Role for BLT1 in adipose tissue inflammation in old mice

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Special thanks goes to my family, friends, and especially my partner. Their constant encouragement supported me in accomplishing this challenging process. Words cannot express my gratitude for their emotional support.

Overview:

This thesis is a combination of two projects. "Role for BLT1 in adipose tissue inflammation in old mice" is a project focusing on the pro-inflammatory mediator LTB4 and its receptor, BLT1, and their relationship with age-induced inflammation. "Glyphosate and adipose tissue inflammation in HFD mice" is a project that collaborates with Dr. Ashutosh Mangalam and Lab at the University of Iowa. Here, I address whether obesity along with glyphosate exposure would cause a more severe inflammatory response. Aging and obesity are highly linked because aging often follows an increase in visceral adipose tissue mass, and increased adipose tissue is one of the main contributors to systemic chronic inflammation. Moreover, the adipose tissue inflammatory response is highly linked to increased pro-inflammatory macrophages and decreased anti-inflammatory macrophages. Both projects highlight how macrophages, obesity, and inflammation are closely connected. Chapter 1 focused on the age-specific changes in BLT1 expression within VAT and its contribution to inflammation in aged mice, while Chapter 2 broadens the discussion to include factors like gut microbiota composition and environmental exposures by glyphosate, which further exacerbate inflammation and metabolic disturbances associated with obesity. Together, these projects provide a comprehensive view of the multifaceted nature of inflammation in aging and obesity.

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CHAPTER 1: Role for BLT1 in adipose tissue inflammation in old mice

Contributions: This chapter is largely derived from a first author manuscript, although portions have been extended to provide additional background or discussion. I wrote all sections and performed all experiments. I'd like to thank Ina and Stephanie for assisting me.

Abstract:

Aging is a complex biological process characterized by obesity and immunosenescence. Immunosenescence indicates the decline in immune function and the increase in chronic-low grade inflammation, called "Inflammaging". Adipose tissue accumulation, particularly visceral adipose tissue (VAT), is associated with an increase in pro-inflammatory macrophages that play an important role in modulating immune responses and producing inflammatory cytokines. The leukotriene B4 receptor 1 (BLT1) is a regulator of obesity-induced inflammation. It's ligand, LTB4, acts as a chemoattractant for immune cells and induces inflammation. Studies showed that BLT1 is crucial for cytokine production during lipopolysaccharide infection of young mice. However, the expression patterns and function of BLT1 in the adipose tissue of older organisms remains unknown. In this study, we investigated BLT1 expression in immune cell subsets within the VAT of aged male and female mice. Moreover, we examined how BLT1 expression alters in response to lipopolysaccharide, in the aging context. Our results demonstrate that aged mice exhibit increased obesity and inflammation, characterized by elevated frequencies of B and T cells, along with pro-inflammatory macrophages in VAT. BLT1 expression is the highest in VAT macrophages and BLT1 showed an increased expression in pro-inflammatory macrophages from aged mice. An in vitro model of bone marrow derived macrophages supported these in vivo findings. Treatment of aged mice with BLT1 antagonist, U75302, followed by lipopolysaccharide-induced endotoxemia resulted in a reduction in pro-inflammatory macrophages and an increase in anti-inflammatory macrophages. This study provides valuable insights into the age-specific changes in BLT1 expression and its impact on immune cell subsets within VAT. This study offers support for its potential for modulating inflammation in aging.

Introduction:

Aging and inflammation

Aging is associated with a severe decline in physiological functions and an increased susceptibility to various chronic diseases. Several factors such as obesity, accumulation of cellular senescence, induced immunosenescence and many other factors are well established to increase in older individuals [1-3]. These different phenotypes could all contribute to a chronic inflammation of aged cells, often referred to as "inflammaging" [4]. Inflammation is an important physiological function triggered by the host system in response to pathogenic infections or tissue injury. Normally, when the proinflammatory factors caused by infections and tissue injuries are eliminated, inflammatory responses would disappear and allow the body to recover to steady state. But in the presence of inflammaging, inflammatory response fails to get back to normal and this low-grade, persistent inflammation plays a significant role in the aging process and age-related diseases. The immune system alters with age, leading to an activation of immune cells and the release of pro-inflammatory cytokines [5]. While inflammation serves a crucial physiological function as a defense mechanism against infections or foreign substances, its prolonged and sustained presence could have been lethal to one's health. Over time, this chronic inflammatory state contributes to the development of age-related diseases, including cardiovascular diseases, neurodegenerative diseases, metabolic dysfunction, and increased susceptibility to infections when compared to the young [6-8]. Based on these findings, numerous researchers have suggested that inflammaging serves as an indicator of accelerated aging, pointing it as one of the essential aspects in the field of the biology of ageing [9].

Currently, the mechanism of inflammaging is still not completely understood due to the complexity of both aging and inflammation, which could be easily impacted by numerous factors. One of the most popular studies is the production of pro-inflammatory cytokines caused by inflammaging. Elevated expression of pro-inflammatory cytokines is detected in serum of the elderly [10]. The high level of pro-inflammatory cytokines in the circulatory system caused an inflammatory environment for tissues and organs. Leading to a chronic-inflammation state of the whole body [4]. Another factor that aggravates inflammaging is the cellular senescence. Cellular senescence is a phenomenon which cells stops to proliferate and forces tissues to remodel due to cellular damage. Although senescent cells could act as a gatekeeper to prevent harmful cells from proliferating and cause damage to the body, senescent cells accumulate over time and is involved in various age-related diseases. Senescent cells contribute to aging by secreting senescence associated secretory phenotype, mainly pro-inflammatory mediators [11]. The age-related dysfunctional of immune response is called immunosenescence. Both innate and adaptive immune responses

would be impacted. Innate immunity has decreased ability to be responsible for nonspecific immune responses, and adaptive immunity was impacted by lower number of memory cells, overall leading to an inefficient elimination of threats and extended production of inflammatory cytokines [12]. Others like DNA damaging, oxidative stress and autophagy are also important factors contribute to inflammaging, but the detailed mechanism has not been well-established [4].

Aging and obesity-induced inflammation

Aging is accompanied by an increased visceral adiposity due to reduced total basal energy expenditure [13, 14] and decreased lipolysis [15]. Moreover, the redistribution of fat from subcutaneous adipose tissue to VAT further contributes to this process of aging [16, 17]. The function of adipose tissue includes regulation of body temperature, storage of energy, and most importantly, modulation of immune responses [18]. Adipose tissue is consist of adipocytes, endothelial cells, and immune cells [19]. Immune cells within adipose tissue, particularly macrophages, play a crucial role in regulating tissue homeostasis and are a major source of inflammatory cytokines such as IL-6 and TNF-α, and contributes to the increased cytokine production observed in aging. The production of inflammatory cytokines is how adipose tissue regulates the immune response [20, 21]. Macrophages can polarize into different types: M1-like pro-inflammatory macrophages and M2-like anti-inflammatory macrophages [22]. M1 macrophages overexpress markers like CD86, CD11c, CD38, and produces pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α [23, 24]. In contrast, M2 macrophages express markers like CD206, CD163, and produces anti-inflammatory cytokines such as IL-10 [23, 25]. Studies describe that, there is a prevalence of alternatively activated M2 type macrophages in lean and young animals. However, with the onset of obesity, there is a recruitment and accumulation of M1 type macrophages. Studies are done based on expression of surface antigens; e.g., CD11c, CD206 and genes; *tnf-α, il-6, arg1,mgll1* [26, 27]. Adipose tissue results in an accumulation of macrophages, and those macrophages exhibit a pro-inflammatory phenotype in obesity and aging, which further supports a systemic increase in inflammation and immunosenescence [26, 28]. Senescent cell accumulation is also a contributor to immunosenescence [1]. Senescent cells express a senescence associated secretory phenotype that promotes the M1 pro-inflammatory polarization of macrophages that further exacerbates the immunosenescence microenvironment [29]. Taken together, the imbalance between pro and anti-inflammatory networks could lead to immunosenescence in aging.

Macrophage inflammaging – the NLRP3 inflammasome

Macrophages are able to recognize cellular pathogens and activate inflammation, which is how macrophages contribute to inflammaging. Pattern-recognition receptors (PPRs) on macrophages are transmembrane or cytosolic receptors that recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), such as harmful stimuli, pathogens or irritants. PAMPs referred to patterns found on bacterial cell walls, DNA, lipoproteins and carbohydrates that caused infection to the body. Whereas DAMPs are endogenous molecules released by stress or damage of cells [30]. PPRs signal downstream and upregulates the nuclear factor-κB (NF-κB), thereby activates the expression of NLRs, mainly NLRP3, to form inflammasomes. The inflammasomes plays a vital role in the release of pro-inflammatory cytokines such as IL-18 and IL-1β, through activation of caspase 1 [31]. It is reported that aging caused an increase in DAMPs that leads to the increase of NLRP3 inflammasome in adipose tissue macrophages [28].

Leukotriene B4 and its receptor, BLT1

Leukotrienes are a part of the eicosanoid family that plays an important role in inflammation, asthma, and anaphylaxis. They are derived from arachidonic acids released from cellular phospholipids by cytosolic phospholipase A2. Arachidonic acids will then convert into leukotriene A4 by metabolization of 5-lipoxygenase, and further hydrolyzed into leukotriene B4 (LTB4) by leukotriene A4 hydrolase; or synthesized into LTC4, LTD4 or LTE4 by leukotriene C4 synthase [32, 33]. LTB4 acts as a chemoattractant of leukocytes, including neutrophils, macrophages, and eosinophils [34-36]. LTB4 can also trigger inflammation by stimulating the secretion of inflammatory cytokines through the interaction with its receptor. Leukotriene B4 receptor 1 (BLT1) and leukotriene B4 receptor 2 (BLT2), G-protein coupled receptors (GPCRs) that bind to LTB4, exhibit distinct characteristics. BLT1 is a high-affinity receptor of LTB4 that is primarily expressed on inflammatory cells, especially myeloid cells [37-39], as compared to BLT2 [40]. Phosphorylation of ERK1/2, JNK1/2 and AKT is induced by the LTB4-BLT1 axis, leading to up-regulation NF-κB and cytokine production, including IL-17, IL-6 and IL-1β [41, 42]. BLT1 is also capable of inhibiting adenylyl cyclase and calcium entry through its interaction with the Gi- and Gq-classes of G proteins [43], which results in activation of NF-κB [44].

Lipopolysaccharides (LPS) are the major component of the gram-negative bacteria outer membrane, which activates acute inflammatory response through toll-like receptor 4 (TLR4) mediated cytokine production [45]. LPS injection is a common way of modeling endotoxemia. Previous studies have shown that aging increases susceptibility and mortality to endotoxemia, due to expression of cytokines like IL-6 from the adipose tissue [8, 46]. Given that LPS increases BLT1 expression, it is crucial to examine the function of BLT1 in regulating the immune response

in the aged mice. [42]. Moreover, obese BLT1-deficient mice exhibit a decreased frequency of M1 macrophages, but an increased frequency of M2 macrophages, suggesting the potential involvement of BLT1 in modulating macrophage polarization [47].

Conclusion

Control of BLT1 could serve as a way to effectively regulate inflammatory responses. While the function of BLT1 is well-established in the young, its role in the aged remains unknown. Here, we investigate the age-specific changes of BLT1 expression on immune cell subsets. We identified an increase in BLT1 expression in pro-inflammatory macrophages during aging. Additionally, we observed that BLT1 can affect the ratio of pro-inflammatory to anti-inflammatory macrophages, thereby regulating the inflammatory response in the LPS-induced endotoxemia model in aged mice. These insights suggested the potential of BLT1 to be used as a therapeutic target to treat age-related inflammation diseases.

Materials and Methods:

Animals: All mice were kept in specific pathogen-free facilities using ventilated cage racks that supplied HEPA-filtered air to each cage, providing free access to sterile water at the University of Minnesota. Sentinel mice in our animal rooms consistently tested negative for standard murine pathogens at various intervals during the studies. 3-8 months old male/female and 22-24 months old male/female C57Bl6/J (wild-type) mice, were treated according to National Institutes of Health guidelines for the care and use of experimental animals, approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Mice were maintained in a controlled environment with fixed temperature (21-23°C), humidity (30-70%), lighting (14h light/10h dark) and free access of drinking water and chow.

Experimental Design: Blinding of investigators was unfeasible during the experiments, given the apparent variations in phenotype resulting from age or LPS treatment. The mice, sourced from the NIA, were either bred in-house or acquired from Jackson Laboratories. Aged mice displaying severe frailty, tumors, or other age-related pathologies were deliberately excluded from the study, constituting less than 5% of the total population of aged mice.

Mouse models:

Lipopolysaccharide (LPS)-induced endotoxemia challenge: Young (3-8 months) and old (18-22 months) mice received intraperitoneal (IP) injections of either sterile phosphate-buffered solution (PBS; Corning) or lipopolysaccharide (LPS; E. coli O111-B4; Sigma, L3024) diluted in sterile PBS. Body temperature was measured using a rectal thermometer before the IP injection and again prior to euthanasia. Euthanasia was performed through exsanguination and cervical dislocation under isoflurane anesthesia, and tissue harvesting occurred subsequent to the euthanasia of the mice.

BLT1 antagonist treatment: Mice were given IP injection with 1mg/kg BLT1 antagonist-U75302 (Cayman Chemical) 1 hour prior to 3mg/kg LPS (E. coli O111-B4; Sigma, L3024) injection. Body temperature was measured via rectal thermometer every 3 hours after LPS injection. After 6 hours of LPS injection, mice were euthanized with isoflurane and tissues were collected. Control mice were injected with same dose of DMSO and PBS, respectively.

Tissue digestion: Visceral adipose tissue (VAT) was obtained after euthanization and weighed. Tissues underwent enzymatic digestion in 0.1% collagenase II (Worthington Biochemicals) in Hanks' Balanced Salt Solution (HBSS) (Life Technologies) for 30 minutes at 37°C, 200rpm

agitation. To ensure consistency, both control and experimental groups were processed and stained on the same day, to minimize procedural variations. The stromavascular fraction was recieved through centrifugation at 1500rpm for 5 minutes, followed by washing in RPMI with 10% FBS. ACK lysis buffer (Quality Biological Inc) was used to remove red blood cells. Cells were quench with RPMI with 10% FBS after 2 minutes in ACK lysis buffer, filtered with a 40uM filter and centrifuge at 1500rpm for 5 minutes to get single cell suspension. Splenocytes and peritoneal fluid cells were isolated into single cells in a similar way, but without digestion in collagenase II.

Flow cytometry: Single cells were stained with Ghost Dye Red 780 Viability Dye (TONBO biosciences) for 30 minutes on ice, avoiding light. Then washed and incubated with FcBlock (BD Bioscience) and surface antibodies for another 30 minutes. Antibodies used are listed in Table S1. The BLT1 staining required a secondary antibody since it is unconjugated. AF647-conjugated anti-mouse IgG1 (Biolegend) was used as the secondary antibody for a 30 minute stain. Control groups were stained with Rabbit IgG Isotype Control (Invitrogen) instead of the BLT1 antibody. For intracellular staining, cells were fixed with the BD CytofixCytoperm kit (554715) for 20 minutes, followed by an intracellular antibody stain for 30 minutes. Flow cytometry analysis was performed on a BD FACSymphony A3 (R6609) cytometer and a FlowJo v10 software.

Bone marrow-derived macrophages (BMDM) culture: Bone marrow-derived macrophages were isolated from femur and tibia of young and aged mice. Cells were lysed in ACK lysis buffer, filtered and resuspended in RPMI +10% FBS +1% antibiotic-antimycotic, supplemented with 25ng/ml of macrophage colony-stimulating factor (M-CSF) (R&D systems) for 5 days. An extra 50ng/ml of M-CSF was added to the cells after 5 days of culture, his supplementation continued for an additional 2 days. For LPS stimulation, cells were stimulated with 1µg/ml LPS for 4 hours. For LTB4 stimulation, cells were stimulated with 100nM LTB4 for 6 hours. For macrophage polarization, cells were being polarized into M1 type macrophages with 1µg/ml LPS and 20ng/ml IFNg, or polarized into M2 type macrophages with 10ng/ml IL-4 for 24 hours.

Statistics: A confidence interval of 95% was used for all statistical tests. All data were assumed to be normally distributed, unless the standard deviation was identified as significantly different between groups. All statistical tests were performed using GraphPad Prism v9 for Windows (GraphPad Software). Data are expressed as mean ± s.e.m. Sample size of independent experiment are described in the figure legends. Statistical analysis was conducted using non-

parametric T-test, 2-way ANOVA with a post-hoc test of Tucky or Sidak, or one-way ANOVA with a post-hoc test of Tukey. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Results:

Aged mice tend to be more obese and have increased inflammation

To examine the physiological difference between young and aged mice, we measured the body weight and VAT mass of young (5-8 months old) and aged (20-22 months old) mice. Aged female mice showed an increase in both body weight and VAT mass (Fig. 1a). Aged male mice showed comparable body weight with the young, but we detected a significant increase of VAT mass (Fig. 1b). Due to the increase of body-fat percentage, both male and female mice exhibit obesity in the form of visceral adiposity (Supplementary Fig. 1a).

As immune cells are a critical component of regulating tissue homeostasis [18], we examined the subsets of immune cells in the young and aged mice. We investigated the frequency and cell counts of VAT immune cell subsets including B cells, T cells, eosinophils, macrophages, and neutrophils in female mice (young: 5-8 months old, old: 22 months old) using multi-parameter flow cytometry. The gating strategy to identify each cell type is shown (Fig. 1c). B cells and CD3+ T cells were increased with aging in both frequencies (Fig. 1d) and cell counts (cells/g tissue) (Fig. 1e). In contrast, eosinophils showed a decrease in frequency (Fig. 1d). We also examined the pro-inflammatory macrophage phenotype, using CD11c, in young and aged mice. We examined the expression level of CD11c on macrophages using mean fluorescence intensity (MFI). Results showed an increased CD11c expression (MFI) on macrophages in VAT from aged female mice (Fig. 1f).

We also quantified immune cell frequency (Fig. 1g) and counts (Fig. 1h) in young and aged male mice. Consistent with the results from female mice, both frequencies and cellularity of B cells and T cells were increased in VAT from aged male mice (Fig. 1g-h). We also detected a decrease in eosinophil and macrophage frequencies (Fig. 1f). There was no difference in CD11c MFI of macrophages in aged male mice, indicating that sex-specific differences in the pro-inflammatory phenotype are present. In conclusion, VAT from aged male and female mice exhibit similar changes in immune cell subsets, including increased B and T cells, but decreased macrophages and eosinophils. The female mice showed a significant increase in CD11c MFI, a marker for pro-inflammatory macrophages.

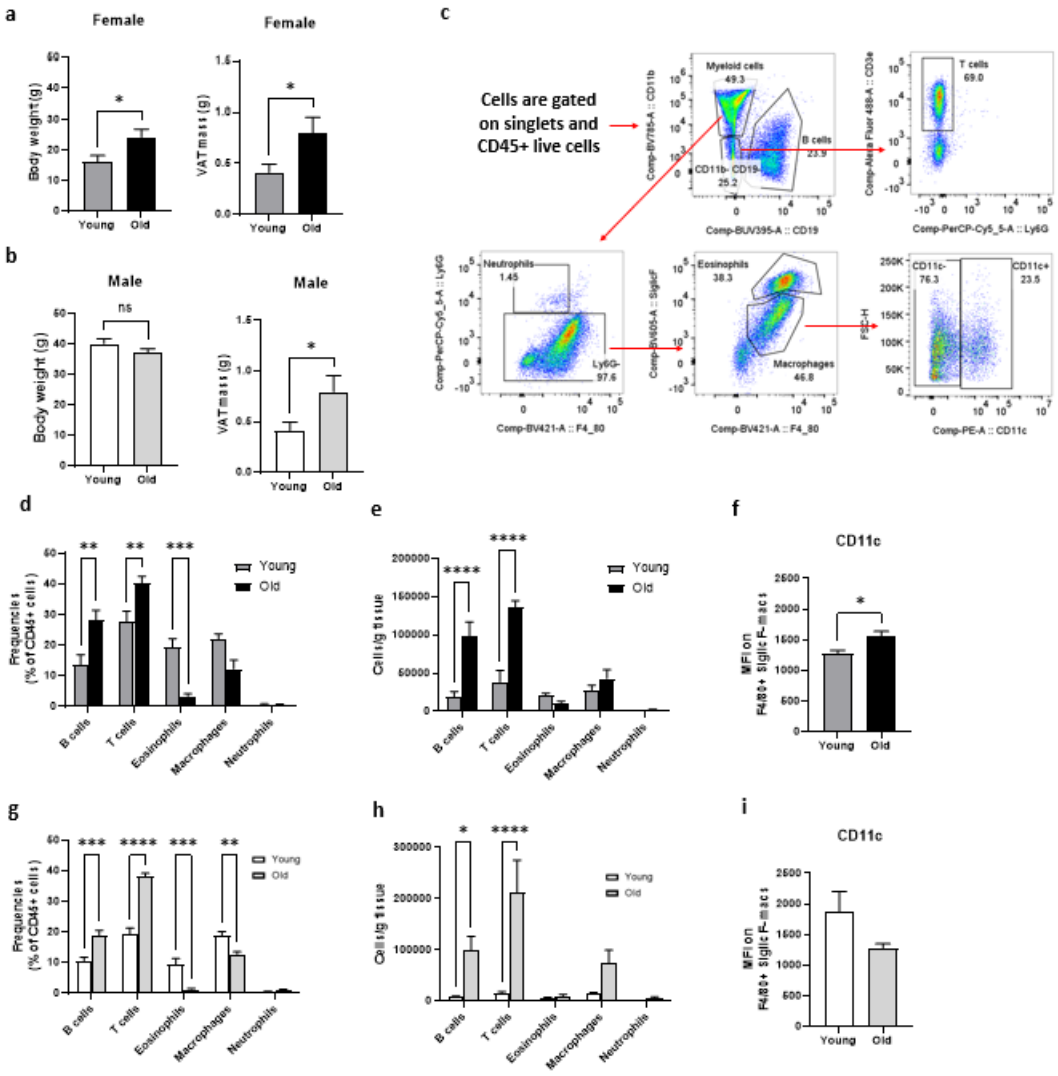


Figure 1 Comparison of immune cell population in VAT of young (5-8 months) and aged (20-22 months) mice. **a** Comparison of young and aged female mice body weight and VAT mass. **b** Comparison of young and aged male mice body weight and VAT mass. **c** Flow gating strategy of immune cell subset. **d** Frequencies of immune cell subset in female mice. **e** Cells/g tissue of immune cell subset in female mice. **f** CD11c MFI of F4/80+ SiglicF- macrophages of female mice. **g** Frequencies of immune cell subset in male mice. **h** Cells/g tissue of immune cell subset in male mice. **i** CD11c MFI of F4/80+ SiglicF- macrophages of male mice. Female (**a, c-f**) and male (**b, g-i**) mice were used in this experiment (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with a post-hoc test of Sidak (**d-e, g-h**) or non-parametric T-tests (**a-b, f, i**). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001..

BLT1 is highly express on VAT macrophages

BLT1 is reported to be highly expressed in neutrophils, macrophages, and eosinophils [37-39]. BLT1 is also reported to be expressed on B2 B cells in the adipose tissue [48]. To examine which immune cell subset in the young and aged mice expresses BLT1, we compared the MFI of BLT1 on B cells, eosinophils, macrophages, and neutrophils of VAT. The antibody used for BLT1 detection (anti-BLT1, clone 7A8) was validated by utilizing BLT1-overexpressing cells and mouse peripheral blood leukocytes that naturally express BLT1. Importantly, this antibody does not cross-react with BLT2 or other GPCRs [49]. Gating strategy is shown, we used isotype control to gate out BLT1+ cells in the immune cell subset (Fig. 2a). All experiments looking at BLT1+ cells used the same gating strategy. We also examined peritoneal fluid (PEC), and spleen, a non-lymphoid and lymphoid tissue that exhibits well-characterized changes with age [50]. The result showed that BLT1 MFI was the highest in VAT macrophages in young mice, as compared to other tissues (Fig. 2b-c). In VAT, BLT1 MFI of B cells and T cells are comparable to the isotype control, indicating that BLT1 is not expressed on those cell types (Fig. 2). Next, we quantified BLT1+ cells in young and aged female mice. However, we did not observe any differences in frequencies and cellularity of BLT1+ macrophages, eosinophils, and neutrophils in VAT from young and aged mice (Fig. 2d). We performed a similar analysis in VAT from male mice. Old mice exhibited an increase in macrophages cells/g tissue that were BLT1+ (Fig. 2e). Overall, this experiment indicates that BLT1 is highly expressed on VAT macrophages compared to other tissues and immune cells.

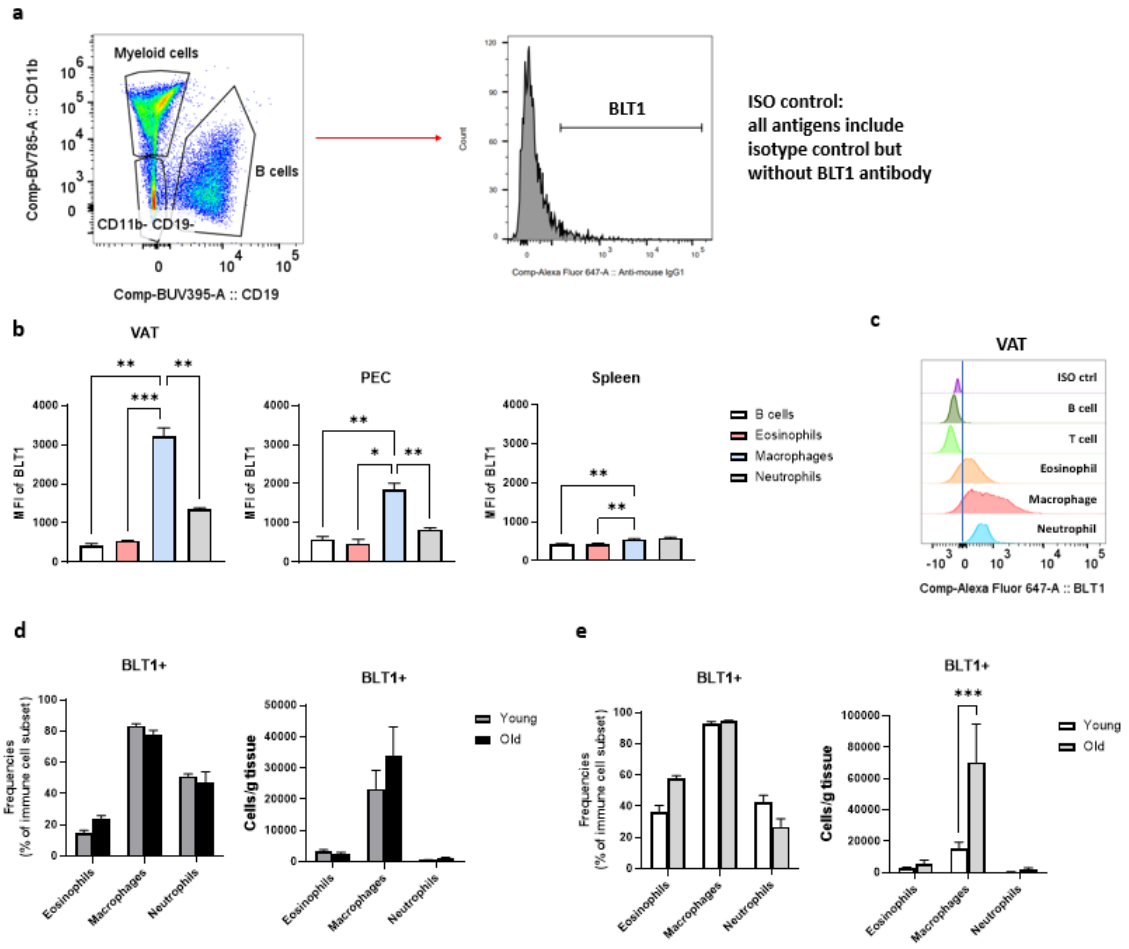


Figure 2 Comparison of BLT1 in immune cell subsets of young (5-8 months) and old (20-22 months) mice. **a** Flow gating strategy to identify BLT1+ cells. **b** BLT1 MFI of immune cell subset in VAT, peritoneal fluid (PEC), and spleen. **c** Histogram of BLT1 in VAT immune cell subset. **d** Frequencies and count of BLT1 in immune cell subset in female young vs. old mice. **e** Frequencies and count of BLT1 in immune cell subset in male young vs. old mice. Female (**a-d**) and male (**e**) mice were used in this experiment (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using paired one-way ANOVA with a post-hoc test of Tukey (**a**), 2-way ANOVA with a post-hoc test of Sidak (**c, e**) or non-parametric T-tests (**d, f**). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Aged BLT1+ BMDMs express a more inflamed phenotype with LPS stimulation, and BLT1 activity increased in aged pro-inflammatory BMDMs as compared to young

The differentiation of bone marrow-derived macrophages (BMDM) is a well-established in vitro system for studying macrophage biology [51]. To address whether inflammation would affect BLT1

expression in young and old macrophages to a comparable extent, we generated BMDMs from marrow of young and aged mice by differentiating them for 7 days in M-CSF (Fig. 3a). We left them untreated or treated with LPS (1mg/ml; 4hours) and used flow cytometry to measure BLT1 MFI and CD86 as a marker of the inflammatory phenotype. CD11c along with CD86, are both reported to be markers highly express on M1 type macrophages, which can both be used to identify pro-inflammatory macrophages [52]. BLT1 MFI and frequency of BLT1+ cells were both increased with LPS treatment in young and old BMDMs (Fig. 3b-c), indicating that LPS increases BLT1 expression to a comparable level in both young and old macrophages. To further examine pro-inflammatory phenotypes, we assessed CD86 expression on the whole macrophage population and on the BLT1+ macrophage subset. When examining the total macrophage population, CD86 MFI was increased in young BMDMs when treated with LPS, but not in old (Fig. 3d). However, when gating through the BLT1+ macrophages, we found that CD86 MFI was significantly increased in the aged BLT1+ BMDMs but not in the young (Fig. 3e), indicating the macrophages that expresses BLT1 are getting more inflamed with LPS stimulation in aging. Although, since BLT1 expression showed comparable values during LPS stimulation in young and aged BMDMs, we wondered whether increased activity could support the inflammatory phenotype of the old BMDMs. We treated BMDMs with LTB4 and examined CD11c as a marker of inflammation. LTB4 stimulation (100nM; 6 hours) increased the frequency of BLT1+ BMDMs from both young and aged mice (Fig. 3f). However, when we gated through CD11c+ to examine the pro-inflammatory macrophages the old mice exhibited an increased expression of BLT1, whereas young mice showed no changes (Fig. 3g). This experiment describes an increased BLT1 activity in aged pro-inflammatory macrophages than young with LTB4 stimulation.

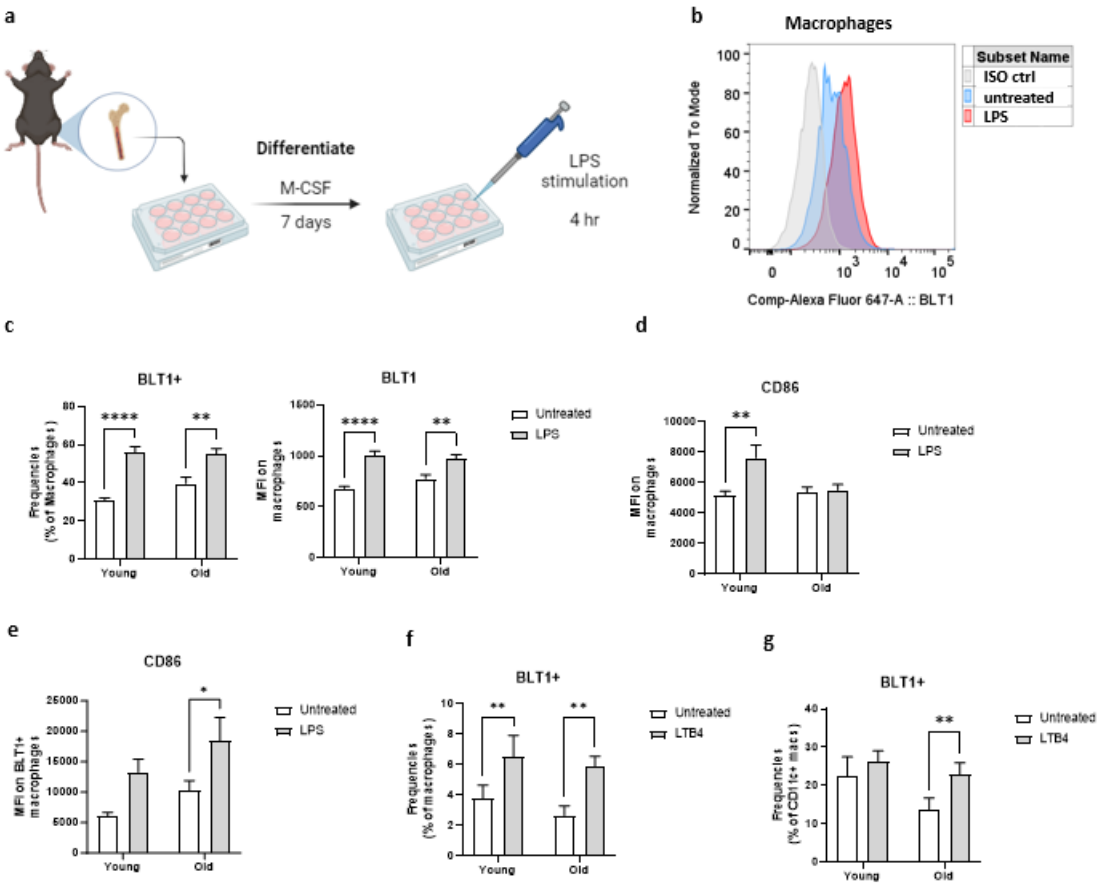


Figure 3 Pro-inflammatory (CD86+) phenotype macrophages in 1µg/ml LPS (**a-e**) or 100nM LTB4 (**f-g**) stimulated bone marrow derive-macrophages (BMDM) of young and old mice. **a** Schematic represents the method used to differentiate BMDMs and treat BMDMs with 1µg/ml of LPS. **b** Representative histogram of BLT1 of macrophages in untreated vs. LPS treated BMDMs. **c** BLT1 frequencies and MFI of macrophages with/without LPS treatment. **d** CD86 MFI of macrophages with/without LPS treatment. **e** CD86 MFI of BLT1+ macrophages with/without LPS treatment. **f** BLT1 frequencies of macrophages with/without LTB4 treatment. **g** BLT1 frequencies of CD11c+ macrophages with/without LTB4 treatment. Male mice were used in this experiment (n=5-6/group). Chart error bars represent mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with a post-hoc test of Sidak. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Old M2 polarized BLT1+ BMDMs showed a less anti-inflamed phenotype than young

Next, we examined if BLT1 was preferentially expressed on M1 or M2 polarized macrophages generated from young or aged BMDMs. BMDMs were polarized to M1 macrophages with LPS + IFNg and M2 macrophages with IL-4 for 24 hours (Fig. 4a). Upon M1 polarization, young and aged

BMDMs showed no difference in CD11c expression (Fig 4b.) But upon M2 polarization, old BMDMs expressed less CD206 compared to young counterparts (Fig. 4c). Aged BLT1+ M2-polarized macrophages showed decreased CD206 expression as compared to young BLT1+ M2 macrophages (Fig. 4d). These data suggest that BLT1+ M2 macrophages may have reduced ability to dampen inflammation upon aging.

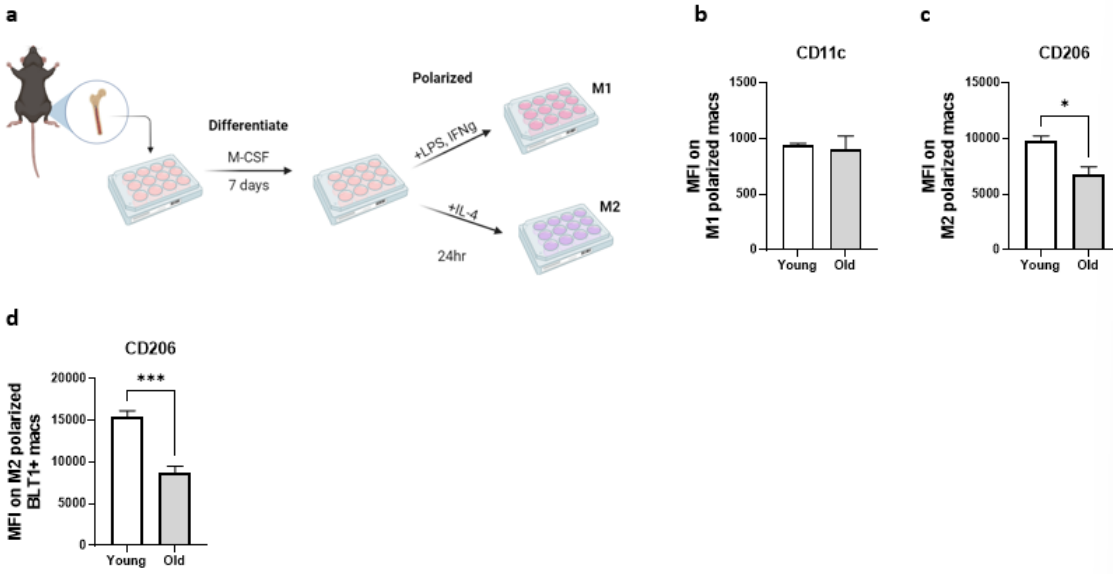


Figure 4 Pro-inflammatory (CD11c+) and anti-inflammatory (CD206+) phenotype macrophages in M1 and M2 polarized BMDMs of young and old mice. **a** Schematic represents the method used to differentiate and polarize BMDMs. CD11c were determined in M1 polarized BMDMs, and CD206 were quantified in M2 polarized BMDMs **b** CD11c MFI of macrophages in M1 polarized BMDMs. **c** CD206 MFI of macrophages in M2 polarized BMDMs. **d** CD206 MFI of BLT1+ macrophages in M2 polarized BMDMs. Male mice were used in this experiment (n=4/group). Chart error bars represent mean ± SEM. Statistical analysis was conducted using non-parametric T-tests. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

The BLT1 antagonist reduces inflammatory response by decreasing pro-inflammatory macrophages and increasing anti-inflammatory macrophages

BLT1 antagonist, U75302, binds the BLT1 to block downstream signaling and was shown to prevent LPS-induced inflammation in young mice [42]. As older individuals have increased susceptibility to endotoxemia [8], we asked if inhibition of LTB4-BLT1 axis with U75302 treatment would reduce severity of endotoxemia in aged mice. To test this, aged male mice (24 months old)

were IP injected with 1mg/kg U75302 1 hour prior to 3mg/kg LPS treatment for 6 hours (Fig 5a). Injection of BLT1 antagonist did not affect hypothermia induced by LPS treatment (Fig. 5b), VAT B cells were decreased from 15% to 10% (Fig. 5c). Lymphocyte activation (CD69) level was comparable between vehicle and U75302 group (Fig. 5d, 5e). We also examined CD11c+ and CD206+ macrophage subsets to identify whether macrophages exhibited a pro- or anti-inflammatory phenotype. We looked at the ratio between CD11c and CD206, both the frequency and MFI of the CD11c/CD206 ratio showed a decrease with the antagonist group (Fig. 5f, 5g). By looking at only CD11c or CD206 frequencies, there is a decrease trend of CD11c+ macrophages and an increase of CD206+ macrophages of the antagonist group compared to the LPS group (Supplementary Fig. 2). These results reveals that the BLT1 antagonist is reducing macrophage inflammation by altering the ratio of pro-inflammatory CD11c+ macrophages and CD206+ anti-inflammatory macrophages.

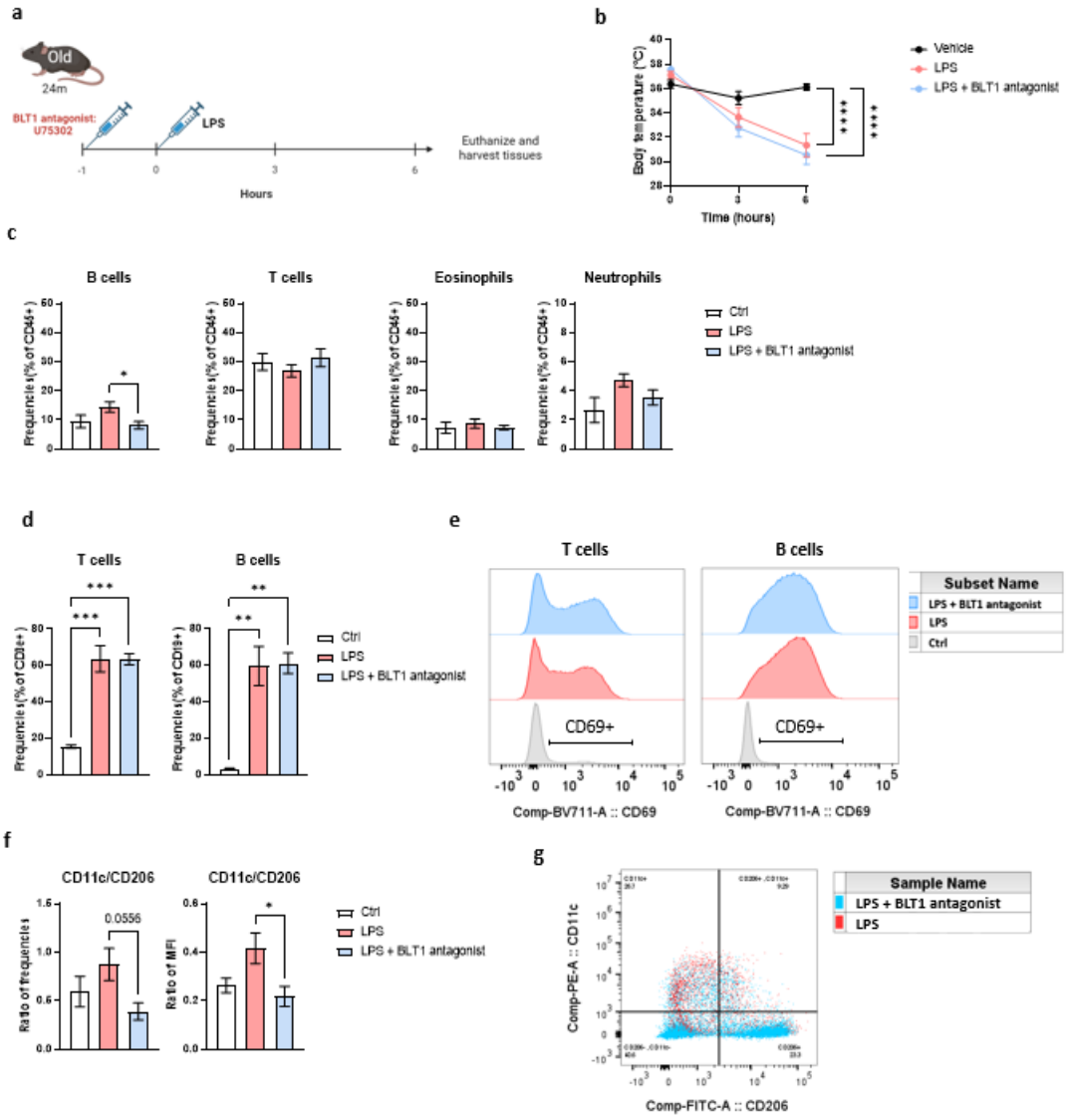
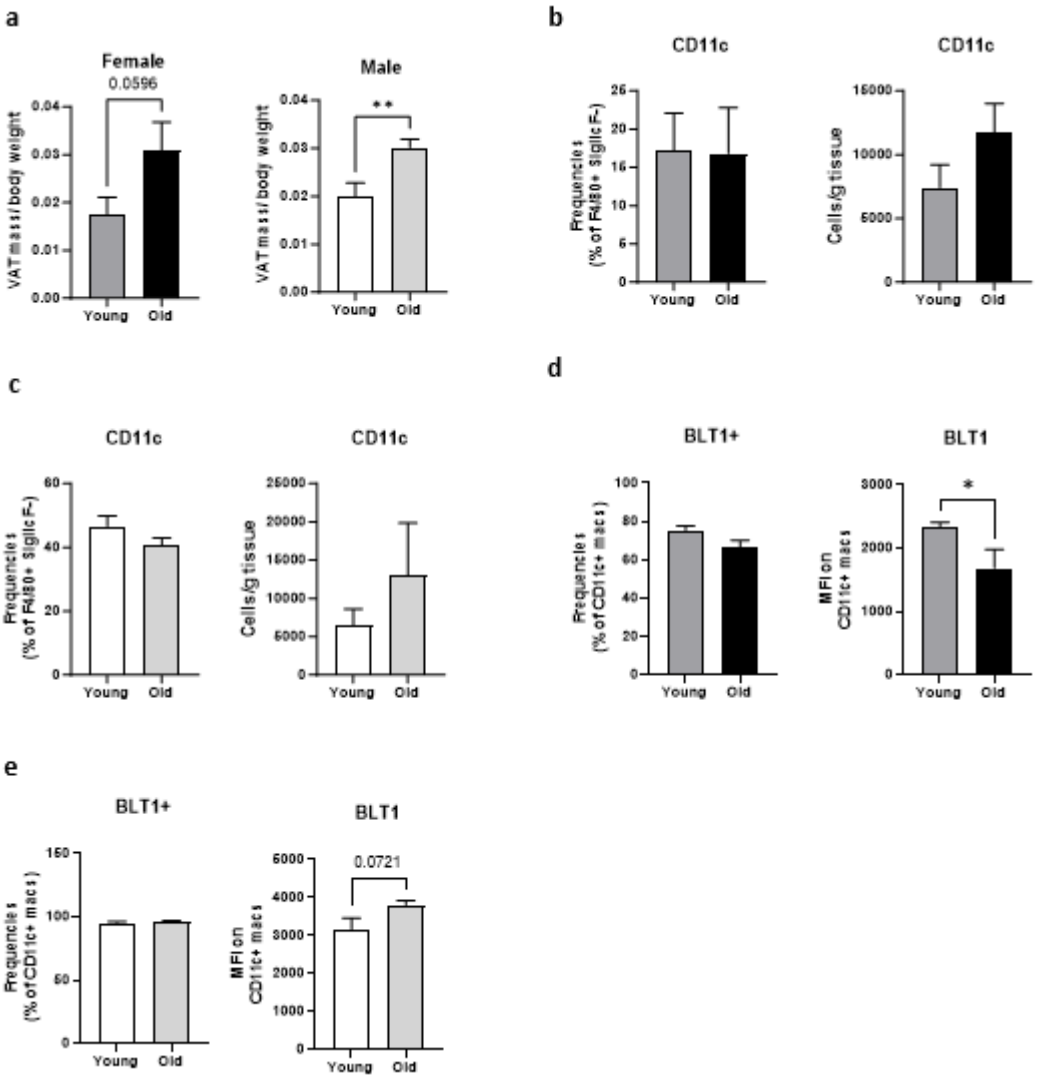
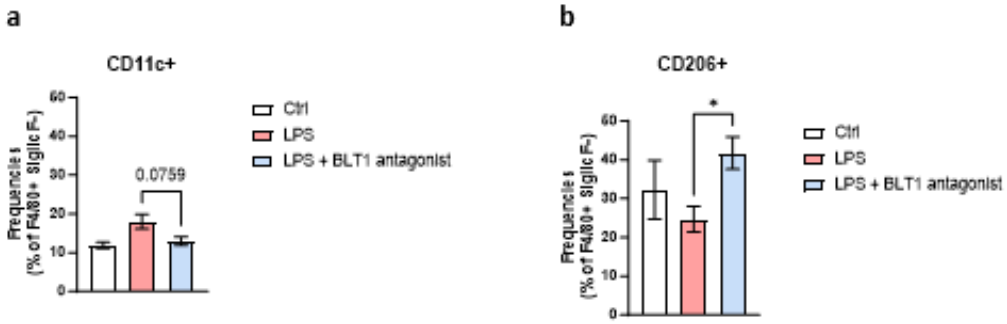


Figure 5 Immune cell population in BLT1 antagonist (U75302) treated old (24 months) mice. **a** Schematic represents the experimental design. Mice were injected with 3mg/kg LPS and 1mg/kg U75302 (BLT1 antagonist) through IP. **b** Body temperature of vehicle, LPS treated and LPS + BLT1 antagonist treated mice. **c** Immune cell frequency of CD45+ live cells in VAT. **d** CD69 population in CD3e+ T cells and CD19+ B cells of spleen. **e** Histogram of CD69 population in CD3e+ T cells and CD19+ B cells of spleen **f** Ratio of CD11c+ and CD206+ to frequency and MFI of F4/80+ SiglicF- macrophages in VAT. **g** Dot plot of LPS vs. LPS + BLT1 antagonist treated mouse in VAT. Male mice were performed in this experiment (n=4 in vehicle, n=7-8 in LPS, LPS + BLT1 antagonist). Chart error bars represent mean ± SEM. Statistical analysis was conducted using one-way ANOVA with a post-hoc test of Tucky. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.



Supplementary Figure 1 Comparison of CD11c frequencies and cellularity of young (5-8 months) and aged (20-22 months) mice. **a** Ratio of VAT mass and body weight of female and male, young and aged mice. **b** CD11c frequencies of F4/80+ SiglicF- macrophages and cells/g tissue of female mice. **c** CD11c frequencies of F4/80+ SiglicF- macrophages and cells/g tissue of male mice. **d** BLT1 frequencies and MFI of F4/80+ CD11c+ cells in female mice. **e** BLT1 frequencies and MFI of F4/80+ CD11c+ cells in male mice. (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using non-parametric T-tests. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.



Supplementary Figure 2 CD11c and CD206 frequencies in BLT1 antagonist (U75302) treated old (24 months) mice. **a** CD11c frequencies of F4/80+ SiglicF- macrophages. **b** CD206 frequencies of F4/80+ SiglicF- macrophages. Male mice were performed in this experiment (n=4 in vehicle, n=7-8 in LPS, LPS + BLT1 antagonist). Chart error bars represent mean ± SEM. Statistical analysis was conducted using one-way ANOVA with a post-hoc test of Tucky. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Discussion:

Overview

The phenomenon of "inflammaging," characterized by chronic low-grade inflammation during aging, significantly influences the immune system and contributes to age-related diseases. Visceral adipose tissue, a key player in immune response, is commonly explored in the context of age-related inflammation [4, 26, 46, 53, 54]. In this study, we examine the expression of the BLT1 on immune cells from VAT of young and aged mice. We complement these analysis with in vitro examination of BMDMs and use of the BLT1 antagonist during endotoxemia challenge. Our findings reveal that BLT1 expression is limited to innate immune cells of the VAT, and that pro-inflammatory macrophages have increased BLT1 expression. Increased pro-inflammatory phenotype are investigated in aged BLT1+ BMDMs, followed by increased activity of BLT1 in aging. M2-polarized BLT1+ macrophages from old mice have reduced CD206. Additionally, BLT1 blockade reverses the LPS-induced M1/M2 ratio in a mouse model of endotoxemia. These findings suggested that BLT1 supports the inflammatory phenotype of aging by altering macrophages.

In depth discussion of figures

Aging, often accompanied by obesity, is a well-established phenomenon that is found in humans and rodents. The aging-associated obesity usually implies to the increase of fat mass, especially VAT. Although we did not observe an increase of body weight in aged male mice, there was a significant elevation in VAT mass compared to young male mice (Fig. 1a-b), and an increased body-fat mass (Supplementary Fig. 1a). Which still implies an obese phenotype in aged male mice. Within aging, studies report B cell and CD3+ T cell values increase [28, 53, 54], while eosinophils decrease [55] in aged VAT. Which is consistent with our findings in both male and female mice (Fig. 1d-e, g-h). Besides CD11c MFI of macrophages, we also examined frequencies of CD11c+ cells in F4/80+ macrophages and CD11c number of cells per gram of tissue (Supplementary Fig. 1b-c), each providing different concepts. Frequencies reflect the percentage of CD11c in F4/80+ macrophages; CD11c counts inform us the actual cell number in 1 gram of tissue; and MFI is related to the fluorescent intensity of CD11c on F4/80+ macrophages. Even though results in female mice did not show an increase of frequency and cell number of CD11c (Supplementary Fig. 1b), the intensity of CD11c presenting on macrophages increased with age (Fig. 1f). This indicates that the levels of inflammatory macrophages did not increase in number, but those inflammatory macrophages are expressing more CD11c.

BLT1 expression in B cells and T cells showed lower mean fluorescence intensity (MFI) than the isotype control, indicating that BLT1 is not expressed on these immune cells (Fig. 2c). We focused on macrophages since our result showed that BLT1 is mostly highly expressed on this cell type. BLT1 MFI on CD11c+ macrophages exhibited a different trend by aging in both males and females, which females showed decreased BLT1 MFI in CD11c+ cells and males showed increase (Supplementary Fig. 1d-e). Sex differences in inflammaging are not a well-known research field due to the complexity of multiple factors including but not limited to the influence of genetics, environment, and sex hormones. Females have been reported to exhibit a lower infection rate than males. Reasons for this may be that sex hormones impact the immune system by binding to genes related to innate immunity, like MyD88, IRF7, and various toll-like receptors (TLRs). Those genes also interact with other transcription factors such as NF-kB to influence immune responses [56, 57]. This gives us insight into how BLT1 might also be a factor that could be impacted by the sex hormones, to influence NF-kB, leading to the decreased intensity of BLT1 expression on aged female mice. But further research about sex differences in BLT1 is needed to prove this hypothesis.

In BMDM experiments, we examined BLT1 in LPS-, LTB4- stimulated or M1 and M2 polarized macrophages. In LPS stimulated conditions, we identified that BLT1+ BMDMs from old mice express higher levels of CD86, as compared to the BLT1+ cells from young mice (Fig. 3e). Moreover, BLT1 was elevated in aged pro-inflammatory macrophages with a LTB4 stimulation (Fig. 3g). This suggests that the LTB4-BLT1 pathway may be highly activated in aged pro-inflammatory BMDMs, as compared to young. In M2 polarized macrophages, both the total macrophage population and the BLT1+ subset showed decreased CD206 in aged cells compared to young (Fig. 4c, e). Suggesting that the aged M2 polarized BMDMs are deficient in the anti-inflammatory phenotype; however additional experiments that fully test M2-functionality would need to be performed.

Research of BLT1 on LPS-injected young mice used a dose of 6 mg/kg or even higher to show differences of LPS-induced and control mice [42, 58, 59]. But previous experiments showed that aged mice started to die within 6 hours of a dose above 3 mg/kg LPS (data not shown). For this reason, we chose to inject 3 mg/kg LPS into the aged mice. Besides macrophages, B cells are the only immune cells that showed a decrease in frequency with the BLT1 antagonist treatment (Fig. 5c). The activation and differentiation of B cells could be promoted through pro-inflammatory cytokines, particularly IL-1β and IL-6 [60]. Both IL-1β and IL-6 are reported to be downregulated by the BLT1 antagonist [42, 58]. Therefore, BLT1 might be able to regulate B cell frequencies in aged mice, by modulating the expression of inflammatory cytokines. Although further experiments should be performed to better understand the relationship between BLT1 and B cells. We examined the CD11c and CD206 ratio in the LPS vs. BLT1 antagonist group (Fig. 5f). By looking at only CD11c or CD206 frequencies, there is a decrease trend of CD11c+ macrophages and an

increase of CD206+ macrophages of the antagonist group comparing to the LPS group (Supplementary Fig. 2). This data supports our hypothesis that BLT1 can reduce inflammation in VAT macrophages by changing the total number of pro and anti-inflammatory macrophages.

LTB4 changes in aging

Despite the importance of LTB4 being a potent pro-inflammatory mediator and could have the ability to regulate age-induced inflammation. Studies of the LTB4-BLT1 axis in aging are limited. Arachidonic acid and its metabolites: LTB4, PGF2α, 20-HETE are found to be increased in aged male mice, indicates that age impacts the metabolic function of arachidonic acid to cause progression of inflammation and metabolic diseases [61]. Another publication reveals the increase of LTB4 in aging is contributing to inflammaging and immunosenescence [62]. Therefore it is possible that increased LTB4 and increased responsiveness of the BLT1 receptor are contributing to the inflammatory environment during aging.

BLT1 in obesity and other concepts

Obesity in often involved in the aging process due to the increased body-fat mass of the elder [13], which is consistent with our data (Supplementary Fig. 1a). Previous works have implicated the LTB4-BLT1 pathway in obesity related inflammation and metabolic dysfunction. Spite et al. described the ability of BLT1 to accumulate macrophages in the adipose tissue which leads to inflammation and insulin resistance. The obese BLT1-null mice have reduced M1 macrophages and pro-inflammatory cytokines; but increased M2 macrophages in the adipose tissue [47]. This is consistent with our data that shows BLT1 antagonist alters inflammatory response by altering the ration of pro- and anti-inflammatory macrophages (Fig. 5f-g, Supplementary Fig. 2). Ying et al. described the accumulation of B2 B cells in the VAT of obese male mice that was dependent on B cell-specific expression of BLT1 [54]. B cells are increased in the VAT of aged, obese mice, but we did not identify any expression of BLT1 on these cells (Fig. 2c). This suggests obesity-induced and age-induced B cell accumulation may occur through separate mechanisms. Horrillo et al. described the increase of LTB4 levels in adipose tissue of obese mice [63]. Ramalho et al. described the BLT1-induced fatty acid accumulation, glycolysis and oxidative metabolism in macrophages of diabetic mice [64]. Our findings examine another important field, BLT1-derived inflammation during endotoxemia in old mice, to provide a more comprehensive knowledge in the relationship of BLT1 and aging.

M1/M2 macrophages and BLT1

Aging is known to cause an increase in M1 macrophages, a decrease in M2 macrophages and increased pro-inflammatory cytokine production [65]. Studies found out that BLT1 expression is significantly higher in the M1-polarized macrophages compared to M2-polarized macrophages in BMDMs [66], which strongly suggested the pro-inflammatory phenotype linking BLT1 and M1 macrophages together. FABP4 (fatty acid binding protein 4) has a high binding affinity to fatty acids. and can also bind to LTA4 and arachidonic acids, the precursors of LTB4. The binding of FABP4 with LTA4 stabilizes LTA4 and helped in its conversion into LTB4 [67]. The study above also found out the depletion of FABP4 gene largely reduced the expression of BLT1 as well as M1 macrophages and increased M2 macrophages [66]. Similarly, BLT1 knockout mice displays an increase in M2 VAT macrophages and decrease in M1 macrophages [35]. These data again demonstrate the importance of BLT1 in regulating the pro and anti-inflammatory macrophages.

Conclusion

The LTB4-BLT1 pathway is a strong inducer of producing pro-inflammatory cytokines, indicating that BLT1 is a potential target for treating infection and chronic inflammatory diseases. This study provides valuable insights into the age-specific changes in BLT1 expression and its impact on immune cell subsets within VAT. The observed association between BLT1 expression and the modulation of inflammation suggests a complex role for BLT1 in the aging immune system. The use of a BLT1 antagonist in mitigating macrophage inflammation opens avenues for further exploration of BLT1 as a potential therapeutic target for treating age-related diseases. Taken together, BLT1 plays a regulatory role in inflammation within the visceral adipose tissue of aged mice by modulating pro-inflammatory and anti-inflammatory macrophages.

CHAPTER 2: Whether simultaneous exposure to glyphosate leads to heightened adipose tissue inflammation in HFD mice

Contributions: This chapter is a collaboration project with Dr. Ashutosh Mangalam and Lab at the University of Iowa. This is a part of a larger project, and the data for the larger project hasn't been fully compiled. I wrote all sections and performed all experiments. I'd like to thank Victor, Declan and Stephanie for assisting me.

Introduction:

Obesity, mainly caused by an increased calorie intake, contributes to the development of metabolic syndrome that leads to type 2 diabetes and cardiovascular diseases [68]. It is noteworthy that the obesity that elevates risk in metabolic diseases is defined by an increase in the fat mass, especially central deposition of adipose tissues, and not the overall body weight [68]. Obesity induces secretion of pro-inflammatory cytokines through adipose tissue is described in Chapter 1. The increase of pro-inflammatory cytokines such as IL-6 and TNF-α is thought to be a risk factor to cardiovascular diseases, due to their ability to promote endothelial dysfunction and atherosclerosis. IL-6 and TNF-α could induce C-reactive proteins (CRPs) in the liver, which studies had shown that CRPs contribute to the pathogenesis of atherosclerotic lesions [69]. Obesity also lead to increased levels of very low-density lipoprotein (VLDL) cholesterol, triacylglycerols and total cholesterol; and a decrease in high-density lipoprotein (HDL) cholesterol levels [70]. Although the main risk of cardiovascular diseases upon all lipoproteins, the low-density lipoprotein (LDL) cholesterol, shown little evidence in its increase in obesity [71]. Type 2 diabetes is also a disease highly linked to obesity. The increasing of free fatty acids (FFAs), and disturbances in adipokine secretion occurs in obesity and could give rise to insulin resistance of patients. FFAs and adipokines, especially inflammatory cytokines and leptin, have the ability to activate inhibitory molecules like SOCS and JNK. This activation can lead to the suppression of insulin signaling, and result in insulin resistance [71].

The gut microbiota consists of various kinds of bacteria in the GI tract that have evolved a beneficial relationship with the host and perform functions such as defense of infection, regulation of immune system and metabolism of nutrient. The gut microbiota mediates the immune system by producing metabolites. Short-chain fatty acid is one kind of the metabolites produced by gut microbiota. They could inhibit histone deacetylases, which leads to inactivation of NF-κB and a reduce production of pro-inflammatory cytokines [72]. Inhibition of histone deacetylases could also promote regulatory T cells to release, which regulatory T cells control inflammation by preventing proliferation of CD4 T cells [73]. Secondary bile acids are another metabolite produced by gut microbiota through primary bile acids. The reduction of primary and secondary bile acids could

also lead to bacterial overgrowth and inflammation [74]. Therefore, interference of gut microbiota could also lead to systemic inflammation and contribute to metabolic diseases [75].

Glyphosate, an herbicide that is widely used for agriculture, has been reported to be able to affect the composition of the gut microbiota, and therefore alters the inflammatory response. Studies of glyphosate on human health has become an urgent issue due to the rising detection of glyphosate in water and food these years [76, 77]. Glyphosate could alter the gut microbiota through inactivating the 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS) enzyme of the shikimate pathway. The inhibition of EPSPS prevents synthesis of gut microbiota metabolites and aromatic amino acids leading to systemic inflammation [78].

Previous studies in the Mangalam lab, which is the collaborator of this project, determined weight gain and abnormal glucose tolerance test with an exposure of glyphosate in high-fat diet (HFD) mice (unpublished data). Suggesting that glyphosate along with HFD could lead to mice developing metabolic diseases, comparing to HFD or glyphosate-exposed mice along. Due to the relationship between obesity and VAT-induced inflammation, we wanted to know whether glyphosate also plays a role in VAT-induced inflammation.

Material and Methods:

Tissue digestion: Mice with treatment of glyphosate and HFD for 90 days were euthanized at the University of Iowa. Visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) and spleen were obtained and stored in RPMI then sent to us. VAT underwent enzymatic digestion in 0.1% collagenase II (Worthington Biochemicals) in Hanks' Balanced Salt Solution (HBSS) (Life Technologies) for 30 minutes at 37°C, 200rpm agitation. To ensure consistency, both control and experimental groups were processed and stained on the same day, to minimize procedural variations. The stromavascular fraction was recieved through centrifugation at 1500rpm for 5 minutes, followed by washing in RPMI with 10% FBS. ACK lysis buffer (Quality Biological Inc) was used to remove red blood cells. Cells were quench with RPMI with 10% FBS after 2 minutes in ACK lysis buffer, filtered with a 40uM filter and centrifuge at 1500rpm for 5 minutes to get single cell suspension. Splenocytes were isolated into single cells in a similar way, but without digestion in collagenase II.

Cell culture: Splenocytes and stromavascular cells were isolated as previously described. 10^6 of cells were plated in 24 well plate with sterile RPMI, supplemented with 10% FBS,1% antibiotic-antimycotic, 2mM L-glutamate, and 10 ng/mL IL-4. Cells were stimulated with 25 ng/mL PMA, 1 µg/mL ionomycin and 1 µg/mL GolgiPlug for 4 hours before harvest and staining.

Flow cytometry: 10^6 of cells were stained with Ghost Dye Red 780 Viability Dye (TONBO biosciences) for 30 minutes on ice, avoiding light. Then washed and incubated with FcBlock (BD Bioscience) and surface antibodies for another 30 minutes. Antibodies used are listed in Table S1. The BLT1 staining required a secondary antibody since it is unconjugated. AF647-conjugated anti-mouse IgG1 (Biolegend) was used as the secondary antibody for a 30 minute stain. Control groups were stained with Rabbit IgG Isotype Control (Invitrogen) instead of the BLT1 antibody. For intracellular staining, cells were fixed with the BD CytofixCytoperm kit (554714) for 20 minutes, followed by an intracellular antibody stain for 30 minutes. For nuclear staining, cells were fixed with the eBioscience Fix/Perm Kit (00-5523-00) for 45 minutes, followed by an intracellular antibody stain for 30 minutes. Flow cytometry analysis was performed on a BD FACSymphony A3 (R6609) cytometer and a FlowJo v10 software.

RNA extraction and gene expression analysis: SAT and VAT were stored in RNA later and sent to us. Tissues were homogenized in Trizol (Invitrogen; cat# 15596026) using the Next Advantage Bullet Blender Storm 24. Chloroform was added to the Trizol mixture and incubated prior to

centrifugation at 12,000 rpm for 15 min at 4°C. RNA extraction on the aqueous phase of the centrifuged homogenate was performed using Invitrogen PureLink RNA Mini Kits according to the manufacturer's instructions. Reverse transcription and qPCR were performed as previously described [79].

Statistics: A confidence interval of 95% was used for all statistical tests. All data were assumed to be normally distributed, unless the standard deviation was identified as significantly different between groups. All statistical tests were performed using GraphPad Prism v9 for Windows (GraphPad Software). Data are expressed as mean ± s.e.m. Sample size of independent experiment are described in the figure legends. Statistical analysis was conducted using non-parametric T-test, 2-way ANOVA with a post-hoc test of Tucky or Sidak, or one-way ANOVA with a post-hoc test of Tukey. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Result:

HFD and glyphosate exposure increases the population of circulating monocytes

First of all, we wanted to understand how the immune cell population is influenced by HFD and glyphosate. We investigated immune cell populations using flow cytometry, and the gating strategy of immune cells was shown (Fig. 6a). We examined the T cell population and also the exhausted T cell marker. Both CD4 and CD8 T cells did not show increase in either diet or glyphosate (Fig. 6b, d). It is reported that obesity could cause an increase of exhausted T cells in adipose tissue [80]. Consistent with previous studies, we observed an increase in PD1+ cells in both CD8 and CD4 T cells (Fig. 6c, f), with PD1 being a marker for exhausted T cell. However, these changes are all related to diet and not glyphosate treatment. CD25 and FoxP3 double-positive cells are the regulatory T cells (Tregs), which also did not show differences in either diet or glyphosate treatment (Fig. 6e). We then assessed myeloid cell population. There is an increasing trend of macrophages (Fig. 6g) and a decrease of eosinophils (Fig. 6i) within the HFD, which is also similar to previous reports [55, 81]. Interestingly, we detected an increase in CCR2+ circulating monocytes in the HFD glyphosate mice (Fig. 6h), indicating that both glyphosate exposure and a high-fat diet could contribute to the increase of circulating monocytes. In conclusion, HFD cause an increase in exhausted T cells and a decrease in eosinophils; and both HFD and glyphosate exposure increases the population of circulating monocytes.

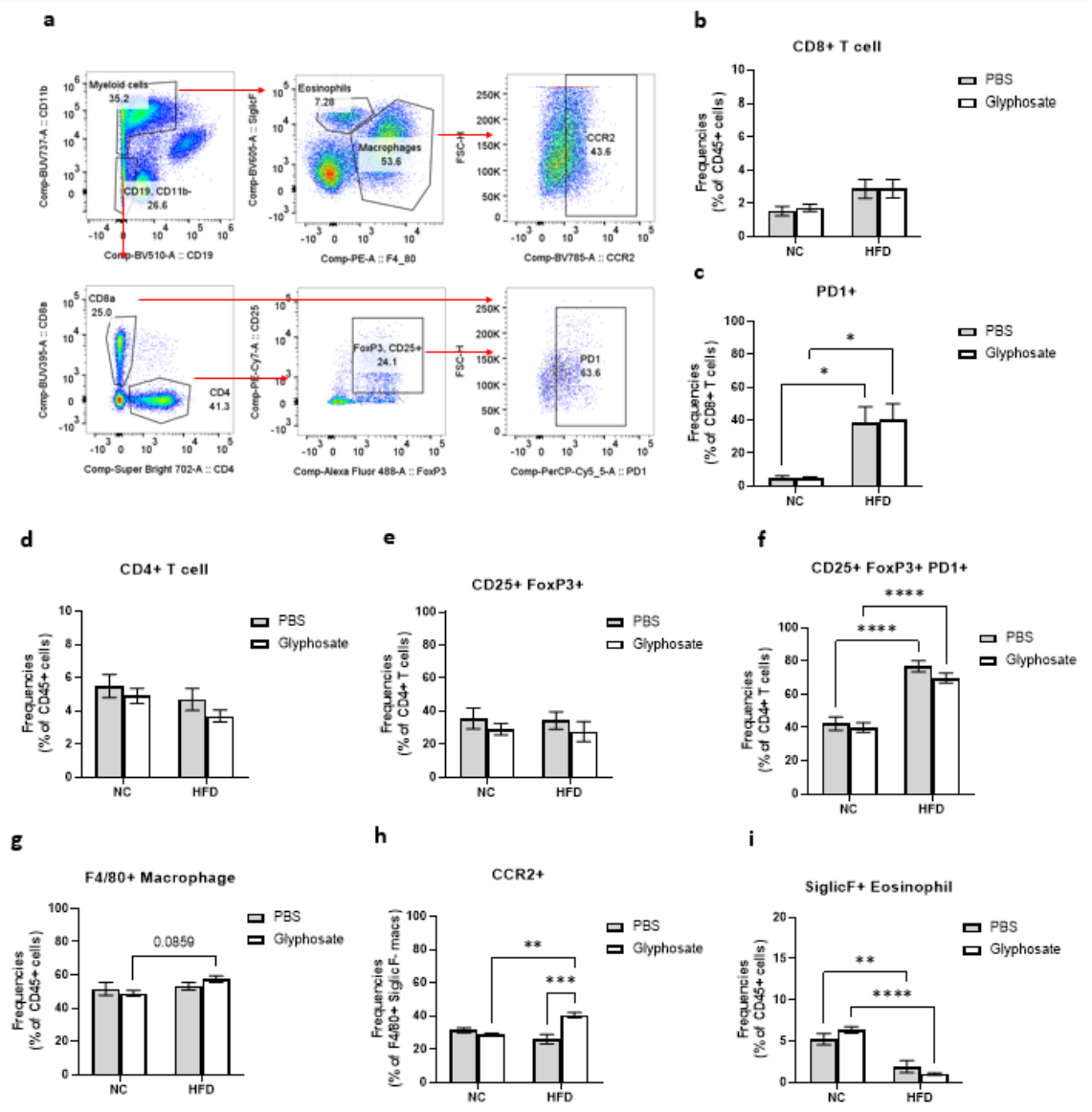


Figure 6 Effects of diet (NC: normal chow, HFD: high fat diet) and glyphosate on immune cells in visceral adipose tissue. **a** Flow gating strategy of immune cell subset. All cells are gated on singlets and CD45+ live cells. **b** CD8+ T cell frequency of CD45+ live cells. **c** PD1+ frequency of CD8+ T cells. **d** CD4+ T cell frequency of CD45+ live cells. **e** CD25+ FoxP3+ frequency of CD4+ T cells. **f** CD25+ FoxP3+ PD1+ frequency of CD4+ T cells. **g** F4/80+ SiglicF- macrophage frequency of CD45+ live cells. **h** CCR2+ frequency of F4/80+ SiglicF- macrophage. **i** SiglicF+ eosinophil frequency of CD45+ live cells. Male mice were used in this experiment (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with a post-hoc test of Tukey. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Obesity increases cytokine production in glyphosate exposure conditions compared to obesity along

Glyphosate exposure is reported to cause systemic inflammation [78], therefore we looked at cytokine production through stimulation with PMA and Ionomycin. The PMA/ionomycin method is widely used to stimulate immune cells. PMA could activate protein kinase C and activates the PI3K pathway; ionomycin is a calcium ionophore which is able to increase intracellular calcium levels. The combination of PMA/ionomycin could stimulate signaling pathways and cause cytokines to be produced from immune cells [82]. GolgiPlug contains brefeldin A, which is a protein transport inhibitor that could block the process of intracellular protein transport. By adding the GolgiPlug, cytokines would accumulate inside the Golgi complex and could be detected through antibody binding and flow cytometry [83]. The gating strategy of cytokines was shown (Fig. 7a). Negative controls, which are cells without stimulation of PMA and ionomycin or without GolgiPlug, showed minimum cytokine detected (Fig. 7b). We looked at TNFa and IFNg cell numbers produced by T cells. TNFa+ and IFNg+ double-positive cells produced by CD4 T cells showed a significant increase in diet of glyphosate exposure mice, and an increasing trend with glyphosate treatment in the HFD mice (Fig. 7d). Although single positives of TNFa and IFNg produced by CD4 T cells did not appear difference with both diet and glyphosate treatment (Fig. 7c, e). As for cytokines produced by CD8 T cells, TNFa+ cells exhibit an increasing trend (Fig. 7f); TNFa+ IFNg+ and IFNg+ cells increased significantly with HFD of glyphosate-treated mice compared to NC diet glyphosate-treated mice (Fig. 7g, h). We also examined IL17a numbers, but both CD4 IL17a+ and CD8 IL17a+ cell numbers are below 100 cells, therefore even though we detected an increasing trend of CD4 IL17a+ cells in the HFD glyphosate mice, we can't conclude IL17a+ cells are elevated within diet (Fig. 7i, j). Last but not least, IL6+ cells produced by myeloid cells (CD11b+) are boosted with both diet and glyphosate exposure (Fig. 7k). Taken together, an interesting finding was obtained: we realized that mice without glyphosate exposure did not reveal any differences in cytokine production within HFD. Whereas all the differences we investigated happen in NC diet and HFD mice with glyphosate exposure. Suggesting that obesity would promote cytokine production in glyphosate exposure conditions compared to obesity along.

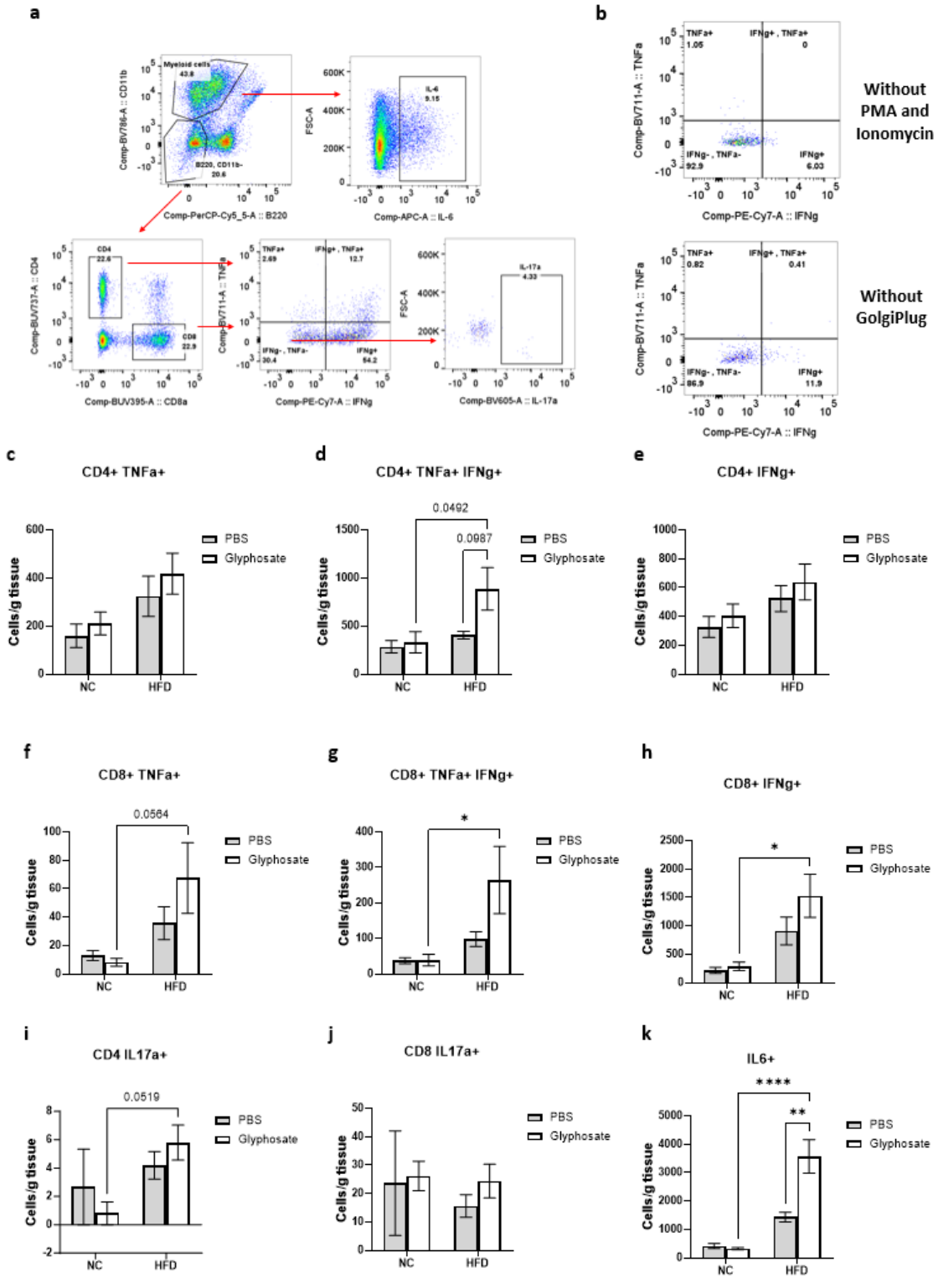


Figure 7 Effects of diet and glyphosate on cytokine production in visceral adipose tissue. **a** Flow gating strategy of immune cell subset. All cells are gated on singlets and CD45+ live cells. **b**

Control groups represent in dot plots were HFD cells without PMA and inomycin and without Golgi-Plug **c** Cells/g tissue of CD4+ TNFα+. **d** Cells/g tissue of CD4+ TNFα+ IFNg+. **e** Cells/g tissue of CD4+ IFNg+. **f** Cells/g tissue of CD8+ TNFα+. **g** Cells/g tissue of CD8+ TNFα+ IFNg+. **h** Cells/g tissue of CD8+ IFNg+. **i** Cells/g tissue of CD4+ IL17a+. **j** Cells/g tissue of CD8+ IL17a+. **k** Cells/g tissue of CD11b+ IL6+. Male mice were used in this experiment (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with a post-hoc test of Tukey. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Memory CD4 T cells increased with diet in the glyphosate exposure group, but neither glyphosate exposure nor diet affected other memory or naïve immune cells

Besides immune cell and cytokine production, we also investigated the population of memory and naïve cells. We used CD73 to gate out memory B cells; CD44 for memory T cells and CD62L for naïve T cells (Fig. 8a). The population of memory and naïve B cells has not been affected by either diet or glyphosate exposure (Fig. 8b, c), same as in CD8 T cells (Fig. 8f, g). We identified an increase in memory CD4 T cell with diet in the glyphosate group (Fig. 8d). The naïve CD4 T cell revealed a decrease in frequency (Fig. 8e). These data suggested that glyphosate might not be a high contributor to memory and naïve cell population.

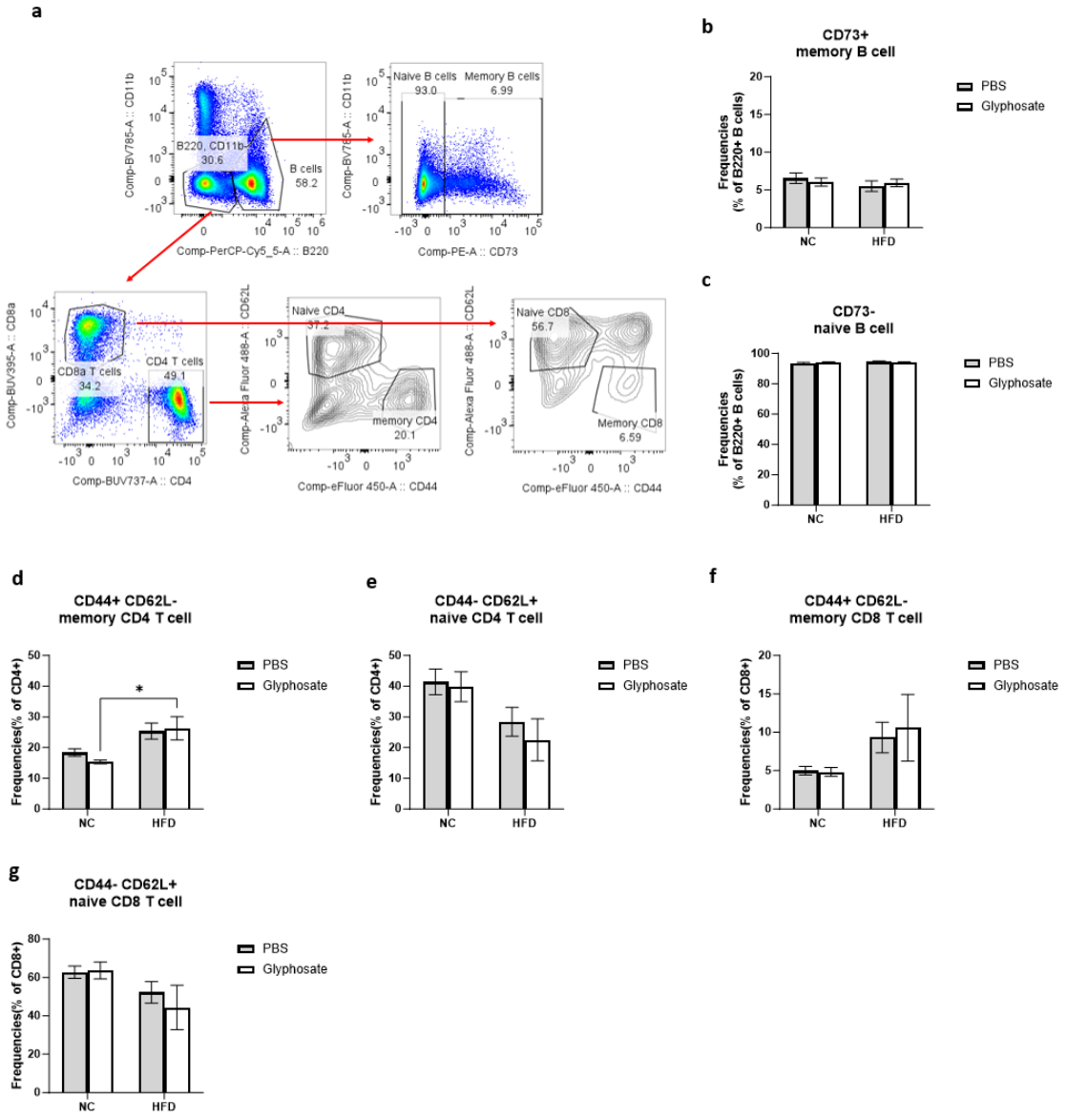
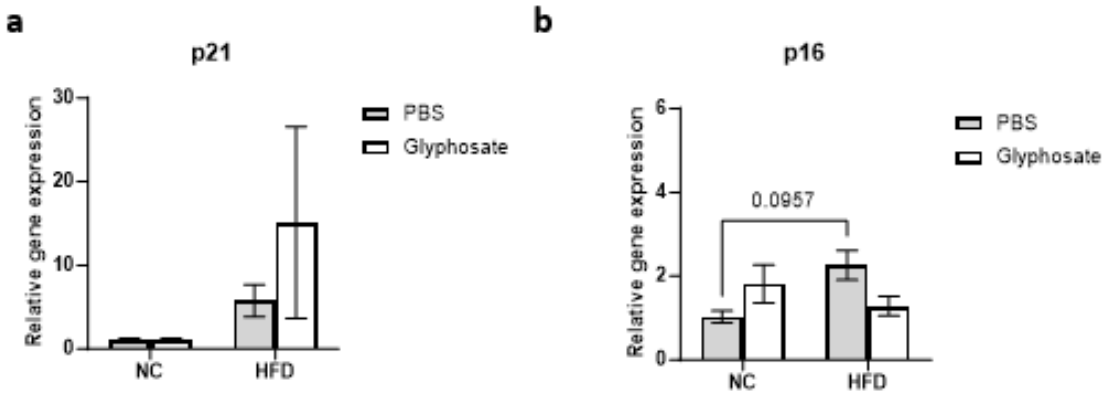


Figure 8 Effects of diet and glyphosate on memory and naïve immune cells in visceral adipose tissue. **a** Flow gating strategy of immune cell subset. All cells are gated on singlets and CD45+ live cells. **b** CD73+ memory B cell frequency of B220+ B cells. **c** CD73- naive B cell frequency of B220+ B cells. **d** CD44+ CD62L- memory CD4 T cell frequency of CD4+ T cells. **e** CD44- CD62L+ naive CD4 T cell frequency of CD4+ T cells. **f** CD44+ CD62L- memory CD8 T cell frequency of CD8+ T cells. **g** CD44- CD62L+ naive CD8 T cell frequency of CD8+ T cells. Male mice were used in this experiment (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with a post-hoc test of Tukey. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001



Supplementary Figure 3 Gene expression of diet and glyphosate on senescence markers in visceral adipose tissue. **a** Gene expression of p21. **b** Gene expression of p16. Male mice were used in this experiment (n=4-5/group, repeated experiment). Chart error bars represent mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with a post-hoc test of Tukey. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Discussion:

The aim of this experiment is to determine whether simultaneous exposure to a high-fat diet and glyphosate leads to heightened adipose tissue inflammation compared to the HFD alone. Several markers exhibited a notable increase with the HFD, including exhausted CD4 and CD8 T cells, and cytokine numbers. Other markers demonstrated an increasing trend with glyphosate exposure, including CCR2+, CD4 TNFα+ IFNg+ and IL6+ cells. Importantly, the increase in cytokines we detected are all found in glyphosate-exposed mice. These findings reveal several interesting aspects that shed light on the potential role of glyphosate in exacerbating inflammation.

This study used flow cytometry to analyze immune cell populations and found that HFD led to an increase in exhausted T cells (PD1+ cells) (Fig. 6c, f) and a decrease in eosinophils (Fig. 6i). Moreover, both HFD and glyphosate exposure resulted in an increase in CCR2+ circulating monocytes (Fig. 6h). Circulating monocytes are reported to be induced during obesity [84]. The increase in CCR2+ cells in glyphosate-exposed mice suggests a potential synergistic effect between HFD and glyphosate in promoting the recruitment of circulating monocytes, a phenomenon known to be associated with inflammation and metabolic diseases [85].

The investigation into cytokine production revealed that obesity, in combination with glyphosate exposure, led to a significant increase in some cytokines. This finding is consistent with the pro-inflammatory effects of both obesity and glyphosate reported in the introduction [20, 21, 78]. Notably, the study observed these differences primarily in mice exposed to glyphosate, suggesting that the presence of glyphosate enhances the inflammatory response caused by obesity. However, the specific mechanisms underlying this interaction need further exploration.

We discovered that obesity increased inflammation is somehow pretty similar to aged increased inflammation. For example, T cell exhaustion is present in aging [86], but we also detected an increase in exhausted T cells in a high-fat diet (Fig. 6c, f). Increasing macrophages and decreasing eosinophils are also shown in aging, which is fully described in Chapter 1, is also what we saw in obesity (Fig. 6g, i). Age-induced cytokine production is a highly known phenomenon [5]. We investigated increased production of multiple cytokines within HFD (Fig. 7). The possible explanation might be that aging usually follows with obesity, especially elevation in VAT mass, therefore the inflammatory response determined in VAT would show alike results. While aging and obesity increases memory cells and declines naïve cells [87, 88], changes in memory and naïve cells are not significant in our data (Fig. 8). CD4 and CD8 T cells did show an increasing trend in memory cells and a decreasing trend in naïve cells, but did not show a significant difference. Reasons may be that we had high variability in our data. We also looked at senescence markers of HFD and glyphosate-exposed mice. Cellular senescence occurs during inflammaging and obesity [11, 89]. We observed an induced trend of p16 in obesity within the non-glyphosate-

exposed mice, but a decrease trend in obesity within the glyphosate exposed mice (Supplementary Fig. 3a, b), indicating that obesity compare with glyphosate exposure may have different mechanisms with obesity alone. Although further research to prove the hypothesis is needed. Taken together, aging and obesity share some similar phenotype during inflammation, which those phenotypes are elevated in obesity combining glyphosate exposure. Again demonstrate that the combination of obesity and glyphosate exposure induces severe inflammatory response compared to either one alone.

In conclusion, this study provides valuable insights into the complex interplay between HFD, glyphosate exposure, and their combined impact on immune cell populations, cytokine production, and memory/naïve cell populations. The results suggest that glyphosate may exacerbate the inflammatory response associated with obesity, potentially contributing to the development of metabolic diseases. Further research is needed to elucidate the molecular mechanisms underlying these interactions and to explore potential therapeutic interventions targeting gut microbiota or immune pathways affected by glyphosate. But overall, this study contributes to the growing body of evidence highlighting the importance of considering environmental factors, such as herbicide exposure, in understanding and addressing the multifaceted nature of metabolic diseases.

Future Direction:

The examination of BLT1 offers insights into its alterations during the aging process and its role in modulating pro- and anti-inflammatory macrophages, thereby influencing the chronic inflammatory characteristic of aging. Further investigation of these findings may be pursued through experimentation on BLT1 knockout mice to better validate the correlation between BLT1 and macrophages within aged adipose tissue. FABP4, a protein implicated in LTB4 formation, have been reported to demonstrate a significant reduction in BLT1 expression in the young knockout mice. Therefore, future investigations may concentrate on evaluating BLT1 expression in aged FABP4 knockout mice. Such research could contribute to the comprehension of the LTB4-BLT1 pathway and its potential alterations during the aging process.

The glyphosate project provides evidence of how obesity along with glyphosate exposure exacerbates the inflammatory phenotype. Further investigations may be warranted to explore alterations in immune cell profiles and senescent markers among aged mice subjected to glyphosate exposure, to augment our understanding of herbicide-induced health repercussions. While the impact of glyphosate exposure on gut microbiota composition is documented, its effects on adipose tissue physiology remain obscure. Hence, treating glyphosate to 3T3-L1 adipocytes could give us more insights into the mechanistic underpins of glyphosate-mediated adipocyte inflammation, thereby enriching our understanding of its potential effect on adipose tissue homeostasis.

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Appendix:

Supplementary Table 1: Antibodies used for flow cytometry

Experiment	Antibody	Fluorophore	Company	Catalogue	Clone
BLT1 y vs. o (Fig. 2)	CD11c	PE	Biolegend	117308	N418
	SiglicF	BV605	BD Biosciences	740388	E50-2440
	F4/80	BV421	Biolegend	123132	BM8
	CD3e	AF488	Biolegend	100321	145-2C11
	Ly6G	PerCP/Cy5.5	Biolegend	127615	1A8
	CD19	BUV395	BD Biosciences	563557	1D3
	CD11b	BV785	Biolegend	101243	M1/70
	CD45.2	BUV737	BD Horizon	612778	104
	LTB4-R1 (BLT1)	unconjugated	Millipore	MABF2769	7A8
	Anti-mouse IgG1	AF647	Biolegend	406618	RMG1-1
LPS stim BMDM (Fig. 3)	F4/80	PE	invitrogen	2049425	BM8
	CD206	FITC	Biolegend	141704	C068C2
	CD86	BV421	BD Biosciences	564198	GL1
	CD45.2	BUV737	BD Horizon	612778	104
	LTB4-R1 (BLT1)	unconjugated	Millipore	MABF2769	7A8
	Anti-mouse IgG1	AF647	Biolegend	406618	RMG1-1
BMDM polarization (Fig. 4)	F4/80	BV421	Biolegend	123132	BM8
	CD206	FITC	Biolegend	141704	C068C2
	CD11c	PE	Biolegend	117308	N418
	CD45.2	BUV737	BD Horizon	612778	104
	LTB4-R1 (BLT1)	unconjugated	Millipore	MABF2769	7A8

	Anti-mouse IgG1	AF647	Biolegend	406618	RMG1-1
BLT1 antagonist (Fig. 5)	CD206	FITC	Biolegend	141704	C068C2
	CD11c	PE	Biolegend	117308	N418
	SiglicF	BV605	BD Biosciences	740388	E50-2440
	F4/80	BV421	Biolegend	123132	BM8
	CD3e	PE-Cy7	invitrogen	25-0031-82	145-2C11
	Ly6G	PerCP/Cy5.5	Biolegend	127615	1A8
	CD19	BUV395	BD Biosciences	563557	1D3
	CD11b	BV785	Biolegend	101243	M1/70
	CD45.2	BUV737	BD Horizon	612778	104
	CD69	BV711	Biolegend	104537	H1.2F3
	LTB4-R1 (BLT1)	unconjugated	Millipore	MABF2769	7A8
	Anti-mouse IgG1	AF647	Biolegend	406618	RMG1-1
Glyphosate immune cell (Fig. 6)	CD45.2	BUV563	Biolegend	109838	104
	CD19	BV510	BD	563557	1D3
	CD11b	BUV737	BD	612801	M1/70
	SiglicF	BV605	BD	45412	90/CD38
	F4/80	PE	invitrogen	12-4801-82	BM8
	CD4	SB702	Biolegend	100451	GK1.5
	CD8a	BUV395	BD	740650	53-5.8
	CD192 (CCR2)	BV785	Biolegend	150628	SA203G11
	PD1	PerCPCy5.5	Biolegend	135208	29F.1A12
	CD25	PECy7	invitrogen	25-0251-82	PC61.5
	FoxP3	AF488	invitrogen	53-5773-82	FJK-16s
Glyphosate cytokine (Fig. 7)	CD45	FITC	BD	612924	30-F11
	CD4	BUV737	BD	612761	GK1.5
	CD8a	BUV395	Biolegend	100744	53-6.7

	CD11b	BV785	Biolegend	101239	M1/70
	IL-17A	BV605	Biolegend	506927	TC11-18H10.1
	IL-6	APC	Biolegend	504508	MP5-20F3
	IFNg	PECy7	Tonbo	60-7311-U100	XMG1.2
	TNFa	BV711	Biolegend	506349	MP6-XT22
Glyphosate memory/naive (Fig. 8)	CD45	AF647	BD	612924	30-F11
	CD4	BUV737	BD	612761	GK1.5
	CD8a	BUV395	Biolegend	100744	53-6.7
	B220	PerCP Cy5.5	invitrogen	45-0452-82	RA3-6B2
	CD73	PE	Biolegend	127205	TY/11.8
	CD44	eFluor 450	invitrogen	48-0441-82	IM7
	CD11b	BV785	Biolegend	101239	M1/70
	CD62L	AF488	invitrogen	53-4801-82	BM8