenous oils derived from omental adipocytes of an MHO or one of two lean individuals (10 μL/mouse). The mice were killed 24 h later, and neutrophils (CD11b[inter] F4/80^{-} Gr-1^{high}), M1-like macrophages (CD11b[inter] F4/80[inter] SiglecF^{-}), and (*B*) M2-like macrophages (CD11b^{high} F4/80^{high}) were identified by flow cytometry. Bar graphs represent mean (+SEM) cell numbers for four mice per group. *C*: Female C57BL/6 mice were immunized as described (*A*). The mice were killed 24 h later, and PECs were isolated. PECs were restimulated with RPMI, Pam3 (5 μg/mL), or HK *E. coli* (10 *E. coli*: 1 PEC). After 24 h, supernatants were analyzed for TNF-α, IL-10, IL-12p40, and IL-12p70 by ELISA. Results are mean cytokine concentrations (+SEM) for four mice per group tested individually in triplicate. PECs from oil-injected mice versus PECs from PBS-injected mice for corresponding stimulus: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *D*: Fatty acid profiles of endogenous oil preparations derived from omental adipocytes of five MHO, five MUO, and five lean individuals were analyzed by gas chromatography following a lipid-extraction and transesterification procedure. Bar graphs and pie charts represent mean (+SEM for bar charts) fatty acid composition as a relative percentage of the total of the quantified fatty acids present in the tissue. n.s., not significant.

Injection of endogenous oils increased total PEC number in both wild-type C3H/HeN mice and TLR4-defective C3H/HeJ mice, but there was no significant difference in total PEC number between the two mouse strains (Fig. 6*A*). The endogenous oils also induced comparable recruitment of neutrophils into the peritoneum of both C3H/HeN and C3H/HeJ mice. Similar results were evident for M1-like macrophages (Fig. 6*B*). Ultimately, TLR4 was dispensable for the immunostimulatory effects of the endogenous oils. Therefore, signaling through TLR4 by free fatty

acids does not appear to be responsible for mediating these effects.

Neutrophil and M1-Like Macrophage Recruitment Into the Peritoneum Following Injection With Endogenous Oils Is IL-1R–Dependent

IL-1α and IL-1β are powerful chemoattractants that may be involved in promoting cell recruitment into the peritoneum (31,42), and the IL-1R has been implicated in compromised insulin sensitivity (43). Both cytokines were elevated significantly at the injection site after 3 h in response to endogenous oils (Fig. 6C). Therefore, this study sought to determine their role in the subsequent recruitment of inflammatory cells.

The endogenous oils increased total PEC number in wild-type mice, whereas cell recruitment into the peritoneum in response to endogenous oils was completely abrogated in $Il1r1^{-/-}$ mice (Fig. 7A). Neutrophil and M1-like macrophage recruitment was entirely dependent on the IL-1R (Fig. 7B). In contrast, injection of 10 μm PS particles effectively recruited both of these inflammatory cell types into the peritoneum (Fig. 7C). Thus the requirement for the IL-1R in recruitment of neutrophils and M1-like macrophages was specific for injection with endogenous oils.

Interestingly, the saturated fatty acid palmitate has been reported to activate the NLRP3 inflammasome (44) and is present within the preparation of endogenous oils derived from necrotic adipocytes (Fig. 5D). However, the endogenous oils increased total PEC number in both C57BL/6 and $Nlrp3^{-/-}$ mice (Supplementary Fig. 3A). Neutrophil recruitment was marginally reduced in $Nlrp3^{-/-}$ mice, whereas M1-like macrophage recruitment was totally independent of this inflammasome (Supplementary Fig. 3B). This result eliminates the possibility that the observed effects are a result of palmitate-mediated NLRP3 activation.

Modulation of PEC Cytokine Secretion by Endogenous Oils Is IL-1R–Dependent

Since the endogenous oils were capable of modulating the local PEC population and the cytokine secretion profile of PECs following ex vivo restimulation with PRR agonists (Fig. 2E), this study next addressed the question of whether this effect was also dependent on the IL-1R.
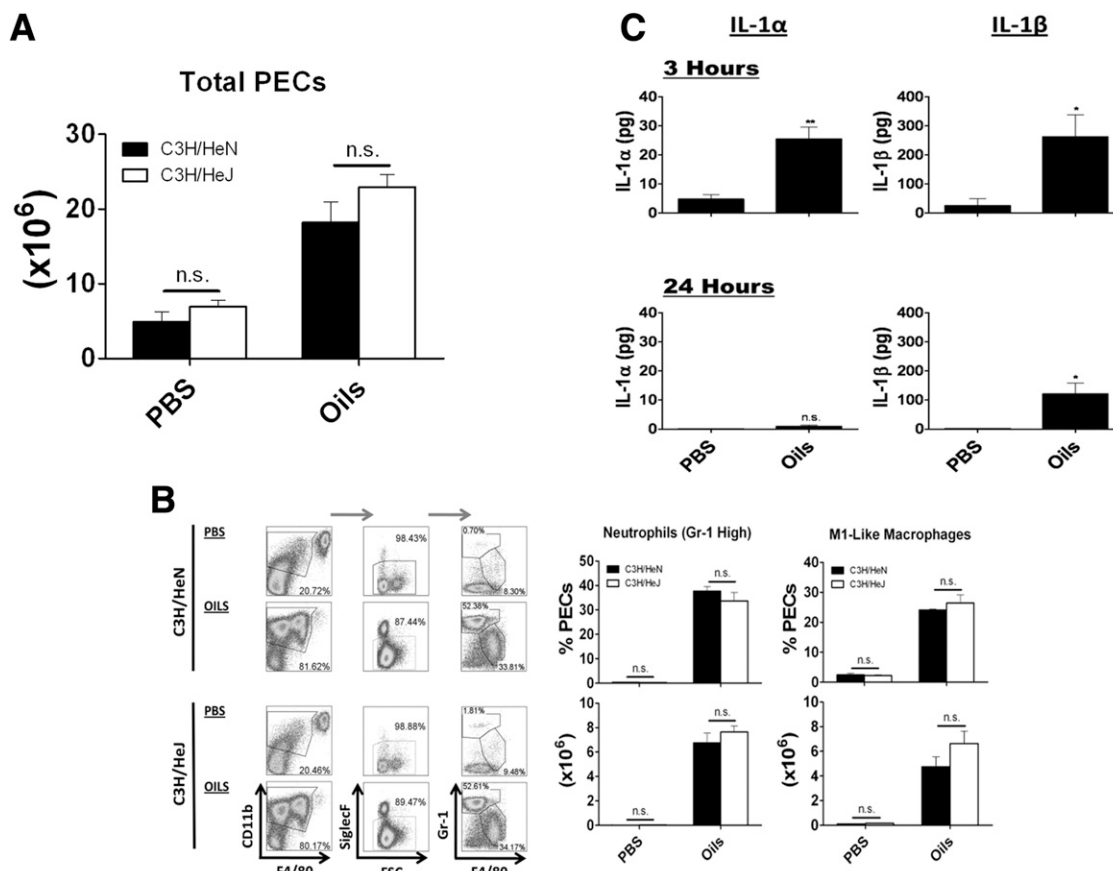
Figure 6—Neutrophil and M1-like macrophage recruitment in response to endogenous oils is independent of TLR4. *A*: Female C3H/HeN and C3H/HeJ mice were immunized intraperitoneally with PBS or endogenous oils (10 μL/mouse). The mice were killed 24 h later, and total PEC numbers were determined. *B*: Neutrophils (CD11b[inter] F4/80^{-} Gr-1^{high}) and M1-like macrophages (CD11b[inter] F4/80[inter] SiglecF^{-}) were identified by flow cytometry. Bar graphs represent mean (+SEM) cell numbers for four mice per group. *C*: Female C57BL/6 mice were immunized as described in *A*. The mice were killed either 3 or 24 h later. PECs were isolated, and PEC supernatants were collected. PEC supernatants were tested for the cytokines IL-1α and IL-1β by ELISA. Results are mean cytokine concentrations (+SEM) for three mice per group. Versus PBS: *, $P < 0.05$; **, $P < 0.01$. n.s., not significant.
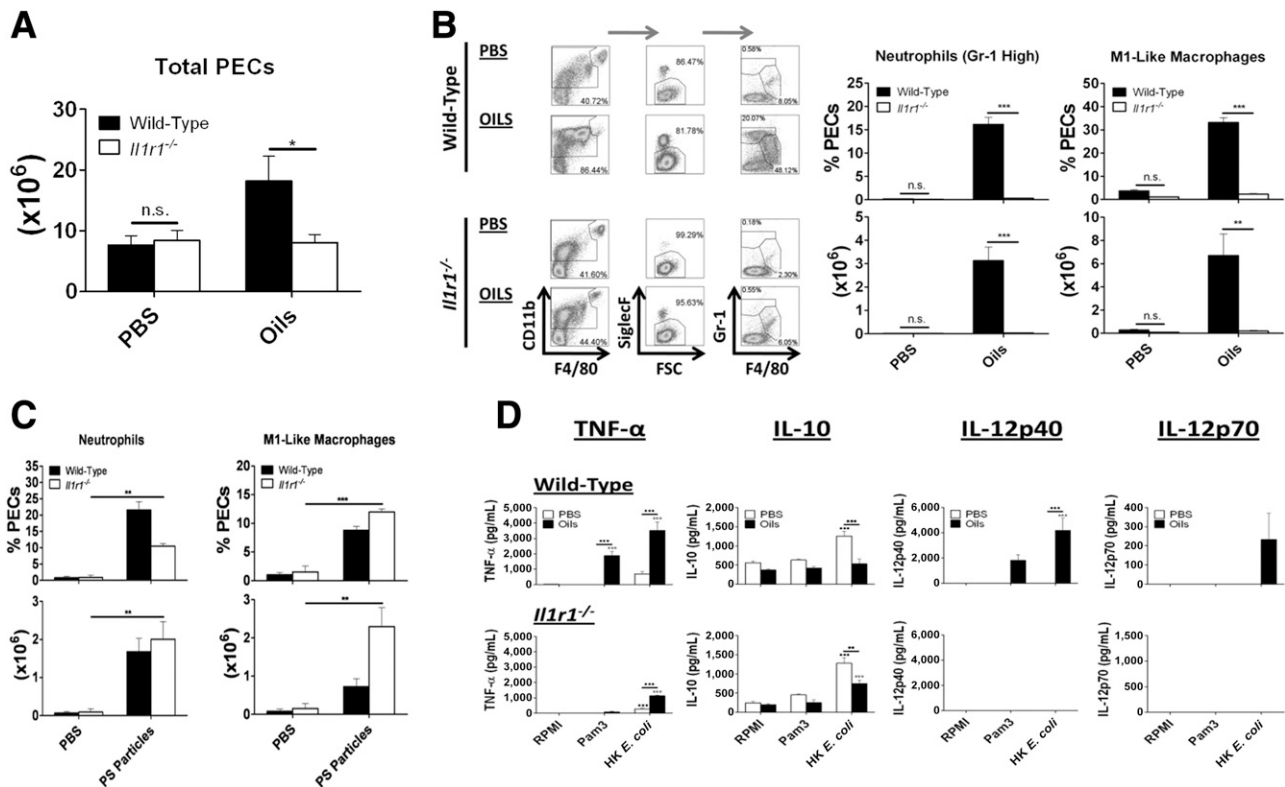
Figure 7—Endogenous oils mediate cell recruitment into the peritoneum through a process dependent on the IL-1R. *A*: Female wild-type C57BL/6 and *Il1r1*$^{-/-}$ mice were immunized intraperitoneally with PBS or endogenous oils (10 μL/mouse) and were killed 24 h later. PEC numbers were determined. *B*: Neutrophils (CD11b^{inter} F4/80$^-$ Gr-1^{high}) and M1-like macrophages (CD11b^{inter} F4/80^{inter} SiglecF$^-$) were identified (four mice per group). *C*: Female C57BL/6 and *Il1r1*$^{-/-}$ mice were immunized intraperitoneally with PBS or 10 μm PS particles (4 mg/mouse). The mice were killed 24 h later. Neutrophils and M1-like macrophages were identified (three mice per group). Bar graphs represent mean (+SEM) cell numbers. Versus wild-type for corresponding treatment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *D*: Female wild-type C57BL/6 and *Il1r1*$^{-/-}$ mice were immunized as described (*A*) and were killed 24 h later. PECs were restimulated with RPMI, Pam3 (5 μg/mL), or HK *E. coli* (10 *E. coli*: 1 PEC). After 24 h, supernatants were analyzed for TNF-α, IL-10, IL-12p40, and IL-12p70 by ELISA. Results are mean cytokine concentrations (+SEM) for four mice/group tested individually in triplicate. Versus PECs from oil-injected mice restimulated with RPMI: ***, $P < 0.001$. Versus PECs from PBS-injected mice restimulated with RPMI: ■■■, $P < 0.001$. PECs from oil-injected mice versus PECs from PBS-injected mice for corresponding stimulus: ●●, $P < 0.01$; ●●●, $P < 0.001$. n.s., not significant.

In contrast to wild-type mice, PECs isolated from *Il1r1*$^{-/-}$ mice injected with endogenous oils failed to secrete detectable levels of IL-12p40 and IL-12p70 following restimulation with PRR agonists. Furthermore, secretion of TNF-α by these cells was completely abrogated following restimulation with Pam3 and significantly reduced when cells were restimulated with HK *E. coli*. However, as in wild-type mice, a reduction in IL-10 synthesis was still evident in PECs isolated from *Il1r1*$^{-/-}$ mice injected with endogenous oils (Fig. 7*D*).

Interestingly, PECs isolated from *Nlrp3*$^{-/-}$ or wild-type mice injected with endogenous oils exhibited an almost identical profile with regard to each of the cytokines tested (Supplementary Fig. 3*C*).

IL-1α Is the Key Cytokine Responsible for Driving Neutrophil and M1-Like Macrophage Recruitment in Response to Endogenous Oils

Both IL-1α and IL-1β are important IL-1 family members that signal through the IL-1R (45). Therefore, the next aim was to determine which of these cytokines was

primarily responsible for mediating the observed effects. Neutrophil and M1-like macrophage recruitment was attenuated in *Il1αβ*$^{-/-}$ mice following injection with endogenous oils (Fig. 8*A*). Intriguingly, however, similar results were also found in *Il1α*$^{-/-}$ mice (Fig. 8*A*), thus confirming that IL-1α is the key IL-1 family member responsible for promoting inflammation in response to endogenous oils. In support of this observation, modulation of the cytokine profile associated with the PEC population following ex vivo restimulation was also IL-1α dependent (Fig. 8*B*).

DISCUSSION

This is the first study to directly test the inflammatory potential of a pure preparation of endogenous oils derived from adipocytes of both lean and obese individuals and demonstrates that these oils have remarkably potent inflammatory effects.

Using a peritonitis model, the endogenous oils increased total PEC number, specifically recruiting M1-like macrophages while simultaneously depleting the M2-like
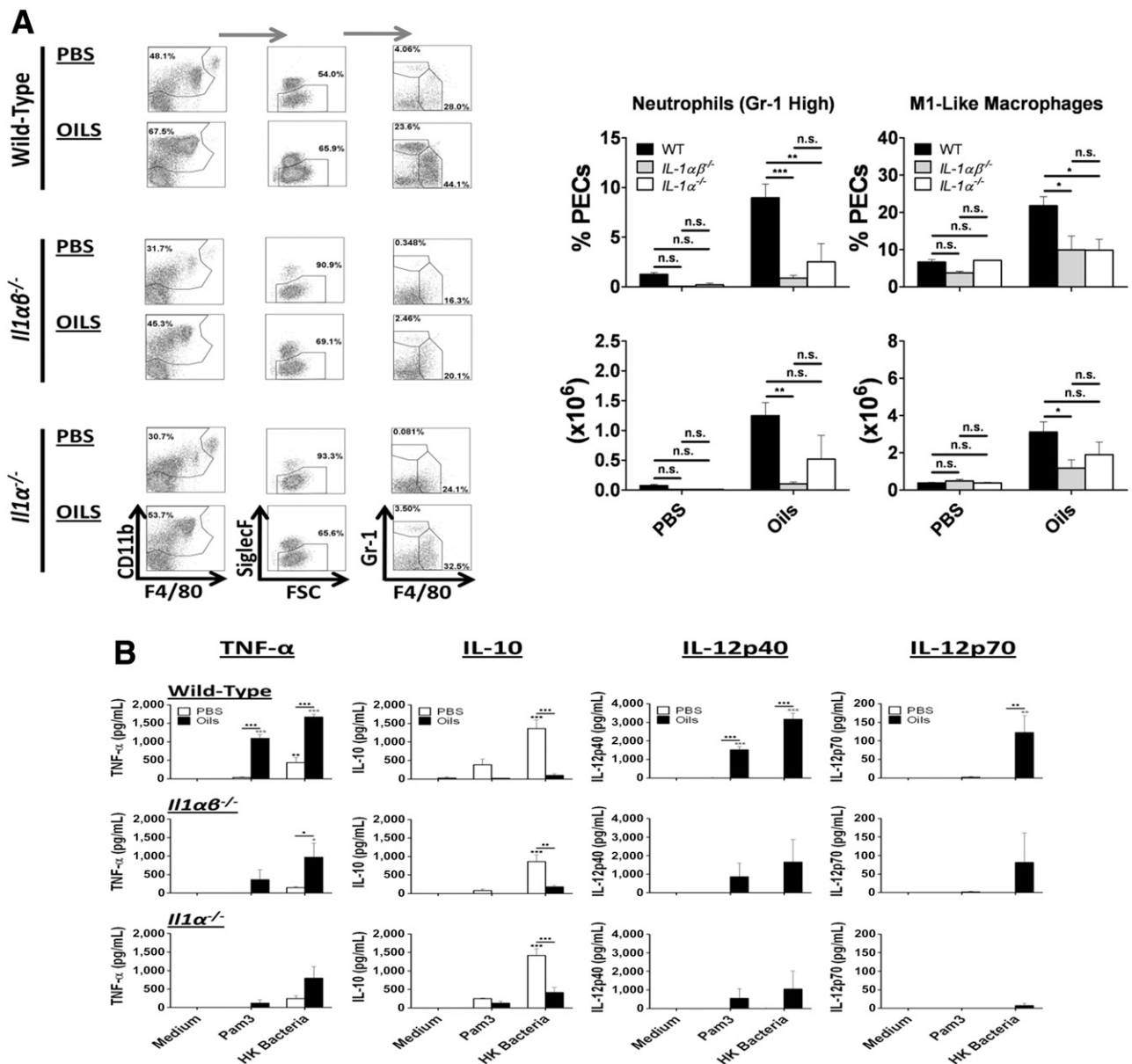
Figure 8—Neutrophil and M1-like macrophage recruitment into the peritoneum in response to endogenous oils is Il-1α–dependent. *A*: Female wild-type C57BL/6, *Il1αβ*⁻/⁻, and *Il1α*⁻/⁻ mice were immunized intraperitoneally with PBS or endogenous oils (10 μL/mouse). The mice were killed 24 h later, and neutrophils (CD11b^inter F4/80⁻ Gr-1^high) and M1-like macrophages (CD11b^inter F4/80^inter SiglecF⁻) were identified by flow cytometry. Bar graphs represent mean (+SEM) cell numbers for four mice per group. Versus wild-type for corresponding treatment: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. *B*: Female wild-type C57BL/6, *Il1αβ*⁻/⁻, and *Il1α*⁻/⁻ mice were immunized as described in *A*. The mice were killed 24 h later, and PECs were restimulated with RPMI, Pam3 (5 μg/mL), or HK *E. coli* (10 *E. coli*: 1 PEC). After 24 h, supernatants were collected and analyzed for the cytokines TNF-α, IL-10, IL-12p40, and IL-12p70 by ELISA. Results are mean cytokine concentrations (+SEM) for four mice per group tested individually in triplicate. Versus PECs from oil-injected mice restimulated with RPMI: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Versus PECs from PBS-injected mice restimulated with RPMI: ■■, *P* < 0.01; ■■■, *P* < 0.001. PECs from oil-injected mice versus PECs from PBS-injected mice for corresponding stimulus: ●, *P* < 0.05; ●●, *P* < 0.01; ●●●, *P* < 0.001. WT, wild-type; n.s., not significant.

resident macrophage population. Comprehensive studies by Ghosn et al. (33) and Spence et al. (32) have recently characterized M1-like and M2-like macrophages and clearly distinguished these coexisting populations using both phenotypic and functional criteria. M1-like macrophages are derived from blood monocytes and are only present at very low levels in the peritoneum of naïve mice. However,

following injection with an inflammatory stimulus such as lipopolysaccharide or thioglycolate, M1-like macrophages become the dominant macrophage population in the peritoneum. M2-like macrophages are resident cells and are the dominant macrophage population in the peritoneum of naïve mice. In contrast to M1-like macrophages, however, the M2-like macrophage population is almost

undetectable in the peritoneum following injection with an inflammatory stimulus (32,33). Injection of endogenous oils strongly promoted the recruitment of M1-like macrophages and depletion of M2-like macrophages and furthermore also potently induced neutrophil recruitment into the peritoneum. Indeed, the fact that endogenous oils are capable of mediating infiltration of two of the most important cell populations in obesity-induced inflammation suggests that these oils may play a prominent role in this process.

The remarkable proinflammatory effects of omental oils were evident following injection of as little as 1 μL of the oil. Taking an average adipocyte diameter and volume as 100 μm and 4/3πr3, respectively, we calculate that 1 μL endogenous oils is the equivalent volume released from only approximately 2×10^3 necrotic adipocytes.

Neutrophil recruitment was only marginally reduced in $Nlrp3^{-/-}$ mice, and M1-like macrophage recruitment was completely NLRP3-independent, thus ruling out a major role for palmitate-dependent NLRP3 activation in this process. In addition, TLR4 is not required for cell recruitment in response to the oils, thereby eliminating the possibility that signaling through TLR4 by free fatty acids is mediating these effects.

Our data suggest that the immunostimulatory activity of endogenous oils is, in fact, mediated by their physicochemical properties rather than by a specific danger signal. Our hypothesis is that most/all triglycerides are, in fact, immunostimulatory but only those that are released from necrotic adipocytes and are therefore exposed to the immune system will signal danger to the host and mediate inflammation. In support of this hypothesis, endogenous oils derived from subcutaneous adipocytes are as potent as those derived from omental adipocytes of the same obese individual, despite the fact that visceral fat is more closely associated with adverse metabolic outcomes than subcutaneous fat (46–48). Furthermore, there is no significant difference in the fatty acid profile or immunostimulatory activity of endogenous oils derived from MHO, MUO, and lean individuals.

We show that IL-1α is the key cytokine responsible for the inflammatory properties of the endogenous oils. Interestingly, Freigang et al. (49) recently demonstrated that oleic acid–mediated neutrophil recruitment into the peritoneum is IL-1α dependent. Furthermore, it was also reported that oleic acid can drive IL-1α production by primary macrophages in vitro and that this cytokine contributes to atherosclerosis in vivo (49). Given the crucial role of IL-1α in responding to endogenous oils demonstrated in the current study, it is conceivable that oleic acid is also playing a significant role in our model. In contrast to previous studies, the purified endogenous oils shown to be highly immunostimulatory here are derived directly from human WAT and thus differ from preparations from atherosclerotic plaques or from fatty acids that have been dissolved in an organic solvent, mixed with an emulsifying agent, and tested in isolation.

We also demonstrate a crucial role for IL-1α in mediating M1-like macrophage recruitment into the peritoneum, an effect that was not previously reported following injection of oleic acid. While Freigang et al. have demonstrated that a number of fatty acids can promote IL-1α release in vitro, none of these fatty acids was capable of driving IL-1β production from lipopolysaccharide-primed macrophages (49). In contrast, our data show that endogenous oils can induce rapid production of IL-1β in the peritoneum, although this does not appear to be decisive in terms of the local inflammatory response. In addition, we found that endogenous oils can prime PECs for secretion of proinflammatory cytokines, which parallels that seen in the inflamed WAT of obese individuals.

These findings open a new avenue of investigation into the mechanism underlying the pathology associated with obesity-induced inflammation. Moreover, the demonstration of the potent inflammatory properties of oils derived from human adipocytes provides a new concept that will inform further research in this field.

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