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Conditional macrophage depletion increases inflammation and does not inhibit the development of osteoarthritis in obese MaFIA mice

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Abstract

Objective—Macrophages are believed to play a critical role in the inflammation associated with the development of osteoarthritis (OA) in obesity. The objective of the study was to investigate whether short-term, systemic depletion of macrophages would mitigate OA following injury in obese mice.

Methods—*CSF-1R-GFP⁺* Macrophage Fas-Induced Apoptosis (MaFIA) transgenic mice that allow conditional depletion of macrophages were placed on a high-fat diet and underwent surgery to induce knee OA. A small molecule (AP20187) was administered to deplete macrophages in MaFIA mice. The effect of macrophage depletion on acute joint inflammation, OA severity, and arthritic bone changes were evaluated using histology and microCT. Immunohistochemistry was used to identify various immune cells. Serum and synovial fluid cytokines were also measured.

Results—Macrophage-depleted mice had significantly fewer M1 and M2 macrophages in the surgical operated-joints relative to controls and exhibited decreased osteophyte formation immediately following depletion. Surprisingly, macrophage depletion did not attenuate OA with obesity; instead, it induced systemic inflammation and led to a massive infiltration of CD3⁺ T cells and particularly neutrophils, but not B cells, into the injured joints. Macrophage-depleted mice also demonstrated markedly increased pro-inflammatory cytokines including granulocyte-colony stimulating factor (G-CSF), IL-1 β , IL-6, IL-8, and TNF- α in both serum and joint synovial fluid, although animals showed trends for decreased serum insulin and leptin levels after depletion.

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Conclusion—Our findings indicate that macrophages are vital in modulating the homeostasis of immune cells in obesity and suggest that more targeted approaches of different macrophage subtypes may be necessary to mitigate inflammation and OA with obesity.

Introduction

Macrophages are an integral part of the innate immune system. Currently, at least two categories of macrophages (M1 and M2) have been identified, although it is now believed that there is a spectrum of macrophage phenotypes. M1 macrophages, also called classically activated macrophages, express high levels of the M1 genes including inducible nitric oxide synthase (NOS-2) and secrete pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-1 (IL-1) (1), while M2 macrophages, i.e. alternatively-activated macrophages, express CD206 and are responsible for releasing anti-inflammatory cytokines such as IL-10 (2).

In addition to defending the host against pathogens via their phagocytic ability, macrophages play a central role in metabolic homeostasis by regulating insulin sensitivity. Indeed, it has been proposed that during infection, pro-inflammatory cytokines secreted from M1 macrophages help tissues develop transient insulin resistance, conserving most glucose for immune cells in order to combat microbes (3). However, although this mechanism is an important adaptive trait for mammals, it could be detrimental to surrounding host tissues if macrophages stay in the prolonged inflammatory phase. For example, excess energy intake, such as diet-induced obesity, often leads to increased cell stress, lipolysis, and apoptosis of adipocytes in fat tissue, along with increased macrophage infiltration (4, 5). Fatty acids (FAs) released from adipocytes along with FAs from the diet lead to phenotypic switch of macrophages toward the M1 pro-inflammatory pathway (6). Additionally, TNF- α secreted by M1 macrophages can further induce insulin resistance and lipolysis of adipocytes. Such interactions between adipocytes and M1 macrophages constitute a vicious cycle, perpetuating chronic systemic inflammation that contribute to metabolic disease in obese individuals (7). A recent animal study has shown that conditional depletion of macrophages using clodronate decreased insulin resistance in obese mice, indicating the role of macrophages in metabolic syndrome (8).

Macrophages may also play a role in the progression of osteoarthritis (OA). OA is a disease of the entire joint involving degradation of articular cartilage, synovitis, and subchondral bone changes. Recent accumulating evidence suggests that, in addition to altered joint loading, inflammation also contributes significantly to OA particularly in obesity (9, 10). For example, we previously demonstrated that obese mice fed high-fat diets rich in pro-inflammatory saturated fatty acid (FAs) and ω -6 FAs, showed significantly more severe OA and macrophage infiltration in the joint synovium after meniscal injury versus obese mice fed high-fat diets rich in anti-inflammatory ω -3 FAs (11). In certain mouse models such as the MRL/MpJ “superhealer” mouse, rapid resolution of macrophage infiltration following joint injury has been associated with reduced inflammation as well as OA (12). It has also been shown that systemic depletion of macrophages decreases joint swelling in a collagen-induced arthritis model (13). Recently, several studies have greatly enhanced our understanding of the significance of macrophages in OA progression but most of them

utilized lean models, i.e. animals that received a standard chow (14–16). Therefore, little is known about whether macrophages in the joints of obese individuals play a similar catabolic role in OA as they do in lean subjects. In this study, we examined the hypothesis that transient, systemic depletion of macrophages can mitigate obesity-associated OA.

Materials and Methods

Detail experimental methods are provided in online supplementary documents.

Animals

All procedures were approved by the Duke University IACUC. Male macrophage Fas-Induced Apoptosis (MaFIA) transgenic mice (C57BL/6-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J, stock #005070) were purchased from Jackson Labs. The MaFIA transgenic mouse model was developed by Burnett et al. by placing the genes encoding enhanced green fluorescent protein (eGFP) and mutant human FK506-binding protein (FKBP)–Fas suicide construct under the macrophage-specific mouse colony stimulating factor 1 receptor promoter (Csf1r). Thus, macrophages in MaFIA transgenic mice can be conditionally driven into an apoptotic pathway through the administration of AP20187 molecule (Clontech), an otherwise inert dimerizing reagent that initiates apoptosis in macrophages by binding to mutant human FBKP (17). Furthermore, these macrophages also express eGFP, providing significant advantages in visualization and quantification of macrophages in MaFIA mice. Beginning at 4 weeks of age, all MaFIA mice were fed a custom-designed high-fat diet (ω -6/ ω -3 ratio = 20.3) (Research Diet, D11120107). The study design is presented in Figure 1A.

Body composition

The body fat content and bone mineral density (BMD) of the mice were measured at 9 and 23 weeks of age using a dual-energy x-ray absorptiometry scanner (DEXA, Lunar PIXImus).

OA induction and macrophage depletion

At 13 weeks of age, all MaFIA mice underwent surgery to destabilize the medial meniscus (DMM) to induce knee OA in the left hind limb as previously described (18). The infrapatellar fat pad was bluntly dissected to reveal the meniscus during the surgery but was not removed as it has been reported that joint fat pad may also play a significant role in inflammation (19, 20). Immediately following DMM surgery, MaFIA mice were treated with either AP20187 solution consisting of 4% ethanol, 10% PEG-400, and 1.7% Tween-20 in water (dose: 10 mg/kg) to systemically ablate macrophages, or with vehicle control solution (17). At one week post-surgery, mice received a second depletion or vehicle as appropriate. The MaFIA mice receiving DMM surgery without macrophage depletion were used as a control group throughout the current study. To determine the effect of macrophage depletion on acute joint inflammation, some mice were sacrificed the day after the second depletion (this time point was designated '0 weeks post-depletion'). To evaluate the effects of macrophage depletion on OA development, mice were sacrificed at 23 weeks of age (this time point was designated '9 weeks post-depletion').

Macrophages quantification

Upon sacrifice, epididymal fat, and both right and left joint capsules were harvested, minced and digested at 37°C with 0.2% collagenase type I (Worthington) for 1 hour. Macrophage percentage was determined by quantifying eGFP⁺ cells using flow cytometry.

OA, synovitis, and osteophytes

Joint sections were stained with hematoxylin, fast green, and Safranin-O to determine OA and osteophyte severity. For synovitis, sections were stained with hematoxylin and eosin (H&E) to reveal infiltrated cells and synovial structure. Three independent, blinded graders scored joint sections for OA using a modified Mankin scoring system, for synovitis using previously established protocol (21), and osteophytes in the joint were graded using Safranin-O/Fast green stained joint slides under high-power field (200× magnification) and a grading scale created based on a review by van der Kraan and co-workers, with slight modifications (11, 22). Scores were averaged among graders for the whole joint.

Bone micro-computed tomography

Joint bone architecture and mineral density were analyzed as previously described (21). Briefly, both hind-limbs were scanned by a micro-computed tomography (microCT) system (Skyscan 1176, Bruker). A hydroxyapatite calibration phantom was used to calibrate bone density values (mg/cm³). Bone morphometric parameters were measured in the distal femoral condyles, the tibial epiphysis immediately distal to the subchondral bone, and the tibial metaphysis in the joints. Parameters reported for the femoral condyles were bone mineral density (BMD; mg/cm³) and cancellous bone fraction (bone volume/total volume, BV/TV, excluding the cortex), while parameters reported for the tibial epiphysis and metaphysis were BMD and BV (mm³). Unless otherwise indicated, to determine how treatment, DMM surgery, and their interaction (surgery × treatment) affected bone remodeling, bone parameters from non-operated (right) and DMM-operated (left) joints were analyzed by two-way repeated measures ANOVA.

Immunohistochemistry (IHC)

DMM-operated joints from each group were immunohistochemically stained for murine macrophages (F4/80, Serotec), M1 macrophages (NOS-2, inducible nitric oxide synthase), and M2 macrophages (CD206), neutrophils (elastase), T cells (CD3, a pan-T cell marker), and B cells (CD22, a pan-B cell marker) (Abcam).

Cytokine analyses

Concentrations of both leptin and insulin in serum samples were measured using the Mouse Metabolic Assay (MSD). Serum IL-27 and TGF-β1 levels were measured using the Mouse TGF-β1 (BioLegend) and IL-27 ELISA kit (R&D). Concentrations of pro-inflammatory cytokines in the synovial fluids were quantified using a multiplexed sandwich ELISA (Mouse Proinflammatory Panel 1 V-plex), and IL-17 was measured using a singleplex ELISA assay (MSD).

Statistics

Statistical analyses are described in each figure caption. Analyses were performed using IBM SPSS, with significance reported at the 95% confidence level.

Results

Macrophage depletion temporarily decreased body weight

Upon macrophage depletion, obese MaFIA mice experienced significant weight loss compared to the non-depleted control mice (Figure 1B). However, throughout the 4 weeks after the depletion treatments, the depleted mice gradually recovered to similar weights as controls. Furthermore, depleted mice also trended toward decreasing percentage body epididymal fat percentage (Figure 1C), but showed significantly increased percent spleen weight at the time of depletion (Figure 1D). There was no significant difference in whole body fat percentage or body BMD evaluated by DEXA scan between depleted and non-depleted control mice at 9 weeks and 23 weeks of age (Supplemental Figure 1).

Macrophage depletion transiently decreased macrophages in visceral fat and DMM-operated joints

Our depletion strategy significantly decreased the amount of macrophages (eGFP⁺ cells in MaFIA transgenic mouse model) in DMM-operated joint capsules (Figure 2A) and epididymal fat (Supplemental Figure 2) at the time of the depletion. At 9 weeks post-depletion, however, the percentage of eGFP⁺ cells in all tissues analyzed was comparable in the control and depleted mice. IHC against F4/80, a marker for general macrophage population in murine, further confirmed that significantly fewer macrophages were present in the DMM-operated (left) joints relative to the non-operated (right) joints in non-depleted mice (Figure 2B). Moreover, control mice trended toward having more NOS-2⁺ cells (M1 macrophages) in the operated-joints as compared to the depleted mice at 0 weeks post-depletion. However, macrophage-depleted mice also exhibited significantly fewer CD206⁺ cells (M2 macrophages) in the DMM joints versus the control mice at 9 weeks post-depletion (Figure 2C and D; see Supplemental Figure 3 and Supplemental Figure 4 for enlarged IHC images against NOS-2⁺ M1 cells and CD206⁺ M2 cells, respectively).

Macrophage depletion altered bone parameters at the time of ablation

Macrophage-depleted mice had less osteophytosis at the end of depletion treatment 8 days after DMM, but had similar degrees of osteophytosis at 9 weeks post-depletion (Figure 3A and B). Furthermore, with the microCT analyses, macrophage-depleted mice had a lower BMD and BV/TV of the femoral condyle and tibia epiphysis as compared to the control mice at 0 weeks post-depletion (Figure 3C and D). There were no significant differences in BMD and BV/TV between the control and depleted mice at 9 weeks post-depletion, although a trend toward a lower BMD of the femoral condyle was observed in the depleted mice.

Macrophage depletion did not prevent OA but increased synovitis

The DMM-operated joints of all mice had significantly higher OA scores compared to their own contralateral non-operated joints only at 9 weeks after depletion. Macrophage depletion in obese mice did not mitigate OA at any time point studied (Figure 4A; for individual components of modified Mankin OA score see Supplemental Figure 5). At both 0 weeks and 9 weeks post-depletion, the operated-joints of all mice had more severe synovial inflammation relative to the non-operated joints (Figure 4B; for individual components of synovitis score see Supplemental Figure 6). Surprisingly, macrophage-depleted mice had significantly more severe synovitis in the operated-joints than the control mice at 9 weeks post-depletion. Given that the number of macrophages was lower in the synovium of the depleted DMM joint (see Figure 2A and B), this result implies a potential regulatory role for macrophages, and that cells other than macrophages may infiltrate into DMM operated-joints, leading to more severe synovitis.

Neutrophils and CD3⁺ T cells were significantly increased after macrophage depletion

No difference in CD22⁺ B cells in the DMM joints between the control and depleted mice was observed at any time point studied (Figure 4C and D). Interestingly, macrophage-depleted mice showed significantly increased infiltration of CD3⁺ T cells (greater than 5-fold) at both time points studied. DMM joints of depleted mice also exhibited more neutrophils (approximate 8-fold increase) relative to those of the control mice immediately following depletion. (see Supplemental Figure 7, Supplemental Figure 8 and Supplemental Figure 9 for enlarged IHC images against CD22⁺ B cells, CD3⁺ T cells and neutrophils, respectively).

Macrophage depletion trended toward decreased serum insulin and leptin concentrations, but significantly increased serum inflammatory cytokine levels at the time of depletion

The mice receiving macrophage depletion trended toward having lower serum insulin and leptin levels relative to the control mice immediately after depletion (Supplemental Figure 10A and B). Furthermore, regardless whether receiving depletion treatment, mice at 9 weeks post-depletion had significantly higher serum insulin and leptin levels compared to mice at 0 weeks post-depletion. Macrophage depletion significantly decreased serum TGF- β 1 concentrations in the depleted mice immediately following depletion (Figure 5A). Surprisingly, macrophage-depleted mice exhibited significantly elevated serum concentrations of granulocyte-colony stimulating factor (G-CSF), IL-1 β , IL-6, IL-8, IL-10, IL-27, and TNF- α at 0 weeks post-depletion (Figure 5B–I). The serum levels of IL-8 and IL-27 in the depleted mice demonstrated a sustained elevation compared to control mice, even at 9 weeks post-depletion (Figure 5F and H). No significant difference was observed in serum interferon- γ (IFN- γ) concentration between depleted and control mice at both time points studied (Figure 5C).

Macrophage depletion increased inflammatory cytokine levels in the synovial fluid of the DMM joint

Macrophage depleted mice trended toward increased IL-1 β and IL-6 levels in the synovial fluid of the operated-joint relative to non-depleted mice at 0 weeks post-depletion (Figure

6A and B). The mice with macrophage depletion also demonstrated significantly higher concentrations of IL-8, IL-10, and TNF- α in the synovial fluid of the DMM-operated joint compared to those cytokines from control mice at the time of depletion (Figure 6C, 6D, 6F). Furthermore, only the macrophage-depleted mice exhibited detectable levels of IL-6, IL-8 and IL-10 in the synovial fluid of the non-operated right joints at the time of ablation. Interestingly, surgery significantly decreased the IL-17 concentrations in the operated joint as compared to that in the non-operated joint in both control and macrophage depleted mice at 0 weeks post-depletion (Figure 6E).

Discussion

Our findings indicate that transient, systemic depletion of macrophages in obese MaFIA mice did not prevent OA development following joint injury. While macrophage-depleted obese mice showed significantly fewer M1 and M2 macrophages in the DMM joints relative to controls, these animals exhibited increased joint synovitis with massive infiltration of neutrophils and CD3⁺ T cells post-depletion. Macrophage-depleted mice also demonstrated markedly increased concentrations of pro-inflammatory cytokines including G-CSF, IL-1 β , IL-6, IL-8, IL-27 and TNF- α in the serum and synovial fluid of DMM joints. Overall, these results suggest that macrophages play a complex immunomodulatory role in inflammation and injury-induced OA in the context of diet-induced obesity.

We found that as early as one-week post-DMM surgery, obese mice already demonstrated deep cartilage clefts and a much more rapid progression of OA as compared to lean mice receiving DMM surgery, which generally may take two weeks to observe mild cartilage fibrillation (23, 24). This result provides further evidence that diet-induced obesity accelerates the rate of OA development in humans and animals (25–28). However, despite the fact that the presence of osteophytes, a clinical hallmark of OA, was less severe in the MaFIA mice immediately after depletion, these obese mice still developed osteophyte scores comparable to the control mice at 9 weeks post-depletion. Low osteophyte formation in the depleted mice at the time of treatment may be related to their low serum TGF- β 1 levels, as it has been shown that macrophages are one of the main sources of this growth factor (15). According to the studies conducted by Burnett et al., macrophages repopulate in about 5 days after AP20187 administration (29). Therefore, it is possible that repopulated macrophages in the joints still contributed to the formation of osteophytes at 9 weeks post-depletion. We also observed that macrophage depleted mice had decreased BMD and BV/TV in their joints compared to non-depleted control immediate post-depletion. This finding is in line with several previous studies showing that the ablation of osteal tissue macrophages led to impaired osteoblast differentiation and mineralization (30, 31).

To our surprise, not only did macrophage depletion fail to decrease OA, but rather exacerbated synovitis and intensified inflammatory cytokine production in the depleted mice. The finding that the number of macrophages was lower in the synovium of the depleted joint implies that cells other than macrophages invaded into the DMM operated-joints of the depleted mice. IHC of various immune cells revealed that CD3⁺ T cells and particularly neutrophils, but not CD22⁺ B cells, massively infiltrated into the operated joint of the depleted mice. This finding suggests that despite their recognized pro-inflammatory

role, macrophages are vital in regulating the homeostasis of immune cells in the joint following injury. Indeed, two recent studies using distinct transgenic mice models capable of macrophage depletion support our observation. Gordy et al. reported that lean mice lacking macrophages in the marginal zone of spleen exhibited significantly increased numbers of neutrophils in circulating blood (32). Similarly, upon removal of macrophages, Lee et al. observed up-regulated Ly-6G expression, a specific marker for neutrophils, in a variety of tissues (33). Both studies described the occurrence of neutrophilia and a systemic inflammatory response following macrophage depletion. Therefore, it is possible that the accumulated neutrophils in the depleted mice are the main source of inflammatory cytokines in synovial fluid, as neutrophils are capable of secreting IL-1, IL-6, and TNF- α . In addition, neutrophils can also produce IL-8, a potent chemokine for themselves, recruiting more granulocytes to inflammatory sites (34–36). Although IL-10, an anti-inflammatory molecule, was also found to be up-regulated in the synovial fluid in our model, its concentration may not be sufficient to counteract the catabolic effects of pro-inflammatory cytokines on the joint due to its relative low quantities compared to IL-1 β (about 10-fold less). In addition, TNF- α levels were also increased nearly 3-fold in DMM joints immediately following macrophage depletion. Interestingly, IL-17 in the synovial fluid of the DMM joints from both groups of mice decreased to below detectable levels after DMM surgery, suggesting this reduction may be associated with injury. However, whether this down-regulation was the result of injury-related cytokines modulating IL-17 producing cells, such as T helper-17 (Th17) cells, requires further investigation. Overall, macrophage depletion in obese MaFIA mice appears to shift the synovial inflammatory response from macrophage-associated to neutrophil-dependent, providing a potential mechanism of OA induction in macrophage-deficient obese mice.

In addition to their potential role in modulating immune cells, joint synovial macrophages can uptake oxidized low-density lipoproteins (oxLDL), inducing anabolic processes by producing TGF- β (37). Recently, the same group further reported that in the lean mice with synovial macrophage-depletion, oxLDL induces catabolic processes including elevated production of pro-inflammatory mediators as well as increased infiltration of monocytes and neutrophils into the joint synovium (38). These results indicate that synovial macrophages can be protective against oxLDL-induced inflammation. In our current study, mice were fed a pro-inflammatory high-fat diet with high ω -6/ ω -3 FA ratio, likely leading to increase systemic LDL levels and metabolic syndrome in these animals. Thus, our observation of elevated joint inflammation following macrophage-depletion may be partly attributed to potential excess oxLDL levels in synovial fluid.

Another important finding in this study is that there was a sustained systemic elevation of IL-27 production in the macrophage-depleted mice. The over-production of IL-27 in our model is also consistent with the presence of neutrophilia in macrophage-depleted mice as it is among the diverse array of secreted factors from neutrophils (39). IL-27 possesses both anti- and pro-inflammatory properties (40). For instance, treatment with IL-27 ameliorates Th17-dependent rheumatoid arthritis (41, 42). In contrast, IL-27 has been shown to promote naive T cell differentiation into Th1 cells that have a prominent role in development of Th1-dependent experimental arthritis (43). Recent studies further demonstrated that IL-27 facilitates a subset of T regulatory cells to produce IL-10 (44, 45). The pleiotropic functions

of IL-27 may provide a potential explanation for increased T cell numbers and IL-10 production in the synovial fluid of the DMM-operated limbs of macrophage-depleted mice.

In the current model, we observed that along with temporarily reduced insulin and leptin levels, obese MaFIA mice lost approximately 30% of their body weight upon macrophage depletion, which is 13% more than what was reported when lean MaFIA mice were used (17). A recent study reported that adipose tissue increased lipolysis in the absence of adipose tissue macrophages (ATMs), suggesting a central role of macrophages in lipid tracking (46). Perhaps, upon macrophage depletion, adipocytes lost regulatory signaling from ATMs and rapidly released large amounts of lipid content, leading to a dramatic weight loss in our obese MaFIA mice. On the contrary, in Burnett and colleagues' lean MaFIA mouse model, the effect of macrophage depletion on body mass was likely to be less profound, potentially due to the lower amount of lipid stored in adipocytes (17). Another possible explanation for the substantial weight loss may be the result of hypothalamic inflammation following macrophage depletion, as it has been reported that mice that underwent macrophage ablation showed elevated inflammatory cytokines in the hypothalamus, causing reduced food intake (33). Our results provide further evidence that macrophages are essential in maintaining energy metabolism.

Conversely, there are studies showing that macrophage ablation in mice did not elicit inflammatory responses. Using CD11c-diphtheria toxin receptor (DTR) transgenic mice, Patsouris et al. depleted CD11c⁺ (another common marker for M1 macrophages) cells by administering diphtheria toxin without inducing severe neutrophilia (47). The discrepancy between their and our findings may result from whether neutrophils were also depleted along with macrophages. In our MaFIA mice, colony stimulating factor 1 receptor (csf1r), the promoter only specific for myeloid cells, was used to express the Fas-FKBP protein that can drive macrophages into the apoptotic pathway upon AP20187 delivery. It has been suggested that granulocytes such as neutrophils may have lower expression levels of Fas-FKBP levels compared to macrophages (48), and thus were not significantly depleted in the csf1r transgenic system. In contrast, a study demonstrated that neutrophils may give rise to a hybrid, neutrophil-dendritic cell population that co-expresses CD11c and Ly6G (49). Furthermore, it has been reported that neutrophils express CD11c in humans (50). Therefore, a subset of neutrophils may likely be removed simultaneously with macrophages in CD11c-DTR system. Nonetheless, there are also limitations of our current study. First, it is unclear whether sustained expression of serum IL-27 at the later time point is the result of the combined secretion of granulocytes and regenerated macrophages, or if it is solely due to excess neutrophils. Moreover, as M2 anti-inflammatory macrophages were also depleted in our MaFIA model, leading to consistently fewer M2 macrophages in DMM synovium of the depleted mice. Thus, whether the disrupted cross-talk between M2 macrophages and neutrophils have a role in elevated synovitis in the depleted mice remains unexplored. Detailed longitudinal studies following general macrophage depletion, and more specific depletion of different macrophage phenotypes such as depleting only M1 or M2 macrophages, may provide answers to these questions.

In summary, contrary to our hypothesis, we found that short-term, systemic macrophage depletion did not mitigate cartilage degeneration following injury in obese mice; instead, it

enhanced joint synovitis by increasing the infiltration of CD3⁺ T cells and neutrophils into the operated joint. These findings emphasize the important regulatory role that macrophages have in modulating the responses of other inflammatory cells, and have significant implications for the potential clinical application of macrophage depletion as a therapy for inflammatory conditions, such as for obese arthritic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Li P, Lu M, Nguyen MA, Bae EJ, Chapman J, Feng D, et al. Functional heterogeneity of CD11c-positive adipose tissue macrophages in diet-induced obese mice. *Journal of Biological Chemistry*. 2010; 285(20):15333–45. [PubMed: 20308074]
2. Aron-Wisnewsky J, Tordjman J, Poitou C, Darakhshan F, Hugol D, Basdevant A, et al. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. *Journal of Clinical Endocrinology & Metabolism*. 2009; 94(11):4619–23. [PubMed: 19837929]
3. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. 2013; 496(7446):445–55. [PubMed: 23619691]
4. Nguyen MA, Favelyukis S, Nguyen A-K, Reichart D, Scott PA, Jenn A, et al. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *Journal of Biological Chemistry*. 2007; 282(48):35279–92. [PubMed: 17916553]
5. Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, et al. Role of the Toll-like receptor 4/NF- κ B pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arteriosclerosis, thrombosis, and vascular biology*. 2007; 27(1):84–91.
6. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of clinical investigation*. 2007; 117(1):175–84. [PubMed: 17200717]
7. Suganami T, Ogawa Y. Adipose tissue macrophages: their role in adipose tissue remodeling. *Journal of leukocyte biology*. 2010; 88(1):33–9. [PubMed: 20360405]
8. Feng B, Jiao P, Nie Y, Kim T, Jun D, Van Rooijen N, et al. Clodronate liposomes improve metabolic profile and reduce visceral adipose macrophage content in diet-induced obese mice. *PLoS One*. 2011; 6(9):e24358. [PubMed: 21931688]
9. Messier SP, Pater M, Beavers DP, Legault C, Loeser RF, Hunter DJ, et al. Influences of alignment and obesity on knee joint loading in osteoarthritic gait. *Osteoarthritis and Cartilage*. 2014; 22(7):912–7. [PubMed: 24857973]
10. Donovan E, Hudson J, Kinter M, Griffin T. Chondrocyte Metabolic Flexibility and Early-Stage Osteoarthritis in Mice Fed a High-Fat Diet. *The FASEB Journal*. 2016; 30(1 Supplement) lb656-lb.
11. Wu C-L, Jain D, McNeill JN, Little D, Anderson JA, Huebner JL, et al. Dietary fatty acid content regulates wound repair and the pathogenesis of osteoarthritis following joint injury. *Annals of the rheumatic diseases*. 2014 annrheumdis-2014-205601.

12. Lewis JS, Furman BD, Zeitler E, Huebner JL, Kraus VB, Guilak F, et al. Genetic and cellular evidence of decreased inflammation associated with reduced incidence of posttraumatic arthritis in MRL/MpJ mice. *Arthritis & Rheumatism*. 2013; 65(3):660–70. [PubMed: 23203659]
13. Li J, Hsu HC, Yang P, Wu Q, Li H, Edgington LE, et al. Treatment of arthritis by macrophage depletion and immunomodulation: Testing an apoptosis-mediated therapy in a humanized death receptor mouse model. *Arthritis & Rheumatism*. 2012; 64(4):1098–109. [PubMed: 22006294]
14. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis research & therapy*. 2006; 8(6): 1.
15. Blom AB, van Lent PL, Libregts S, Holthuysen AE, van der Kraan PM, van Rooijen N, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. *Arthritis & Rheumatism*. 2007; 56(1):147–57. [PubMed: 17195217]
16. Blom AB, van Lent PL, Holthuysen AE, van der Kraan PM, Roth J, van Rooijen N, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. *Osteoarthritis and cartilage*. 2004; 12(8):627–35. [PubMed: 15262242]
17. Burnett SH, Kershen EJ, Zhang J, Zeng L, Straley SC, Kaplan AM, et al. Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. *J Leukoc Biol*. 2004; 75(4):612–23. [PubMed: 14726498]
18. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2007; 15(9):1061–9.
19. Clockaerts S, Bastiaansen-Jenniskens YM, Runhaar J, Van Osch G, Van Offel J, Verhaar J, et al. The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. *Osteoarthritis and Cartilage*. 2010; 18(7):876–82. [PubMed: 20417297]
20. Wu, C-L., Little, D. *Animal Models of Meniscal Injury in Post-Traumatic Arthritis*. Post-Traumatic Arthritis; Springer US: 2015. p. 41-62.
21. Louer CR, Furman BD, Huebner JL, Kraus VB, Olson SA, Guilak F. Diet-induced obesity significantly increases the severity of posttraumatic arthritis in mice. *Arthritis and rheumatism*. 2012; 64(10):3220–30. [PubMed: 22576842]
22. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2007; 15(3):237–44.
23. Loeser RF, Olex AL, McNulty MA, Carlson CS, Callahan M, Ferguson C, et al. Disease progression and phasic changes in gene expression in a mouse model of osteoarthritis. *PLoS One*. 2013; 8(1):e54633. [PubMed: 23382930]
24. Fang H, Beier F. Mouse models of osteoarthritis: modelling risk factors and assessing outcomes. *Nature Reviews Rheumatology*. 2014; 10(7):413–21. [PubMed: 24662645]
25. Collins KH, Hart DA, Reimer RA, Seerattan RA, Herzog W. Response to diet-induced obesity produces time-dependent induction and progression of metabolic osteoarthritis in rat knees. *Journal of Orthopaedic Research*. 2015
26. Lu B, Driban JB, Xu C, Lapane KL, McAlindon TE, Eaton CB. Dietary Fat and Progression of Knee Osteoarthritis Dietary Fat Intake and Radiographic Progression of Knee Osteoarthritis: Data from the Osteoarthritis Initiative. *Arthritis care & research*. 2016
27. Leyland KM, Judge A, Javaid MK, Diez-Perez A, Carr A, Cooper C, et al. Obesity and the Relative Risk of Knee Replacement Surgery in Patients With Knee Osteoarthritis: A Prospective Cohort Study. *Arthritis & Rheumatology*. 2016; 68(4):817–25. [PubMed: 26556722]
28. Berenbaum F, Griffin TM, Liu-Bryan R. Metabolic Regulation of Inflammation in Osteoarthritis. *Arthritis & Rheumatology*. 2016
29. Burnett SH, Beus BJ, Avdiushko R, Qualls J, Kaplan AM, Cohen DA. Development of peritoneal adhesions in macrophage depleted mice. *Journal of Surgical Research*. 2006; 131(2):296–301. [PubMed: 16289593]
30. Chang MK, Raggatt L-J, Alexander KA, Kuliwaba JS, Fazzalari NL, Schroder K, et al. Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate

- osteoblast function in vitro and in vivo. *The Journal of Immunology*. 2008; 181(2):1232–44. [PubMed: 18606677]
31. Alexander KA, Raggatt L-J, Millard S, Batoon L, Wu AC-K, Chang M-K, et al. Resting and injury-induced inflamed periosteum contain multiple macrophage subsets that are located at sites of bone growth and regeneration. *Immunology and cell biology*. 2017; 95(1):7–16. [PubMed: 27553584]
 32. Gordy C, Pua H, Sempowski GD, He Y-W. Regulation of steady-state neutrophil homeostasis by macrophages. *Blood*. 2011; 117(2):618–29. [PubMed: 20980680]
 33. Lee B, Qiao L, Kinney B, Feng G-S, Shao J. Macrophage Depletion Disrupts Immune Balance and Energy Homeostasis. *PloS one*. 2014; 9(6):e99575. [PubMed: 24911652]
 34. Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. *Nature Reviews Rheumatology*. 2014; 10(10):593–601. [PubMed: 24914698]
 35. Scapini P, Cassatella MA. Social networking of human neutrophils within the immune system. *Blood*. 2014; 124(5):710–9. [PubMed: 24923297]
 36. Simard J-C, Noël C, Tessier PA, Girard D. Human S100A9 potentiates IL-8 production in response to GM-CSF or fMLP via activation of a different set of transcription factors in neutrophils. *FEBS letters*. 2014; 588(13):2141–6. [PubMed: 24796790]
 37. de Munter W, van der Kraan PM, van den Berg WB, van Lent PL. High systemic levels of low-density lipoprotein cholesterol: fuel to the flames in inflammatory osteoarthritis? *Rheumatology*. 2016; 55(1):16–24. [PubMed: 26231344]
 38. de Munter W, Geven E, Blom A, Walgreen B, Helsen M, Joosten L, et al. Synovial macrophages promote TGF- β signaling and protect against influx of S100A8/S100A9-producing cells after intra-articular injections of oxidized low-density lipoproteins. *Osteoarthritis and Cartilage*. 2017; 25(1):118–27. [PubMed: 27514996]
 39. Rinchai D, Khaenam P, Kewcharoenwong C, Buddhisa S, Pankla R, Chaussabel D, et al. Production of interleukin-27 by human neutrophils regulates their function during bacterial infection. *European journal of immunology*. 2012; 42(12):3280–90. [PubMed: 22965735]
 40. Hunter CA, Kastelein R. Interleukin-27: balancing protective and pathological immunity. *Immunity*. 2012; 37(6):960–9. [PubMed: 23244718]
 41. Hirahara K, Ghoreschi K, Yang X-P, Takahashi H, Laurence A, Vahedi G, et al. Interleukin-27 priming of T cells controls IL-17 production in trans via induction of the ligand PD-L1. *Immunity*. 2012; 36(6):1017–30. [PubMed: 22726954]
 42. Moon S-J, Park J-S, Heo Y-J, Kang C-M, Kim E-K, Lim M-A, et al. In vivo action of IL-27: reciprocal regulation of Th17 and Treg cells in collagen-induced arthritis. *Experimental & molecular medicine*. 2013; 45(10):e46. [PubMed: 24091748]
 43. Cao Y, Doodles PD, Glant TT, Finnegan A. IL-27 induces a Th1 immune response and susceptibility to experimental arthritis. *The Journal of Immunology*. 2008; 180(2):922–30. [PubMed: 18178832]
 44. Hall AOH, Beiting DP, Tato C, John B, Oldenhove G, Lombana CG, et al. The cytokines interleukin 27 and interferon- γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity*. 2012; 37(3):511–23. [PubMed: 22981537]
 45. Hall A, Silver JS, Hunter CA. The immunobiology of IL-27. *Adv Immunol*. 2012; 115(1):1–44. [PubMed: 22608254]
 46. Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R, et al. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *The Journal of clinical investigation*. 2010; 120(10):3466. [PubMed: 20877011]
 47. Patsouris D, Li P-P, Thapar D, Chapman J, Olefsky JM, Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell metabolism*. 2008; 8(4):301–9. [PubMed: 18840360]
 48. Hume DA. Applications of myeloid-specific promoters in transgenic mice support in vivo imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity. *Journal of leukocyte biology*. 2011; 89(4):525–38. [PubMed: 21169519]

49. Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, Mayuzumi N, et al. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. *Blood*. 2013; 121(10):1677–89. [PubMed: 23305731]
50. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers J-W, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *The Journal of clinical investigation*. 2012; 122(1):327. [PubMed: 22156198]

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Figure 1.

(A) Experimental design. To evaluate the effect of macrophage (MΦ) depletion on acute joint inflammation, five mice were sacrificed at 14 weeks of age immediately after the second MΦ depletion (this time point refers to 0 week post-depletion). To investigate the effect of MΦ depletion on OA severity, twelve mice were sacrificed at 23 weeks of age (this time point refers to 9 weeks post-depletion). (B) Body weight over time. MΦ depletion significantly decreased body weight of the depleted mice at the time of depletion (black arrows); however, the depleted mice gradually recovered their weight after the depletion treatments. MΦ depletion also trended toward decreasing (C) percent body epididymal fat, but significantly increased (D) percent spleen weight in the depleted mice at 0 weeks post-depletion. n = 4–12 mice/group. * p < 0.05 compared to corresponding control group. All data are presented as mean ± SEM. (B) Statistical significance was determined by one-way repeated measures ANOVA. (C) and (D) Statistical significance was determined by student's t-test within each time point. DMM: destabilization of medial meniscus. DEXA: dual-energy x-ray absorptiometry. Control: obese MaFIA mice without MΦ depletion. MΦ-depleted: obese MaFIA mice with MΦ depletion.

Figure 2.

(A) Flow cytometry analysis for percent eGFP⁺ cells (macrophages, MΦs) in DMM-operated (left) and non-operated (right) hind limbs from the control and MΦ-depleted mice. Depleted mice had significantly lower eGFP⁺ cells in the DMM joint versus the control mice immediately following depletion. At 9 weeks post-depletion, although the DMM joints had significantly more eGFP⁺ cells than non-operated joints, no significant difference in the percentage of eGFP⁺ cells was observed in the DMM joints of the control and depleted mice. (B) F4/80 IHC against MΦs in DMM-operated joints. Consistent to our flow analysis, DMM joints from the depleted mice had significantly lower MΦ numbers than those from the control mice. (C) Representative IHC images of NOS-2⁺ cells and CD206⁺ cells in the synovium at the femoral condyle of the DMM-operated joints. (D) Quantification of NOS-2⁺ cells and CD206⁺ cells in the synovium of the entire DMM joints. MΦ-depleted mice trended toward having fewer NOS-2⁺ cells immediately after ablation. At 9 weeks post-depletion, depleted mice also had significantly fewer CD206⁺ cells in the DMM joint versus the control mice. $n = 4-12$ mice/group. Red arrowheads indicate cells that were positive for staining. Different letters are significantly different from each other, $p < 0.05$. Statistical significance was determined by two-way ANOVA followed by Fisher's *post-hoc* analysis. All data are presented as mean $\pm$ SEM. WT: wide type C57B6/J mice used for gating control. DMM: destabilization of medial meniscus. Right: right non-operated joints. Left: left DMM operated-joint. Control: obese MaFIA mice without MΦ depletion. MΦ-depleted: obese MaFIA mice with MΦ depletion.

Figure 3.

(A) 3D reconstruction of MicroCT of left (DMM-operated) hind-limbs (white arrows indicate osteophytes). (B) Osteophyte score of the DMM-operated joints. Mice with macrophage (MΦ) depletion had significantly lower osteophyte score than the control mice at 0 week of post-depletion. MΦ depletion significantly decreased (C) bone mineral density and (D) cancellous bone fraction (bone volume / total volume, BV/TV) of femoral condyle and tibial epiphysis of the DMM-operated joints at the time of depletion. (A), (C) and (D) Data analyzed by MicroCT. n = 5–12 mice/group. Different letters are significantly different from each other, p < 0.05. Statistical significance was determined by two-way repeated

measures ANOVA using right (non-operated) joints as the contralateral control. All data are presented as mean $\pm$ SEM. DMM: destabilization of medial meniscus. Right: right non-operated joints. Left: left DMM operated-joint. Control: obese MaFIA mice without M ϕ depletion. M ϕ -depleted: obese MaFIA mice with M ϕ depletion.

Figure 4.

(A) Safranin-O and fast green histology for the DMM-operated joints. Severe cartilage loss was found in the control and macrophage (M Φ)-depleted mice at 9 weeks post-depletion. The DMM-operated joints of the M Φ -depleted mice were not significantly different from those of the control mice at 9 weeks of post-depletion. (B) H&E histology of the medial femoral condyle of the DMM-operated joints. Yellow arrowheads indicate massively infiltrated cells. The DMM-operated joints from M Φ -depleted mice had significantly higher synovitis scores than those from the control mice at 9 weeks post-depletion. (C) Representative IHC images of CD22⁺ B cells, CD3⁺ T cells, and neutrophils in the

synovium at the femoral condyle of the DMM-operated joints. Red arrowheads indicate cells that were positive for staining. **(D)** Quantification of CD22⁺ B cells, CD3⁺ T cells, and neutrophils in the synovium of the entire DMM joints. M ϕ -depleted mice exhibited significantly more CD3⁺ T cells in the DMM joints versus the control mice at both time points. Immediately following M ϕ depletion, the DMM joints of the depleted mice also had significantly more neutrophils than those of the control mice. However, the control and depleted mice had comparable CD22⁺ B cells in their DMM-operated joints. **(A and B)**, n = 5–12 mice/group. * p < 0.05 for the joints within each group. Different letters are significantly different from each other, p < 0.05. Statistical significance was determined by two-way repeated measures ANOVA using right (non-operated) joints as the contralateral control. **(C and D)**, n = 4–10 mice/group. Different letters are significantly different from each other, p < 0.05. Statistical significance was determined by two-way ANOVA followed by Fisher's *post-hoc* test. All data are presented as mean $\pm$ SEM. DMM: destabilization of medial meniscus. Right: right non-operated joints. Left: left DMM operated-joint. Control: obese MaFIA mice without M ϕ depletion. M ϕ -depleted: obese MaFIA mice with M ϕ depletion.

Figure 5.

Serum cytokine analyses. The depleted mice exhibited significantly lower serum (A) TGF-β1 concentration, but higher serum (B) granulocyte colony-stimulating factor (G-CSF), (D) IL-1β, (E) IL-6, (F) IL-8, (G) IL-10, and (I) TNF-α compared to control mice at 0 week post-depletion. Macrophage (MΦ) depletion also significantly increased serum (H) IL-27 levels in the depleted mice at both time points. n = 5–12 mice/group. * p < 0.05 for the joints within each group. Different letters are significantly different from each other, p < 0.05. Statistical significance was determined by two-way ANOVA followed by Fisher's *post-hoc*

test. All data are presented as mean $\pm$ SEM. IFN- γ : interferon- γ . Control: obese MaFIA mice without M ϕ depletion. M ϕ -depleted: obese MaFIA mice with M ϕ depletion.

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Figure 6.

Synovial fluid cytokine analyses for the DMM-operated (left) joints and non-operated (right) joints. At 0 week post-depletion, macrophage (M ϕ)-depleted mice showed significantly higher (C) IL-8, (D) IL-10, and (F) TNF- α in the DMM joints as compared to the control mice. The depleted mice also trended toward increasing (A) IL-1 β , and (B) IL-6 in the DMM joint versus control mice immediately following depletion. DMM surgery significantly decreased IL-17 concentrations in the operated joint as compared to that in the non-operated joint in both control and M ϕ -depleted mice at 0 week post-depletion. Labels without bars indicate the cytokine measured was below detectable levels. n = 5–12 mice/group. * p < 0.05 for the joints within each group. Statistical significance was determined by two-way ANOVA followed by Fisher's *post-hoc* test. All data are presented as mean $\pm$ SEM. DMM: destabilization of medial meniscus. Right: right non-operated joints. Left: left DMM operated-joint. Control: obese MaFIA mice without M ϕ depletion. M ϕ -depleted: obese MaFIA mice with M ϕ depletion.