High fat diet modulates conventional dendritic cell development through Interferon regulatory factor 8

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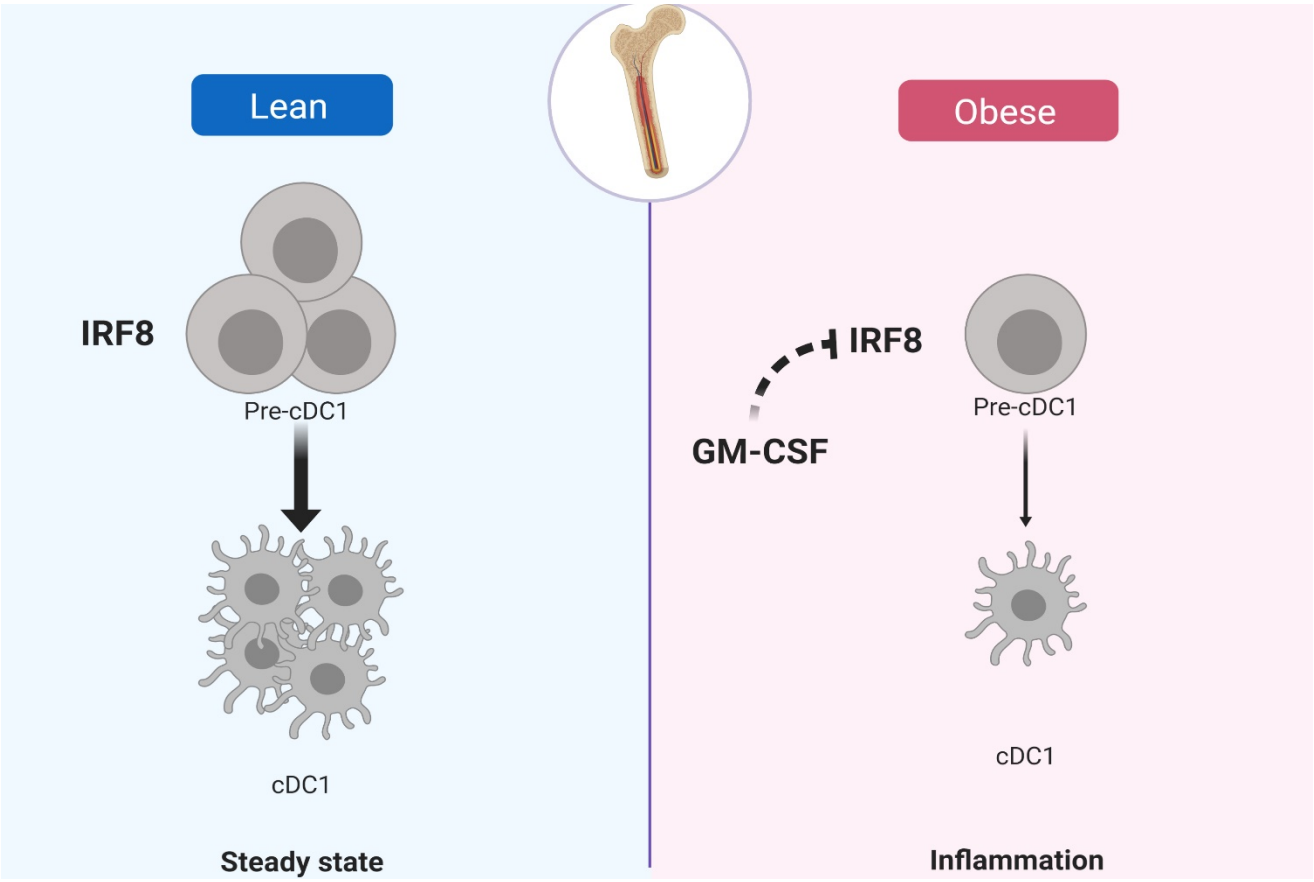
Contents

Abbreviations

ADT	Adipose tissue
APC	Antigen-presenting cells
BM	Bone marrow
BMI	body mass index
BMAT	bone marrow adipose tissue
cDCs	Conventional dendritic cells
cDC1	Conventional dendritic cells type 1
cDC2	Conventional dendritic cells type 2
CD	Control diet
CDP	Common dendritic cells precursors
CMP	Common myeloid progenitors
CLP	Common lymphoid progenitors
cMoP	Common monocyte progenitors
DC	Dendritic cells
DAMPs	Damage associated molecular patterns
DIO	Diet induce obesity
ELISA	The enzyme-linked immunosorbent assay
eWAT	epididymal white adipose tissue
Flt3L	FMS-like tyrosine kinase 3 ligand
FACS	Flow cytometry

GMP	Granulocyte macrophage progenitors
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSCs	Hematopoietic stem cells
HFD	High fat diet
IRF	Interferon regulatory factor
IHC	Immunohistochemistry
ISH	In situ hybridization
LN	Lymph nodes
MCMV	Murine cytomegalovirus
MDP	Macrophage and DC precursors
NK cells	Natural killer cells
PAMPs	Pathogen associated molecular patterns
pDC	Plasmacytoid dendritic cells
preDCs	Dendritic cells precursors
s.e.m	standard error of the mean
STAT	signal transducer and activator of transcription
TFs	Transcription factors
T2D	Type 2 diabetes
WAT	white adipose tissue

Overview



Overview Figure:

High fat diet alters the cDC subsets. Development of Pre-DC1 to cDC1 during the high fat diet is controlled by Granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF suppresses suppressing the transcription expression factor Interferon regulatory factor 8 (IRF8) that is crucial for cDC1 development and survival.

Summary

During obesity-associated inflammation, myeloid cells play an essential role in initiating and maintaining inflammation during diet-induced obesity. In recent years, more evidence has linked conventional dendritic cell (cDC) to the state of inflammation in obesity. Identification of cDC subsets in the context of obesity has been challenging due to ambiguous marker expression. This study's objective was to understand how $XCR1^+$ cDC1 develops under diet-induced obesity (DIO). To understand the development of cDC1, I analyzed the progenitors of cDCs in the bone marrow and blood to understand the potential mechanisms of how DIO influence DC progenitors. I analyzed the transcription factors controlling DC progenitors to understand how DIO can impact their cell fate. I developed a new gating strategy to define cDC subsets in the ADT based on $XCR1^+$ (cDC1) and $CD172a^+$ (cDC2) to compare our findings with previous studies that, in contrast, defined cDCs in the ADT as $CD103^+$ (cDC1) and $CD11b^+$ (cDC2). This work reveals how DIO modulates cDC1 development and maturation, showing that cDC development is sensitive to nutritional changes. Also, the results present in my thesis work imply that disease complications during obesity like lung infection and tumor may be due to defect in cDC1 development. Finally, cDC1 can play a role in the control of inflammation in adipose tissue during obesity.

Introduction

1. Dendritic cells.

1.1 Definition and functions of DCs.

Dendritic cells (DCs) are proficient antigen-presenting cells (APCs) derived from bone marrow precursors and can be found in most tissues across the body[1-5]. They patrol the tissue's environment to distinguish infected cells or infiltrate pathological tissues to take up antigens and activate effector cells. DCs are essential for controlling infectious diseases and reducing tumor clearance by initiating cellular and humoral immune responses. DCs take up antigens using surface receptors like C-type lectin receptors, endocytosis receptors, and phagocytosis receptors[6]. Additionally, DCs express a range of extracellular and intracellular pattern recognition receptors for recognizing different PAMPs or DAMPs[2, 7-9]. When DCs uptake antigens, they get activated, upregulate costimulatory molecules, and produce a range of cytokines and chemokines. In the final stage, they migrate to lymph nodes where they present antigens to $CD8^+$ and $CD4^+$ T cells and subsequently activate those for different immune responses, which play an essential role in linking the innate and adaptive immune systems by inducing tolerance or inflammation through T cells priming[3, 7, 10]. During homeostasis, dendritic cells maintain peripheral tolerance due to their capacity to process self-antigens and signal tolerance to the T cells pool. The malfunction of DCs leads to diseases such as autoimmunity, allergies, and cancer[3, 11-15].

1.2 DC classification and subsets.

DCs can be classified according to their surface markers and their subset specific functions as classical/conventional dendritic cells (cDCs) that can be further subcategorized into a cDC1 ($CD8\alpha^+$,$CD103^+$, |$XCR1^+$, $CD24^+$, $Clec9^+$), a cDC2 ($CD11b^+$

,CD172α^{+}, SIRPα^{+}), and a plasmacytoid DC (pDC) (CD11c^{lo}CD11b^{-}B220^{+}Gr-1^{+}Siglec-H^{+}) lineage [2, 3, 13, 16-18].

Within cDCs, cDC1 are found at lower numbers compared to cDC2 across various organs. In humans and mice, cDC1 can be identified by the expression of X-C Motif Chemokine Receptor 1 (XCR1). In humans, cDC1 express CD141^{+} (BDCA3) CD11b$^{-/low}$), while in mice, they express CD8α^{+} in lymphoid organs or CD103 in mucosal tissues. However, cDC1s in mice and humans show many common signatures, markers, and functions; human cDC1 express different markers and secrete various cytokines compared to mice[2, 19-21]. Besides, cDC1 can be identified by other markers, including the C-type lectin Clec9A (also named DNGR1), CD36, CD24, and Nectin-like Protein 2 (Necl2, also called CADM1).

cDC1 can modulate many immune system functions during homeostasis by maintaining central and peripheral tolerance by deleting autoreactive CD8^{+} T cells[2, 6, 13, 16, 22]. Upon stimulation by antigen uptake, cDC1 start type 1 immune response by identifying intracellular pathogens and inducing a range of immune cells including ILC1, NK cells, and T helper cell 1, or mediating cross-presentation of extracellular antigen in response to a tumor or viral infection to CD8^{+} T cells to induce a T cell cytotoxic response[6, 22]. cDC1 are potent cytokine-secreting cells; they can produce a high level of IL-12 upon infection to stimulate cells like NK, NKT and help the phenotypic shift of naïve CD4^{+} T cells toward a Th1 phenotype[2, 16, 19, 23, 24]. cDC1 infiltrate tumors and take up antigens released by dying tumor cells and, when signaled by type I IFN, can induce strong and specific T cell responses [25]. . cDC1 elicit an antitumor immune response through the expression of Clec9a[26]. It mediates the cross-presentation of tumor-associated antigens with activating antigen-specific CD8^{+} T cell responses in vivo[26-28]. Studies showed that mice lacking cDC1 globally or locally in the tumor microenvironment could not mount appropriate antitumor responses[23, 29, 30]. It was also reported that XCR1, a chemokine receptor specifically expressed by cDC1 using XCR1-XCL1/2 axis, provide a potential immune-modulating system for interacting between XCL1/2 expressing cells (CD8^{+} T cell and NK cells) and cDC1 which in mice and humans was found to be critical for antitumor response[10, 20, 31, 32]. During viral

infection, cDC1 induce protective cytotoxic T lymphocytes (CTL) responses against various viral infections by developing tissue-resident memory T cells and inducing $CD8^+$ T cells' retention in the lymph nodes[20, 33-35]. Hence, overall, cDC1 play several critical roles in increasing innate and adaptive immune responses to different pathogens across tissues.

cDC2 represent the majority of cDCs in lymphoid organs and peripheral organs and can be identified by the expression of CD172α (SIRPα) and CD11b in mice, while in humans, they express CD1c*,* BDCA1, CD11b [2, 5, 13, 17, 36, 37]. cDC2 are more heterogeneous than cDC1, and they were found to express different markers according to their location. In lymphoid organs, cDC2 represent approximately 80% of spleen DCs and are defined as $CD11c^{hi}$ $MHCII^+$ $CD8^-$ $CD11b^+$ $CD172\alpha^+$. However, this cDC2 subset is heterogeneous, including two populations that can be separated using the markers C-type lectin domain family 12 member A (Clec12A) and Endothelial cell-selective adhesion molecule (Esam). The majority of these cells are $CD4^+$ $Clec12A^{-/lo}$ $Esam^{hi}$, and the minor population is defined as $Clec12A^+$ $Esam^{lo}$ [2, 16, 36]. It seems that the $Esam^{hi}$ cDC2 population is derived from CDP[6, 22]. In tissues, cDC2 populations constantly express CD11b, so they can be both $CD103^+$ or $CD103^-$, and in the lungs and intestines, they may also express CD24[2, 6, 38].

cDC2 are efficient at antigen representation for the activation and expansion of $CD4^+$ T cells and promoting a varied range of T cells such as the activation of Th2 cells against parasites and during asthma and the induction of T-helper type 17 (Th17) immune responses to extracellular bacteria[5, 36, 39-42]. cDC2 can produce the cytokine IL-23, a member of the IL-12 family, and enhance the expansion of Th17 cells[15, 18, 43]. In humans, they are the primary sources of IL-12 and IL-10[12, 16]. cDC2 can respond to various danger signals ranging from nucleotides to polysaccharides by expressing a range of TLRs. They also express high levels of nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing (NLRPs) and other inflammation-associated signaling molecules compared to the other DCs subsets[14, 32, 44, 45]. Different cDC2 subsets have different functions in many sites. In the gut and

thymus, they are also known to strongly induce Th-17 response along with regulatory T cell induction[15, 46].

pDCs are a unique and rare type of DCs subset, and, at steady state, they lack the characteristic DC morphology[5, 16, 36, 39]. In mice, they are identified by expressing B220, SiglecH, and BST2[2, 5, 39], while in humans, pDCs constitute 0.1%–0.5% of human peripheral blood mononuclear cells (PBMCs), and they are identified by expressing CD303 (BDCA2), CD304 (BDCA4), and CD45RA[47].

pDCs are poor stimulators of T helper (Th) cells, but upon activation with methylated DNA or viral infection, they produce enormous amounts of type I IFN to control viral infection[13, 47, 48]. The importance of pDC in fighting virus infection was shown by depleting pDCs that decreased the ability to clear virus infection and demonstrated the significance of IFN secreted by pDCs for antiviral immunity[47, 49]. pDCs are able to respond to a large number of RNA and DNA viruses, including vesicular stomatitis virus G (VSV G), hepatitis C virus (HCV), hepatitis A virus HAV, lymphocytic choriomeningitis mammarenavirus (LCMV), Epstein-Barr virus (EBV), human immunodeficiency viruses (HIV), and murine cytomegalovirus (MCMV)[49-51]. In mucosal immunity and colitis models, pDC play a critical role through regulatory T cell generation and induction to protect against viral infections[52, 53].

Depending on their location, DCs can be divided into lymphoid-resident (resident DCs) and tissue-derived (migratory DCs) populations, which migrate from the peripheral tissues to the lymph nodes. Lymphoid-resident DCs enter the lymph nodes (LN) from the blood and receive antigens through lymphatic drainage or transfer from other cells to present the antigens and control T cell activation. While migratory DCs are located within tissues during activation, they take up antigens and migrate to LN to directly stimulate T cells or transfer antigens to resident DCs and enhance T cell activation indirectly [15–18]. In tissues, immature DCs maturate upon contact with the antigen, which leads to the upregulation of MHC class I, MHC class II, costimulatory molecules CD80, CD86, and migratory c-c chemokine receptor 7 (CCR7) as well as to an increased production of cytokines [1, 8, 9].

1.3 Development of DCs from hematopoietic stem cells.

DCs develop in the bone marrow (BM) from hematopoietic stem cells; they are produced in a continuous process according to the necessity for replacing mature DCs in lymphoid and peripheral tissues or due to increased demand caused by inflammatory conditions [5, 39, 54]. The development of the DCs in the BM occurs in a stepwise mode, starting from the long-term hematopoietic stem cells. Long-term hematopoietic stem cells (HSCs) differentiate into short-term HSCs and multipotent progenitors. These multipotent progenitors can differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CMPs can further develop into granulocyte-macrophage progenitors (GMPs) and into macrophage and DC precursors (MDPs) [5, 55]. At the MDP stage, these cells have the potential to produce common monocyte progenitors (cMoP) or common DC precursors (CDPs). CDPs generate Pre-DCs that later develop into cDCs or pDCs [56, 57]. pDCs were found to leave the bone marrow upon completing development, in contrast to cDCs, which leave the bone marrow as precursors (Pre-cDCs)[5, 39, 57, 58]. In mice, several Pre-DCs subsets have been identified using Siglec-H and Ly6C expression. pre-DCs subpopulations are committed to the cDC1 lineages, defined as SiglecH^{+}Ly6C^{-} (called Pre-DC1) or cDC2 lineages, defined as SiglecH^{-} Ly6C^{+} (called Pre-DC2)[59]. In BM, DCs progenitors first committed to the cDC1 or cDC2 lineages then distributed to lymphoid and non-lymphoid tissues. According to the specific microenvironment of the tissues , the Pre-DCs will differentiate further into cDC1s and cDC2s[5, 19, 39, 59, 60].

1.4 Transcription factors control the development and differentiation of DCs.

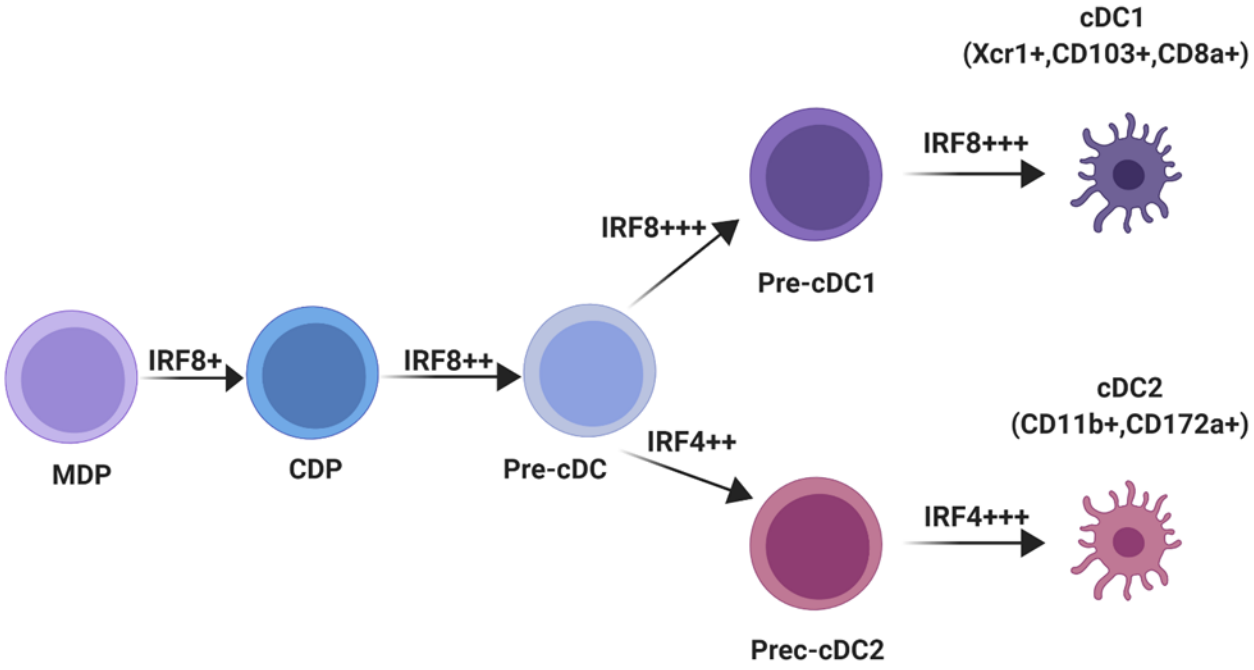
Transcription factors (TF) are proteins involved in the process of DNA transcription into RNA by binding to regulatory sequences to stimulate or suppress transcription of the target gene[61]. The development and terminal differentiation of DCs subsets are

controlled by specific transcription factors (TF), including PU.1, IRF8, E2-2, ID2, IRF4, Batf3, and other TFs. Still, numerous studies have indicated the crucial role of interferon regulatory factor-4 (IRF4) and interferon regulatory factor-8 (IRF8) in the development of cDCs[37, 62] (**Graphic illustration 1)**.

cDC1 development is mainly depending on IRF8 expression, while no defect was observed for cDC2. In mice, complete IRF8 deletion leads to a deficiency in DCs development, while one copy of IRF8 ($Irf8^{+/-}$) affects cDC1 development [15, 59, 64]. The deletion of IRF8 in the Pre-DC stage didn't affect the equilibrium between Pre-DC1s and Pre-DC2s commitment. Still, it was found to induce Pre-DCs to gain a Pre-DC2 phenotype leading to the acceleration of cDC2 development and increased cDC2 population [5, 38, 42, 58, 64]. IRF8 is essential for differentiation, function, and survival of cDC1 in humans and mice [15, 24, 59, 62, 64, 67]. IRF8 is also essential for CDPs initial commitment toward the Pre-DCs lineage and early myeloid development stages. HSCs express low amounts of IRF8, which increases stepwise at the MDP stage, get upregulated in CDPs, and then are maintained in cDC1s [64].

cDC2 subsets are mainly dependent upon IRF4 and transcriptional regulation mediated by Zeb2 [43, 59] in contrast to the IRF8, BATF3, and ID2 control development of cDC1 [5, 16, 24, 59, 63-65]. In the terminal stage of cDCs lineages, uncommitted pre-DCs separate according to the TF modulations into IRF8/BATF3-dependent Pre-DC1 or IRF4-dependent Pre-DC2 and E2-2/IRF8-dependent pre-pDCs [37, 55, 59, 66]. IRF4 is mainly expressed in cDC2 but not cDC1 and was not essential in cDC2 differentiation from preDC2 or CDP commitment toward pre-DCs but was required to control cDC2 survival and migration end stages[16, 43, 46, 62, 65]. Also, IRF4 was found to suppress the production of IL-12 in cDCs by suppressing cDC1 production and development[67].

In contrast, all of the evidence shows that the level and the activation timing of TFs are essential in controlling the development of different DC subpopulations and their maintenance and functions; any disparity in the TF would lead to a shift in progenitors' development toward different subsets.



Graphic illustration 1: Transcription factors control DCs development and terminal selection.

IRF8 and IRF4 are the major transcription factors known to be involved in DC lineage commitment. Development occurs starting from MPD depending on IRF8 expression in hematopoietic progenitor. Progressive gaining or Loss or decrease of IRF8 or IRF4, redirect commitment to different DCs subsets.

1.5 Cytokines control CDC development.

The development and differentiation of cDCs are dependent on different cytokines. Two essential cytokines were found to be vital for DC development; these are FMS-like tyrosine kinase 3 ligand (Flt3L) and granulocyte-macrophage colony-stimulating factor (GM-CSF), both of which are vital for the development of DC progenitors in different stages[2, 56, 68-70]. In the earliest bone marrow progenitor population, Flt3 and GM-CSF receptors expression are relatively low. Still, at late-stage progenitors, expression increased slightly for both receptors on MDP and even more on CDP[71]. The experiments in vitro found that cDCs' differentiation from hematopoietic progenitor cells depends mainly on Flt3L and GM-CSF[72, 73]. Differentiation of progenitors using Flt3L only produce all DC subsets cDC1, cDC2, and pDC. Simultaneously, GM-CSF-only cultures lack pDC, and most cells produced were found to be cDC2 with very low production of cDC1[69-71, 73].

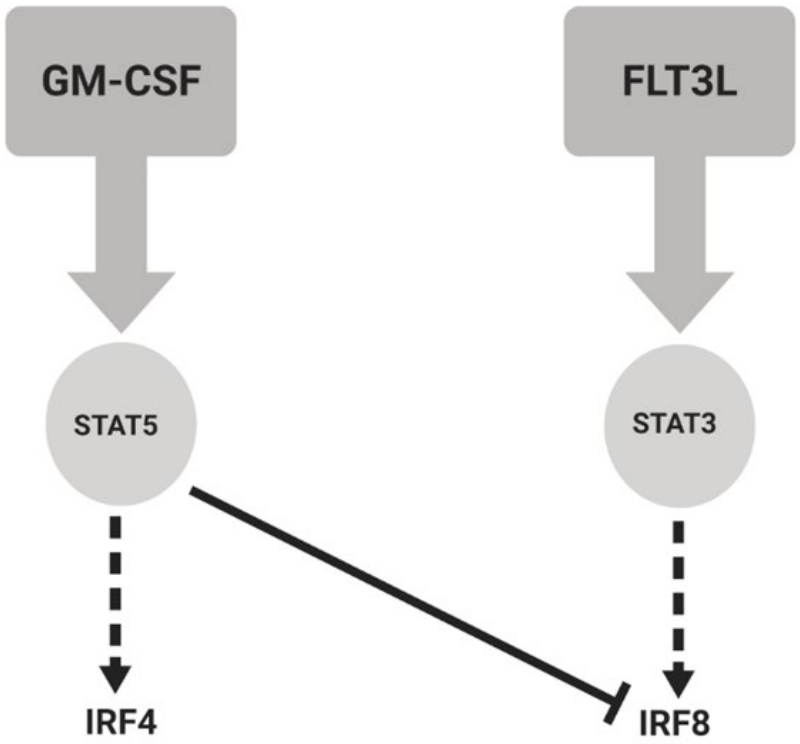
Flt3L is produced by many cells like stromal cells, endothelial cells, and some T cell subsets[43, 54, 69, 74]. The Flt3 receptor (CD135), which is expressed on BM precursors, gives rise to DCs; only precursors expressing Flt3 can develop into DCs. In vivo, the increase of Flt3L led to the expansion of DCs, and cDC1 were more affected by changes in Flt3L levels than cDC2 and pDC [71-73, 75, 76].

GM-CSF is not essential for DCs development at steady-state, as mice lacking the GM-CSF receptor (GM-CSFR) have only slightly reduced numbers of DCs[77]. In contrast, mice need Flt3L to produce most DCs progenitors and steady-state DCs. During steady-state, GM-CSF is found in a very low concentration. Upon inflammation, a high level of GM-CSF is produced by many immune cells and non-immune cells to induce the recruitment of more myeloid cells such as dendritic cells, macrophages, and neutrophils into tissues[68, 70, 71, 77, 78]. In obesity, a high level of GM-CSF in tissues and circulation was detected in both humans and mice[79]. An increase in GM-CSF during diet-induced obesity (DIO) induces the recruitment of more myeloid cells like dendritic cells, macrophages, and neutrophils into tissues. Weight reduction in humans and mice

was found to be sufficient to lower GM-CSF levels and decrease the recruitment of inflammatory immune cells.

1.6 STATs regulate TFs during DC development through cytokine signaling.

The signal transducer and activator of transcription (STAT) protein belong to intracellular TFs and control the development and function of the immune system[80-82]. DC development under STATs is controlled by the level and type of cytokines[83]. STAT3 and STAT5 were found to play a central role in regulating IRF8 and IRF4 activation through cytokine signaling during the development of DCs. GM-CSF induces activation of both STAT3 and STAT5, but it was found that STAT5 and not STAT3 had a crucial function in the induction of DC development driven by GM-CSF[36, 68, 73, 83, 84]. In DC progenitors, GM-CSF signaling mediates downstream STAT5 activation, which blocks IRF8 and induces IRF4, which triggers shifts toward cDC2 with only a low proportion of cDC1 production. In contrast, Flt3L stimulates the expression of PU.1 and STAT3m, which were found to stimulate IRF8 expression directly and thereby IRF8-dependent DCs development, including cDC1 and pDCs[82, 83, 85] (**Graphic illustration 2)**.



Graphic illustration 2: Cytokine-STAT control transcriptional regulators for DC development.

The influence of GM-CSF and Flt3L on STATs signaling that regulates DC TF expression and developmental responses.

2.Obesity

2.1 Obesity complications and development.

The incidence of obesity continues to rise worldwide at an alarming rate[86]. Obesity, defined as a body mass index (BMI) ≥ 30 kg/m2, is characterized by fat depot accumulation in adipose tissue[87]. This accumulation prompts the induction of low-grade chronic inflammation across the body called metainflammation, which causes diseases like diabetes, autoimmune diseases, infections, and many others[86, 88, 89]. By 2025, it

is expected that more than 300 million people will have type 2 diabetes (T2D) due to obesity, which makes obesity the disease of the century[86, 88, 89].

2.2 The functions and composition of adipose tissue.

Adipose tissue (ADT) consists of different types of cells, such as adipocytes, preadipocytes, endothelial cells, immune cells, and fibroblasts. Adipose tissue has various functions, but it mainly regulates systemic metabolism by controlling how lipids are stored and energy utilized in the body[88, 90, 91]. Lipids are stored in adipocytes as triglycerides and released as glycerol. Fatty acids are transported in the blood to organs and utilized as a source of energy to modulate whole-body energy balance[92, 93].

2.3 Adipose tissue classification.

Adipose tissue is classified into two subtypes: white adipose tissue (WAT) and brown adipose tissue (BAT)[90, 94, 95]. WAT located under the skin is called subcutaneous WAT (SCAT) and represents the highest percentage of WAT in the body. The other type is Visceral WAT (VAT), which surrounds the inner organs and is less innervated and vascularized[90, 91, 94]. In the case of obesity, the main difference between these two types of WAT is that VAT leads to poor insulin sensitivity and other obesity-related diseases. On the other hand, SCAT is protected against this effect due to its inability to adapt and expand during hyperplasia[95-98]. Most of the inflammatory cytokines and adipokines that lead to inflammation during obesity are produced from VAT and result from the expansion of adipocytes [99, 100]. Mice have visceral adipose tissues similar to humans, including the mesenteric, perirenal, pericardial, and retroperitoneal fat depots. However, there is a well-developed epididymal fat pad (eWAT) in mice, which is comparable in terms of location to the large gluteofemoral subcutaneous depot in humans [101, 102].

Brown adipocytes are smaller than white adipocytes and contain multitudes of small (multilocular) lipid droplets that store energy in a smaller space than white fat[103]. They are loaded with iron-rich mitochondria, giving them their brown look, producing energy as heat via high levels of uncoupling protein 1 (UCP1) to reduce hypothermia and obesity through lipid utilization. Brown fat is a type of "healthy" fat[94, 103]. Brown adipocytes are

smaller than white adipocytes and contain multitudes of small (multilocular) lipid droplets that store energy in smaller spaces than white fat[94, 103]. Humans with higher levels of brown fat may have lower body weights[96, 103]. BAT tissue may increase over WAT by different methods like diet changes, exercise, and chemical molecules, making it one of the approaches for treating obesity[103].

Other types of adipose tissue are found in other sites across the body. One important example is bone marrow adipose tissue (BMAT), representing 10% to 70% of the bone marrow's volume. BMAT looks like WAT, but it displays both WAT and BAT phenotypes[104]. Within the bone marrow's environment, BMAT secretes cytokines and adiponectin to modulate hematopoiesis and osteogenesis. In obesity, BMAT expands and secretes many inflammatory cytokines and adiponectin, which act as a source of circulating inflammatory mediators, negatively regulating hematopoiesis and inducing the activity of osteoclasts to reduce bone density[104-106].

2.4 Adipose tissue endocrine functions.

In recent years, a great deal of evidence suggests that storing energy is not the only function of adipose tissue, but it is a multifaceted organ which acts as an essential endocrine and immune regulatory component in maintaining body homeostasis[91, 99, 101, 105]. Adipocyte secreted factors are adipokines, molecules that control several biological processes by autocrine, paracrine, and endocrine pathways. Adipokines released from the adipose tissue play different roles, including appetite control, regulation of body fat storage and energy consumption, glucose tolerance, insulin sensitivity, cell growth, and inflammation. The adipose tissue orchestrates different biological processes in other organs such as energy metabolism, immunological response, and neuroendocrine function using these signaling molecules [93, 96, 98, 99, 101, 104, 107].

Cytokines are another group of signaling molecules that are released from the adipose tissue. Cytokines influence both local and systemic metabolism differently from adipokines[108]. They are secreted directly from adipocytes or other cells within the adipose tissue, like preadipocytes, immune cells, and fibroblasts. Adipose tissue

cytokines play a central role in regulating immune function[109]. In obesity, the alteration of released cytokines from the non-inflammatory type toward the inflammatory. This leads to a corresponding change in immune cells within the adipose tissue to adopt an inflammatory phenotype and later altered the entire immune system [88, 97, 110].Generally, in both normal and dysregulated conditions, adipocytes- and the adipose tissue-released factors can affect local cellular and systemic events through endocrine exchanges[99, 100, 111, 112].

3. Adipose tissue immune cell crosstalk.

The full spectrum of immune cells, including myeloid and lymphoid cells, are found within adipose tissue[113]. In normal conditions, immune cells like Th2, Treg, and eosinophils play essential roles in adipose tissue by removing apoptotic cells, maintaining tissue homeostasis, and supporting anti-inflammatory responses [113, 114]. However, obesity-associated excessive fat accumulation within adipocytes leads to producing hormones and adipokines, including leptin, adiponectin, TNF-α, IL-1β, IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1), which extensively change the density and function of immune cells by inducing activation of accumulating inflammatory immune cells like neutrophils, pro-inflammatory M1 macrophages, dendritic cells, and T cells [88, 96, 107]. Inflammatory immune cells within the ADT secrete mediators that promote the local and systemic pro-inflammatory status and impair insulin signaling within the ADT and other organs across the body [96, 98]. These cytokine effects are mediated by the stimulation of IκB kinase β (IKKβ) and JNK1, which is expressed in immune and insulin-targeted cells. Obesity-induced inflammation initiates in the ADT and then spreads to other tissues, which leads to low-grade systemic inflammation by increasing circulatory inflammation cytokines [96, 98, 113]. That means that crosstalk between adipocytes and immune cells is essential to maintain the tissues' metabolic homeostasis and normal function. The crosstalk between adipocytes and immune cells is possible because of the signaling molecules (cytokines and adipokines) produced by both cells. The expression of effector receptors for these molecules facilitates such communication.

3.1 Adipokines secreted by adipocytes modulate immune cells.

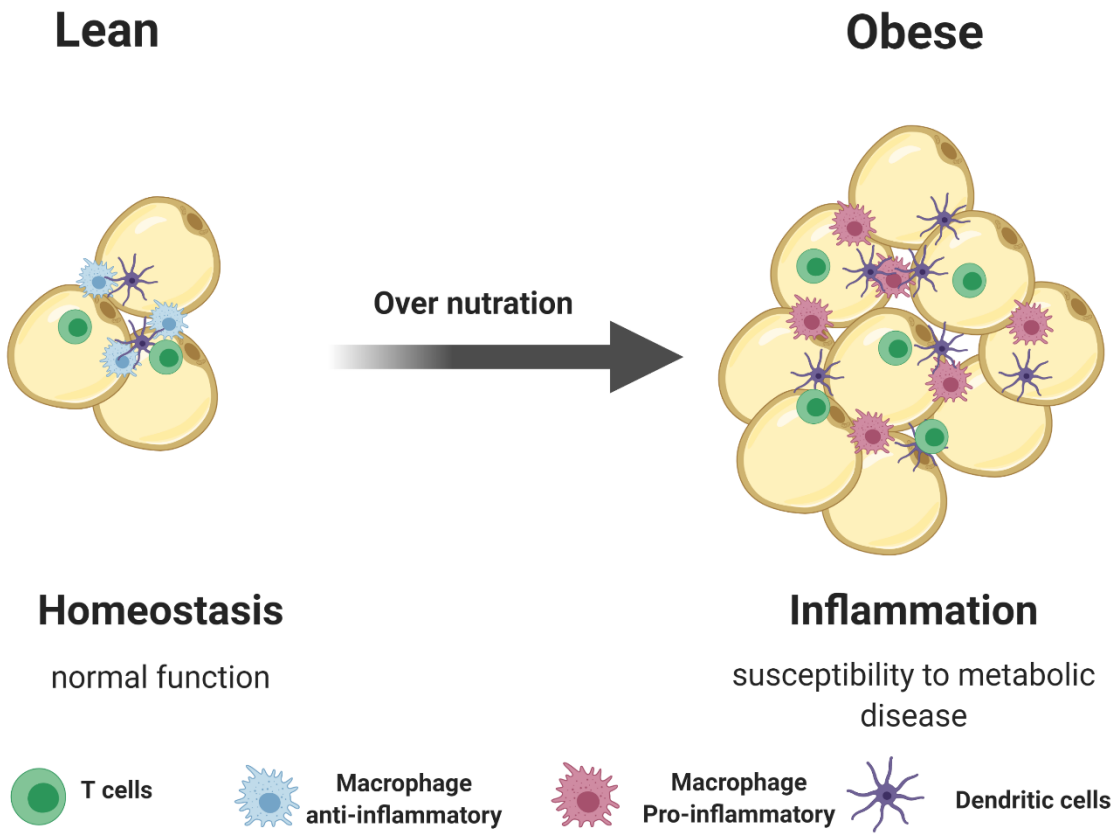
Adipokines secreted by adipocytes have both anti-inflammatory and inflammatory functions. Anti-inflammatory adipokines like Adipolin/CTRP12, Omentin-1, CTRP9, and adiponectin were found to protect against the development of obesity-related complications by regulating insulin signaling and suppressing inflammatory cytokines in tissues [115]. Inflammatory adipokines such as leptin, visfatin, and resistin were described as indicators that are positively related to body weight, fat mass, promotion of insulin resistance, and pro-inflammatory properties (induction of inflammatory cytokines and class switch of immune cells to inflammatory phenotypes) [93, 96, 98, 107, 112]. Leptin is the first adipokine discovered[116]. Typically, leptin's main function is to control the metabolism by playing a central role in satiety, hormone regulation, food intake, energy, and insulin sensitivity [116]. [93, 111, 116, 117]. Recently, many studies revealed that leptin modulates the immune system[107, 117-119]. It has been shown that decreased leptin production is associated with increased susceptibility to infection, whereas increased leptin secretion induced immune-mediated disorders such as autoimmune diseases[120]. As such, leptin can be considered a mediator of the inflammatory response by stimulating cytokines and factors influencing immune function and development like migration and activation of macrophages, inducing NK cells cytotoxicity, activation of neutrophils, and inducer of T cells proliferation. The leptin receptor has been found in many immune cells (neutrophils, monocytes, and lymphocytes), directly affecting them. This mode of communication was observed with many other adipokines. Overall, this can explain how adipose tissue can, directly and indirectly, communicate with immune cells.

3.2 Immune cells modulate adipose functions via cytokines.

Crosstalk between immune cells and adipose tissue was observed in the opposite direction. In normal conditions within adipose tissue, immune cells release cytokines that are important in adipocytes' development and tissue homeostasis maintenance [99, 100, 121]. In obesity, excessive fat accumulation leads to extensive changes in the amount and function of immune cells within the ADT by increasing the number and activity of

inflammatory cells which abundantly produce inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IFN-γ, while at the same time reducing non-inflammatory cells and anti-inflammatory cytokines like IL-10, IL-15, IL-2, IL-5, and IL-25[99, 100, 108, 111]. Cytokines released from immune cells influence adipocytes' essential functions by affecting glucose levels, insulin sensitivity, adipokines' release, and the development of adipocytes[97, 100]. Cytokines are involved in adipocyte development regulation by modulating adipogenic transcription factors' synthesis and activity[100, 122]. In leanness, anti-inflammatory cytokines such as IL-10 released from Tregs were found to promote insulin signaling in adipocytes via suppressing inflammatory cytokine synthesis[123]. In contrast, in obesity, increased release of inflammatory cytokines, like TNFα, IL6, and IFNγ, interferes with the normal function of adipocytes by preventing the normal development of preadipocytes to fully differentiated adipose cells; this promotes an inflammatory phenotype of the adipocytes through modulating transcription factors and causes a reduced insulin-stimulated glucose uptake[108, 112, 113] (**Graphic illustration 3)**.

Collectively, this demonstrates bidirectional crosstalk between adipocytes and immune cells. The alteration between types and the secreted amount of signaling molecules produced from both cells is dependent on the state of inflammation. Obesity leads to a complete shift toward the release of inflammatory cytokines, which affects cells locally within adipose tissue and ultimately released in the bloodstream, modulating remote cells in different organs, well away from their site of origin, the adipose tissue.



Graphic illustration 3: Obesity induces adipose tissue inflammation.

Lean adipose tissue contains regulatory non-inflammatory immune cells. In contrast, obese adipose tissue is infiltrated with pro-inflammatory immune that produce high amounts of inflammatory cytokines.

4. Myeloid cells during obesity.

Myeloid cells are essential immune cells in ADT hemostasis[124]. Myeloid cells are part of the immune system, including monocytes, dendritic cells, tissue macrophages, and granulocytes. Their primary function is to mediate cellular activation, differentiation, ontogeny, and tissue-specific modulation, including tissue homeostasis regulation, immune surveillance, and inflammation[97, 113]. Myeloid cells play an essential role in innate immunity; upon infection, they are quickly recruited into infected tissues via various chemokine receptors. They are stimulated for phagocytosis and secretion of inflammatory cytokines to induce the recruitment of more inflammatory cells. Alteration in myeloid cell development and functions may cause abnormal disease development [125, 126].

Myeloid cells were among the first leukocytes identified to accumulate in obese adipose tissue and circulation. Many studies showed that the content of ADT myeloid cells was linked to insulin resistance, suggesting a mechanistic contribution of myeloid cells to the development of metabolic disease due to obesity. Diet-induced obesity was found to promote myelopoiesis in hematopoietic stem cells[111, 125].

4.1 Obesity induces myelopoiesis.

In humans and mice, obesity increases circulating neutrophils, classical monocytes ($Ly6c^{hi}$ in mice and $CD14^{+}$ $CD16^{+}$ in humans), and adipose tissue macrophage (ATM, indicating the influence of obesity on myeloid cell regulation[126]. Activation and recruitment of neutrophils and inflammatory macrophages to the adipose tissue correlated with an increased release of inflammatory cytokines and insulin resistance associated with developing metabolic diseases like diabetes[97, 99, 113]. ATMs represent most of the innate immune cells in the adipose tissue, and in the obese adipose tissue, it makes up around 40% of the non-adipocyte fraction. Around ten years ago, two studies showed that obesity is associated with increased infiltration of inflammatory macrophages, which coincides with an increase in the size of the adipose tissue that leads to further recruitment of inflammatory macrophages to the tissue. Increased

inflammatory macrophage infiltration in adipose tissue is considered a hallmark characteristic of adipose tissue inflammation[121, 127-129].

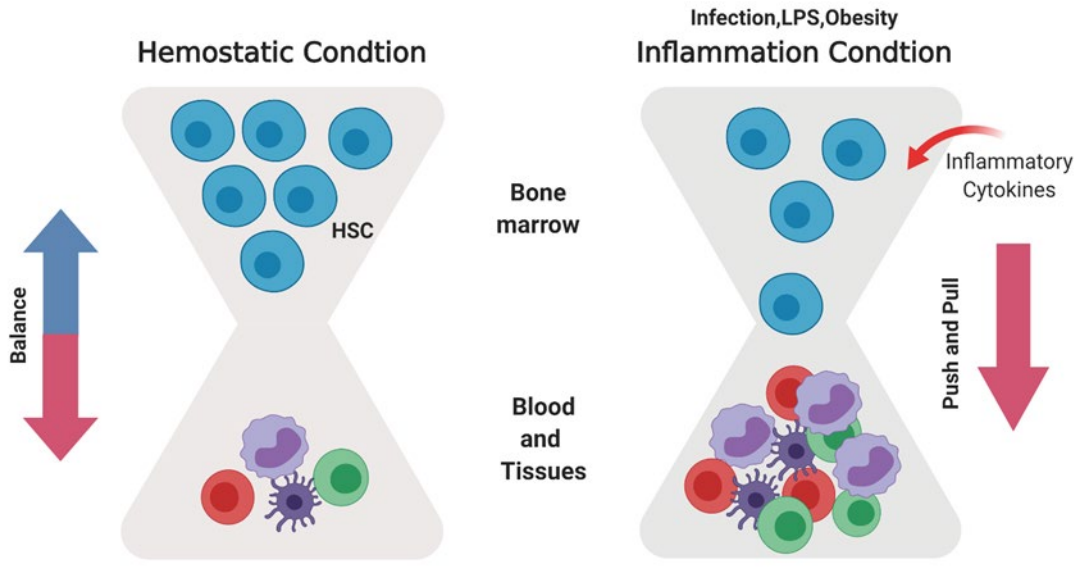
4.2 DCs altered during obesity.

Due to myeloid cells' increased infiltration during obesity, more immune cells are recruited to the ADT from the bone marrow[124, 129]. During peripheral inflammatory responses like inflammation and obesity, hematopoietic stem cells (HSCs) increase myeloid cells' production through direct and indirect pathways to potentiate peripheral immune responses[130, 131] (**Graphic illustration 4)**. In obesity, consistent with that, obese patients show a surge in circulating hematopoietic progenitors, indicating that inflammation in obesity can trigger the activation of HSCs[132] (**Graphic illustration 4)**. In obesity, inflammatory cytokines released from adipose tissue reaches the bone marrow and shifts the differentiation of HSCs toward inflammatory myeloid cells[112, 127, 132]. ADT signals HSCs to proliferate, expand, and increase myeloid cells' production by releasing inflammatory cytokines like IL-1β, GM-CSF, CSF1, or adipokines (leptin)[124, 133]. Macrophages represent the largest immune cell population in the adipose tissue. Other myeloid cell populations also respond to changes in obesity-associated metainflammation and are found to accumulate in ADT upon inflammation, induced due to fat accumulation. DCs (both cDCs and pDCs) are found in the adipose tissue[100, 113, 134, 135]. Most studies on ADT dendritic cells are focused mainly on conventional DCs (cDCs) and their role in T cell polarization and define them as $CD11c^{+}$ high cells[125, 136].

Nevertheless, pDCs were also recently found to be recruited to the ADT upon obesity. They play a crucial role in the initiation of metainflammation under a high-fat diet. pDCs are the significant producers of type I IFNs in the body[137]. In obesity, induction of type I IFN was linked to both the adipose tissue and systemic insulin resistance. Under a high-fat diet, accumulation of pDCs in the ADT and liver was found to correlate with an increase in the number of proinflammatory macrophages, and that mice lacking pDCs were protected from inflammation and developing insulin resistance, suggesting that pDCs

recruit adipose-resident macrophages to the ADT and lead to their polarization to the proinflammatory phenotype[113, 137, 138].

cDCs can modulate function and recruitment of other immune cells in different tissues; however, their role in regulating ADT inflammation or homeostasis is dependent on the actual adipose tissue metabolic state[121, 126, 135, 136]. When steady, cDCs were shown to promote an anti-inflammatory phenotype and slow down the onset of obesity-induced inflammation and insulin resistance by producing or inducing anti-inflammatory cytokines. Moreover, cDCs gain a tolerogenic phenotype by upregulating pathways involved in adipocyte differentiation[134, 139]. In contrast, diet-induced obesity in mice causes a shift to a proinflammatory phenotype leading to the activation of Th1 and Th17 responses and the activation and recruitment of macrophages[136, 140, 141]. Similar to mice, cDCs were found to correlate positively with body weight in subcutaneous adipose tissue (SAT) in humans[126, 142]



Graphic illustration 4: Inflammation regulates hematopoietic stem cells. Adapted from Katherine Y. King et al., 2011

Inflammation promotes the differentiation of hematopoietic stem cells (HSCs) at the expense of self-renewal activity, leading ultimately to depletion of the HSC population.

5. Difficulties in studying cDCs role during obesity.

Despite all the studies discussing the role of DCs in inflammation in obesity, the underlying mechanism of how cDCs contribute to ADT inflammation is still unclear. One other problem in studying ADT-DC is that gating strategies (DCs are identified as $CD11c^{+}$ $F4/80^{-}$ cells) could lead to contamination by NK cells and monocytes. Studies analyzing all adipose tissue DC subsets usually focus on cDC2 since cDC2 ($CD11b^{+}$) accounts for 80-90% of total cDCs in ADT; this led to masking a minor subset of cDC1 ($CD103^{+}$)[112, 113, 139, 143]. Even though cDC1 exist in the ADT at an exceptionally low percentage, it is unclear how cDC1 contribute to obesity. Recently, a study found that mice lacking cDC1 ($Batf3^{-/-}$) become obese and diabetic as they age faster than wild-type mice, which shows the protective role of cDC1 in obesity[144]. It also showed that diet-induced obesity promotes myelopoiesis in hematopoietic stem cells, but no study reveals the effect on direct cDCs progenitors or its onset.

Method and Material

Methods

Mice

Starting at the age of 8 weeks male C57BL/6J mice were fed nutrient-matched diets with 13 kcal% fat (D12450B, ssniff-Spezialdiäten GmbH, Germany, contain energy 17.8[MJ/kg]) as control diet (CD) or 60 kcal% fat (D12492, ssniff-Spezialdiäten GmbH, Germany, contain energy 24[MJ/kg]) as high fat diet (HFD). Each cage housed five animals. Food was replaced and twice a week, animals were weighed once a week . Mice were bred in the house (LIMES Institute, Bonn, Germany). Mice were housed under specific pathogen-free/SPF conditions.

Preparation of cell suspensions.

Spleen and adipose tissue were firstly cut down with scissor into small pieces; then they were digested for 45 minutes in PBS (Phosphate Buffered Saline) (1X, pH 7.4) containing 10% BSA and collagenase type IV (0.2 mg/ml; working activity of 770 U/mg) (Sigma) at 37°C degrees. Bone marrow was flushed from the femur and tibia of back limbs using flow cytometry buffer without any digestion. The cell suspension was then filtered through a 70-μm cell strainer and mashed with a syringe's plunger into Falcon tubes. RBCs were lysed with ammonium-chloride-potassium buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM 0.5 M EDTA), and cells were washed, pelleted, and used for flow cytometry.

Flow cytometry

All antibodies were used at pre-titrated, optimal concentrations antibodies table 1. For surface staining of live cells, a buffer containing 5% BSA in 1x PBS was used for antibody staining. FACS measured using Symphony or LSR II flow cytometer (Becton Dickinson, USA). For transcription factor intranuclear staining, cells were permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, UK) according to the manufacturer's protocol, and stained with monoclonal antibodies targeting IRF4, IRF8

(Thermofisher, US) combined with surface antibodies and live and death staining Fixable Viability Dyes (FVD) (eBiosciences, UK). For cytokines staining (GM-CSF: Rat monoclonal, Thermofisher , US) using intracellular staining Set (eBiosciences, UK) and following manufacture protocol. For phosphorylated STAT (Phospho-STAT5: mouse monoclonal, eBiosciences, UK) staining, cells were permeabilized using Phosflow™ Perm Buffer IV (Becton Dickinson, USA) and stained by following manufacturer protocol. Detection of Apoptosis Using the BD Annexin V kit following manufacturer protocol (BioLegend Way, US) combined with surface staining.

ELISA

For detection of GM-CSF in bone marrow supernatant, bone marrow from one leg was flushed with 150 ul of PBS, then centrifuged for 10min at 1000rpm 4° C, and the supernatant was collected. Mouse GM-CSF DuoSet ELISA kit (R&D Systems, US) used as manufacture protocol with half area plate and measured using HTS -high throughput sampler- plate reader.

Histology and tissue preparation

Samples from bone marrow and adipose tissue were collected and fixed using 10% Neutral buffered formalin (NBF) overnight, then processed to Standard Protocol for Formalin-Fixed Paraffin-Embedded Tissue. For Bone marrow, an additional step was added. After fixing overnight bone, it was decalcified overnight using Immunocal™ Decalcifier (statlab, US) and washed for 15min with tap water. Finally, bones are processed to paraffin embedding step.

H&E staining and histological scoring

Five-micrometer sections from epididymal adipose tissue (eWAT) were stained with hematoxylin eosin staining protocol described before [145]. Afterward, slides were imaged using Zeiss Axio microscope. (Zeiss, Germany). Then adiposoft plugin from (ImageJ, image processing program)[146], as described before[147], was used to quantify the size and numbers of adipocytes in eWAT. Three samples from each diet

group (8 weeks of diet) were used as a representative to quantify the size and numbers of adipocytes.

Immunohistochemical staining.

To stain cDC1 in BM, I used CLEC9a antibody (Polyclonal Sheep, R&D Systems, US). The first paraffin Five-micrometer sections of BM were deparaffinized using xylol and a series of ethanol dilutions. Next, sections were blocked using 10% sheep serum for 30 minutes. Sections were then incubated with the primary antibody at 4°C overnight. The next day, sections were washed with 1x PBS and incubated with secondary antibody Sheep IgG HRP-conjugated Antibody (R&D Systems, US) for 45 minutes at RT. Slides were washed, and the signal was developed using the HIGHDEF® brown IHC chromogen (Enzo Life Science, US). Finally, slides were mounted and covered for imagining using the Zeiss Axio microscope. (Zeiss, Germany).

In situ RNA hybridization (RNAscope)

ISH was performed using an RNAscope 2.5 HD Assay-RED Kit (Advanced Cell Diagnostics) following the manufacturer's protocol with minor changes. In brief, paraffin-embedded formalin-fixed tissue from bone marrow was cut into Five-micrometer sections. Then, sections were deparaffinized, and endogenous peroxidase was blocked using H2O2 from the kit, followed by RNAscope Target Retrieval at 95°C for 5min using a microwave. Finally, slides were incubated with RNAscope Protease Plus for 5 min at 40°C. A custom designed XCR1 probe (gene ID: 329244) was hybridized to the tissues for 2h T 40ºC. The signal was amplified using the RNAscope HD 2.5 detection Kit according to the manufacturer's protocol. Signal was detected using a mixture of Fast-RED solutions. After slides were counterstained with Gill's II hematoxylin (Sigma), slides were dried in a 60 °C dry oven for 15; slides were mounted using VECTASHIELD® HardFSet (Vector lab., US), then imaged using the Zeiss Axio microscope. (Zeiss, Germany).

Statistics

The data were analyzed with GraphPad Prism software (version 8) and are presented as dot plots in which each experimental group's mean is presented in addition to the individual samples. Statistical significance was calculated using Students unpaired t-test; the mean ± SEM is shown. The *p* values are indicated as follows: *P0.05, **P0.01, and ***P0.005.

Materials

Table1. Antibody list

Marker	Clone	Company
CD19	6D5	BioLegend
CD3	17A2	BioLegend
CD45R/B220	RA3-6B2	BioLegend
TER-119	TER-119	BioLegend
NK-1.1	PK136	BioLegend
Ly-6G	1A8	BioLegend
CD45	30-F11	BioLegend
Ly-6G/Ly-6C (Gr-1)	RB6-8C5	BioLegend
CD11b	M1/70	BioLegend
CD64 (FcγRI)	X54-5/7.1	BioLegend
MERTK (Mer)	2B10C42	BioLegend
MHC-ClassII I-A/I-E	M5/114.15.2	BioLegend
XCR1	ZET	BioLegend
CD11c	N418	BioLegend
CD24	M1/69	BioLegend
CD172a (SIRP alpha)	P84	Thermofisher
CD115 (CSF-1R)	AFS98	BioLegend
Siglec H	551	BioLegend
Ly-6C	HK1.4	BioLegend
CD117 (c-Kit)	2B8	BioLegend
CD135	A2F10	BioLegend
IRF8	V3GYWCH	Thermofisher

IRF4	3E4	Thermofisher
GM-CSF	MP1-22E9	Thermofisher
Phospho-STAT5 (Tyr694)	SRBCZX	Thermofisher

Table2. Material list

name	Catalog	Company
High fat diet: 60% of energy from fat)	D12450B, E15742	ssniff-Spezialdiäten GmbH
Control diet: 13% of energy from fat),	D12492, E15748-04	ssniff-Spezialdiäten GmbH
ROTI ® Cell 10x PBS	9150.1	Carl Roth GmbH + Co. KG
Collagenase Type 4	C5138	Sigma-Aldrich
Deoxyribonuclease I	DN25-100MG	Sigma-Aldrich
Albumin Bovine Fraction V, pH 7.0	11930.04	SERVA Electrophoresis GmbH
NH4Cl	A9434	Sigma-Aldrich
(Ethylenedinitrilo)tetraacetic acid, EDTA	T9285	Sigma-Aldrich

KHCO3	237205	Sigma-Aldrich
Fixation/Permeabilization Diluent	00-5223-56	eBioscience™
Fixation/Permeabilization Concentrate	00-5123-43	eBioscience™
Permeabilization Buffer	00-8333-56	eBioscience™
Perm Buffer IV 10×	560746	Becton Dickinson
BD Phosflow™ Lyse/Fix Buffer	558049	Becton Dickinson
Fixable Viability Dye	65-0865-14	eBioscience™
Mouse GM-CSF DuoSet ELISA	DY415-05	R&D Systems
Transparent half-width 96-well microtiter plates	734-1624	VWR International
TWEEN® 20	P9416	Sigma-Aldrich
H2SO4	057559	Sigma-Aldrich
10% Neutral buffered formalin (NBF)	HT501128-4L	Sigma-Aldrich
Ethanol	H225-H319	Carl Roth GmbH + Co. KG
Low Melting Paraffin	CN49.2	Carl Roth GmbH + Co. KG
Immunocal™ Decalcifier	1414-32	statlab
xylol	534056-4L	Sigma-Aldrich
VectaMount	vec-h-5000	vectorlab

Animal-Free Blocker® and Diluent	SP-5035	vectorlab
BLOXALL™ Blocking Solution	SP-6000-100	vectorlab
Hematoxylin Solution, Gill No. 1	GHS1128-4L	Sigma-Aldrich
Eosin Y-solution 0.5% aqueous	1.09844	
RNAscope® 2.5 HD Detection Reagents-RED assay	322350	Bio-Techne
VECTASHIELD® HardFSet	H-1400-10	vectorlab

Results

1.High fat diet induces weight gain and increases adipocyte number and size in mice.

High fat diet (HFD) is usually used to study diet induced obesity (DIO). HFD consumption leads to consistent induction in mice body weight, adipocytes hyperplasia (cell number increase), hypertrophy (cell size increase), and increased infiltration of inflammatory immune cells in adipose tissue [148, 149]. To study DIO, I fed mice with either HFD as 60 kcal% from fat or nutrient-matched low-fat control diet (CD) as 14 kcal% from fat starting at 7-8 weeks of age. Following 1–15 weeks of exposure to diet, obese mice (HFD) had around 45% increase in body weight (fig1, A), which is consistent with previous findings [150]. Next, I found that HFD induced adipocytes hypertrophy compare to CD due to increased fat accumulation in the adipocytes within epididymal adipose tissue (eWAT) [151] (fig1, B). Furthermore, in addition to adipocytes hypertrophy, I observed increased accumulation of macrophages in adipose tissue under HFD surrounding adipocytes in the so-called “crown-like structure,” which is a hallmark of increased infiltration of inflammatory macrophages during obesity [127, 128] (fig1, D). It is known that increased fat consumption leads to an augmentation of fat depots in peripheral organs, such as the liver, bone marrow, and fat depots [151]. Indeed, by analyzing histology sections from bone, I observed that HFD induced adipocyte hyperplasia in bone marrow as well (fig1, C). These results collectively show that our HFD-induced DIO induces expansion of adipose tissue and increases immune cell infiltration, in agreement with previous studies.

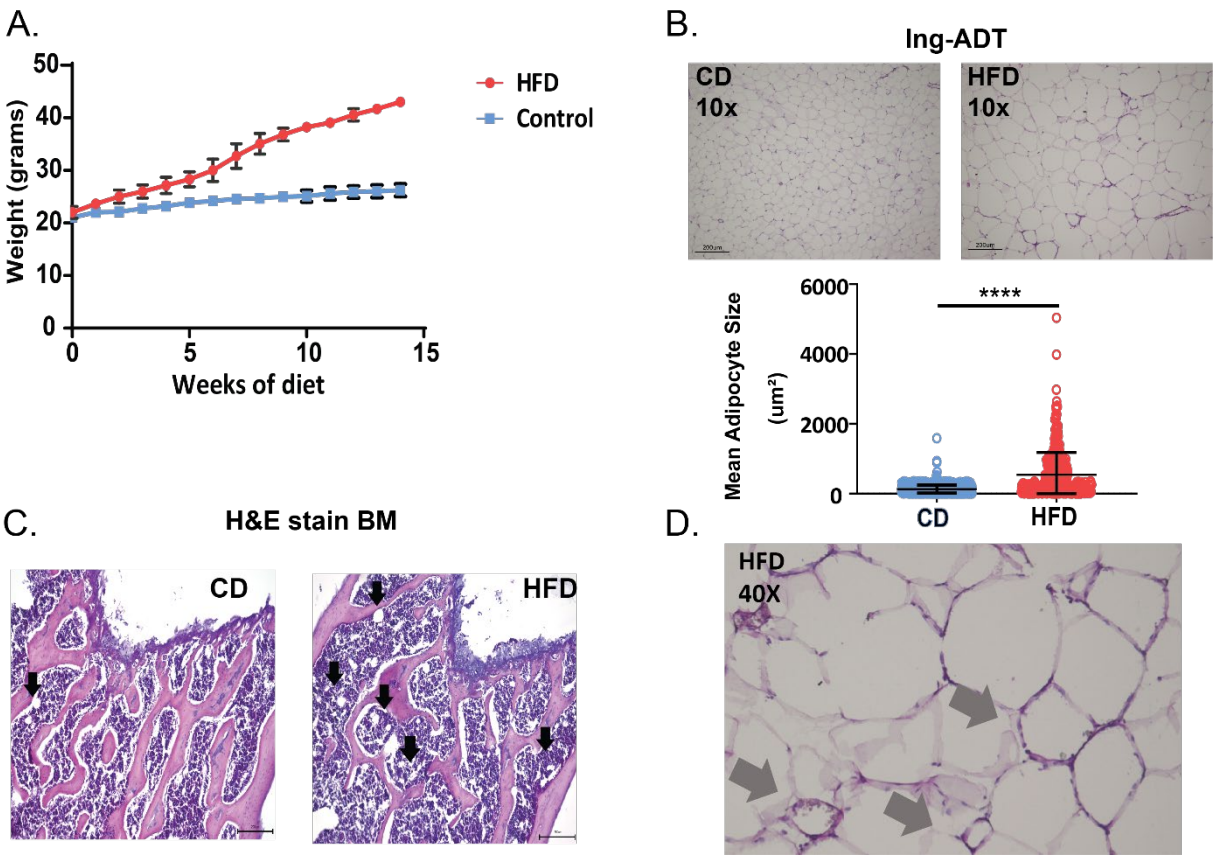


Figure 1. High fat diet increases body weight and adipocytes hyperplasia with hypertrophy.

(A) Bodyweights gain of mice under CD or HFD until 15 weeks starting from 7-8 weeks of age.

(B) Representative images of H&E staining of histological sections from eWAT (epididymal adipose tissue) and quantification of adipocyte size from HFD and CD mice after 8 weeks of diet (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean ± s.e.m. Scale bar, 200 μm .

(C) Representative images of H&E staining of histological sections from bone marrow after 8 weeks of diet from CD and HFD show adipocyte hypertrophy (arrows) in the HFD group. Scale bar, 200 μm .

(D) Representative images of H&E staining of histological sections from HFD mice after 8 weeks of diet show invasion of inflammatory macrophages (arrows) (Crown-like structure).

2. High fat diet alters the myeloid cell compartment in blood, spleen and ADT .

Obesity changes immune cell types and numbers by mainly inducing myelopoiesis[69, 124]. To see if our DIO module, as previously reported, induce myeloid cells production [125].Using flow cytometry, I analyzed different organs including spleen, eWAT, blood, and bone marrow , to define monocytes, neutrophils, dendritic cells, plasma dendritic cells (fig2,3,4.A). First, around 3 weeks of the diet, I observed induction of neutrophils numbers in blood and increased accumulation in ADT. However, neutrophil numbers were decreased in the spleen (fig2,3,4.B). In the late stage of the diet, I still could observe increases in neutrophils numbers in eWAT around 20 weeks and blood around 14 weeks. These results are in line with previous reports showing that obesity induces neutrophils production at the early stage of HFD and increases their ADT accumulation induces tissue inflammation [79, 113, 125]. Next, HFD is known to induce monocytosis [152], which I observed by increased accumulation of monocytes around 20 weeks in eWAT but decreased numbers in the spleen around 8 weeks and no change in 3 or 14 weeks of diet. In blood, I found that these migratory monocytes were mainly ly6C^{-} monocytes (fig2,3,4. B). Collectively, this shows that our HFD induces myelopoiesis and increases myeloid cells recruitment in lymphoid and non-lymphoid organs.

3. High fat diet triggers cDCs reduction in the spleen and adipose tissue.

Previous studies that focused on the role of cDCs during obesity did not separate cDC subsets. In addition, with no clear strategy to sperate cDCs cells from other immune cells populations expressing similar markers like macrophages, NK, or pDCs causing contamination with other cells. all together this will lead to potential misinterpretation of cDCs role during HFD [144, 153]. To avoid this, I used a defined and unified gating

strategy in different organs to determine cDCs using MHCII^{+} and CD11c^{+} in combination with cleaning strategy to remove pDC and Lineage-positive cells (fig2,3.A). Throughout obesity, it was reported that obesity increase DCs numbers [102, 129, 143]. In contrast to previous reports, I found that HFD led to a significant reduction in cDC numbers in the spleen and eWAT that started 3 weeks in the spleen and 20 weeks in eWAT (fig2,3.B). Hence, these results suggested that this alteration may be due to one specific cDC subset.

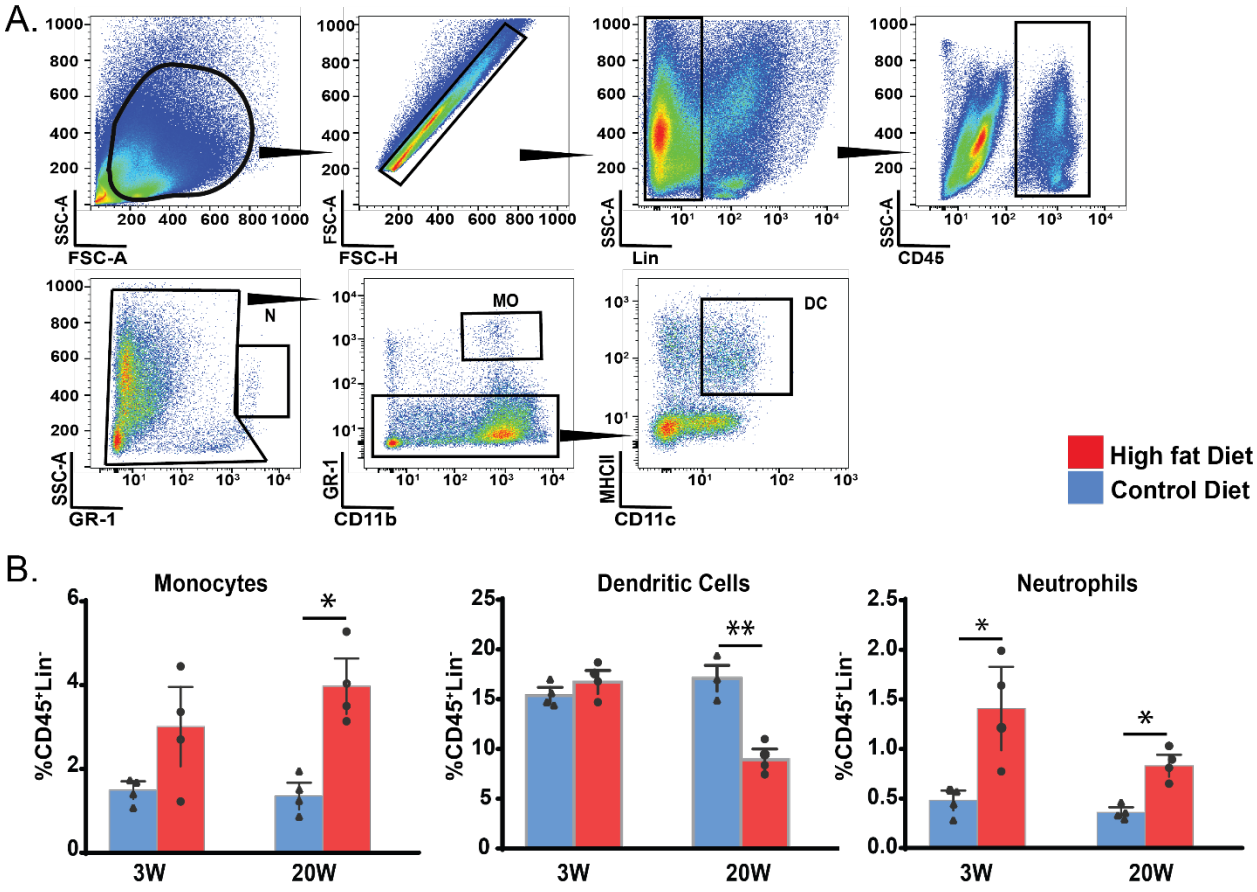


Figure 2. High fat diet alters myeloid cells in epididymal white adipose tissue.

(A) Gating strategy for flow cytometry analysis of Monocytes, Dendritic cells, and Neutrophils as myeloid cells in mouse spleen. All cells were gated as viable Lin (CD3, CD19, NK1.1, Ter119, B220)$^{-}$ and CD45^{+}; within this population, neutrophils were gated GR1^{+} SSC-A ^{high}. monocytes were gated as GR1^{+} CD11b^{+}; dendritic cells were gated as GR1^{-} MHCII^{+} CD11c^{+}.

(B) Population percentages of lin$^-$ and CD45$^+$ from CD and HFD mice after 3 or 20 weeks of diet for Monocytes (left), Dendritic cells (middle) , Neutrophils (right) (n = 4/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

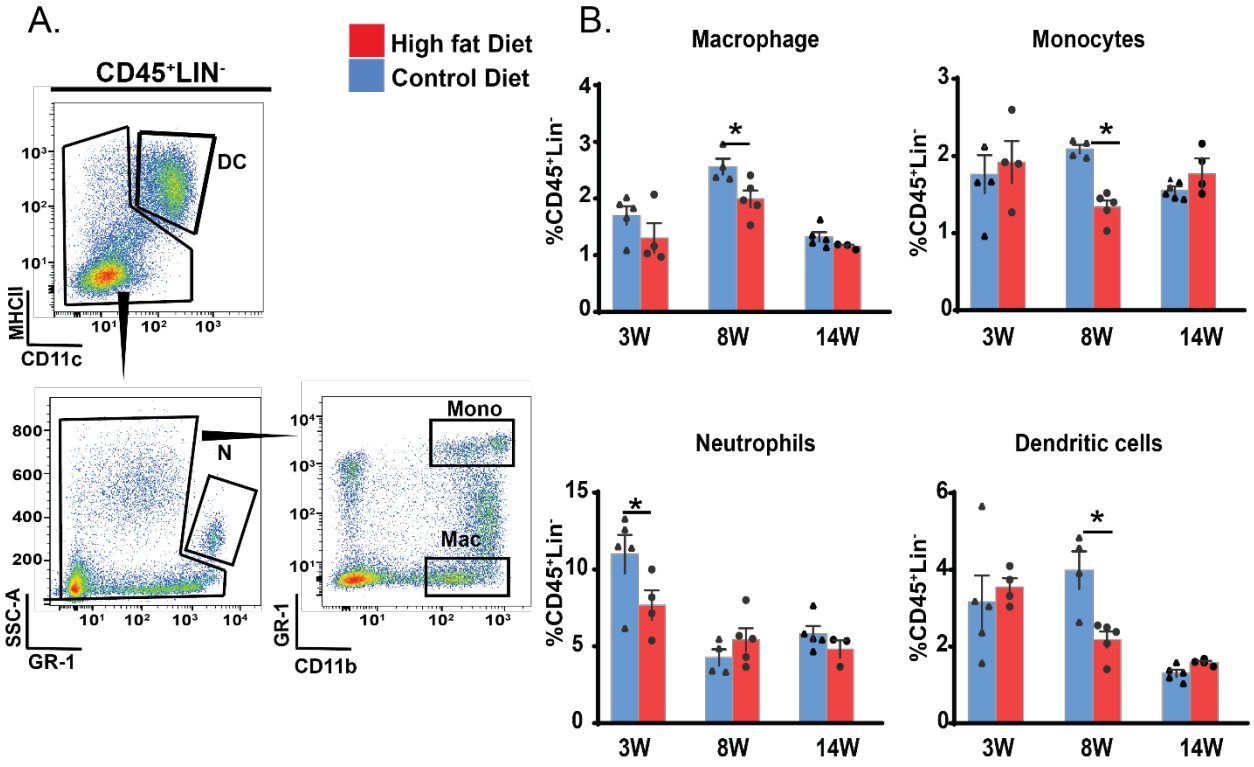


Figure 3. High fat diet alters myeloid cells in the spleen.

(A) Gating strategy for flow cytometry analysis of Macrophages, Monocytes, Dendritic cells, and neutrophils as myeloid cells in mouse spleen. All cells were gated as viable Lin (CD3, CD19, NK1.1, Ter119, B220)$^-$ and CD45$^+$; within this population, dendritic cells were gated as MHCII+ CD11C+, neutrophils were gated as GR1$^+$ SSC-A ^{high}, monocytes were gated as GR1$^+$ CD11b$^+$, macrophages as GR1$^-$ CD11b$^+$.

(B) Population percentages of Lin $^-$ and CD45$^+$ from CD and HFD mice after 3 or 8 weeks or 14 weeks of diet for Macrophages (top left), Monocytes (top right), Neutrophils (bottom left), Dendritic cells (bottom right) , (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

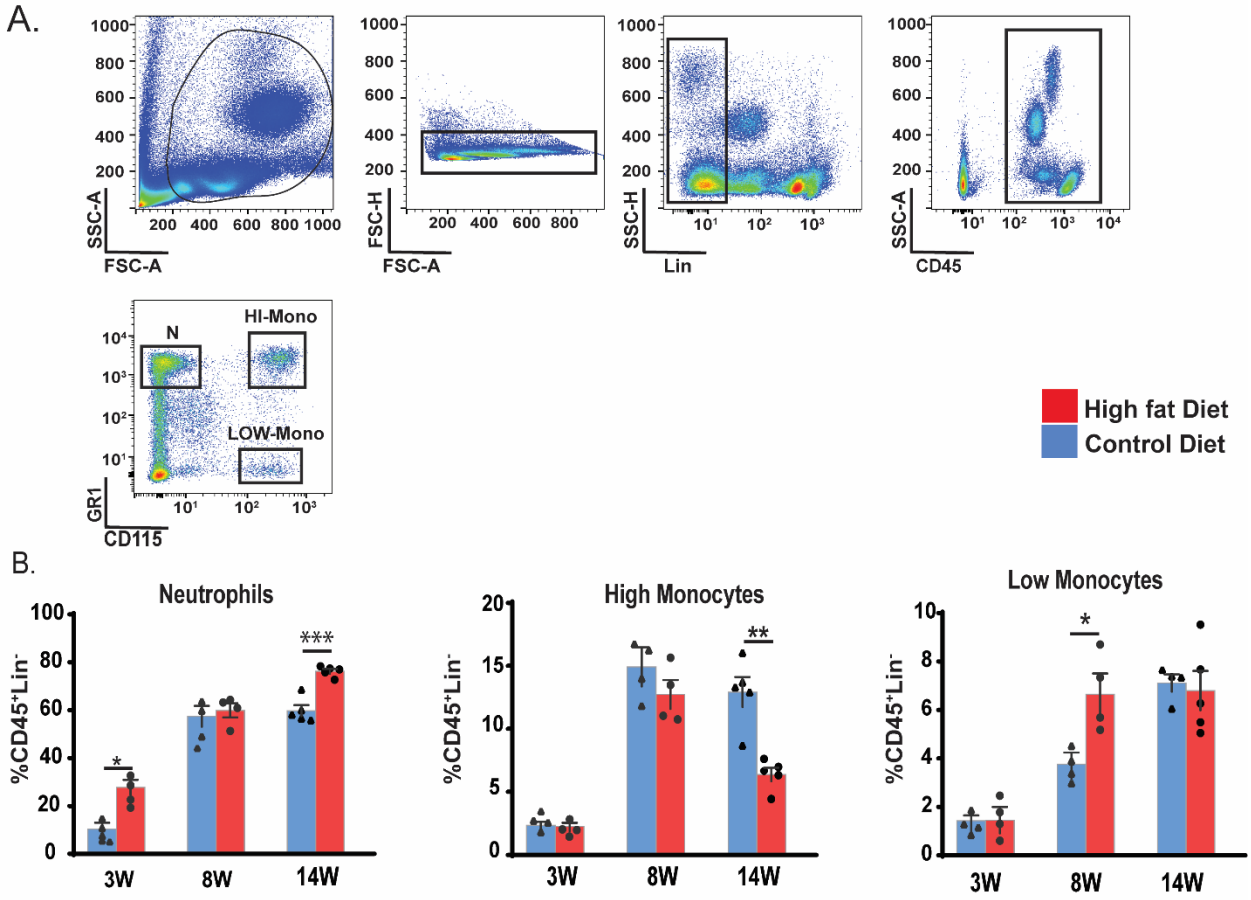


Figure 4. High fat diet alters myeloid cell composition in blood.

(A) Gating strategy for flow cytometry analysis of Neutrophils, ly6C^{+} Monocytes, ly6C^{-} Monocytes in mouse blood. All cells were gated as viable Lin (CD3, CD19, NK1.1, Ter119, B220)$^{-}$ and CD45^{+} within this population, Neutrophils were gated Gr1^{+} CD115^{-}. ly6C^{+} Monocytes were gated as GR1^{+} CD115^{+}. ly6C^{-} were gated as GR1^{-} CD115^{+}.

(B) Population percentages of Lin $^{-}$ and CD45^{+} from CD and HFD mice after 3 or 8 or 14 weeks of diet for Neutrophils (left), ly6C^{+} Monocytes (central), ly6C^{-} Monocytes (right), (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

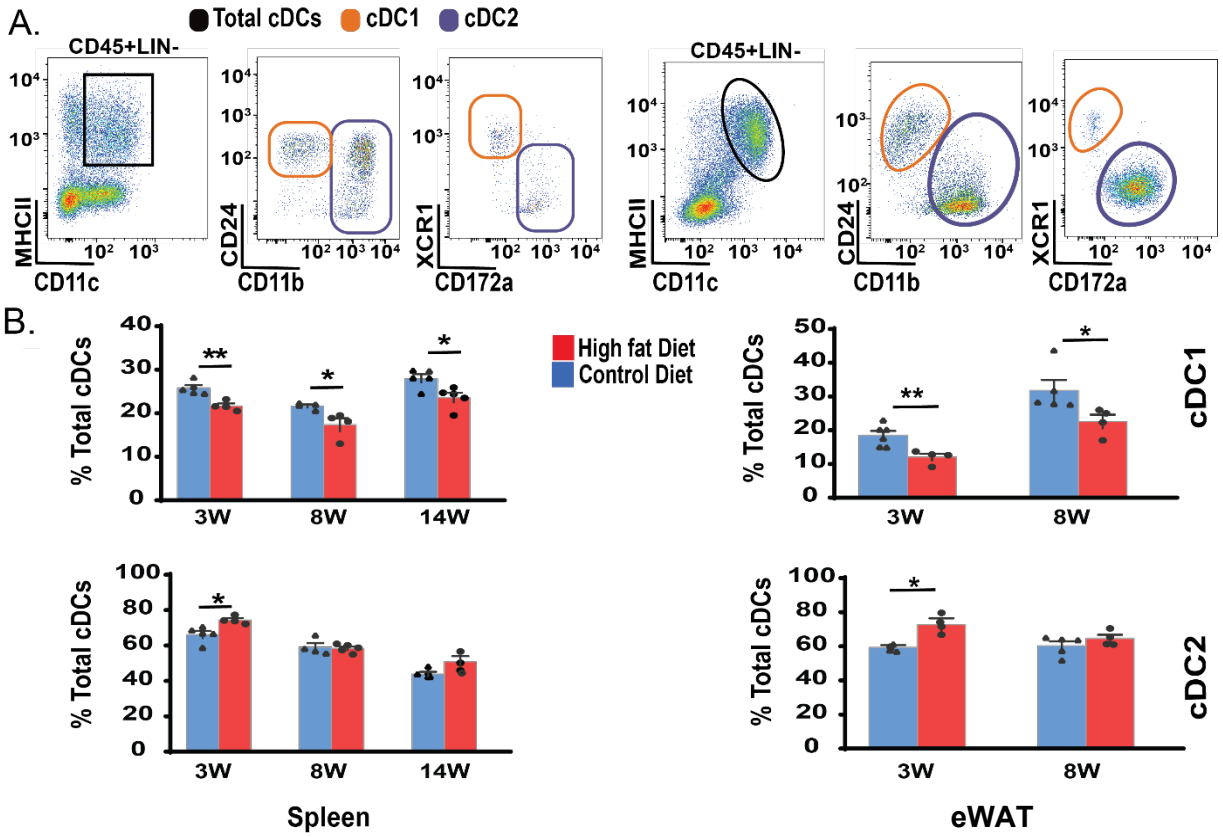


Figure 5. High fat diet induces cDC2 and reduces cDC1 in spleen and epididymal white adipose tissue.

(A) Gating strategy for flow cytometry analysis of different subsets of cDCs ,cDC1 and cDC2 in mouse spleen (left) and eWAT(right). All cells were gated as viable Lin (CD3, CD19, NK1.1, Ter119, B220, CD64) $^{-}$ and CD45^{+}, total cDCs were gated as MHCII^{+} CD11c^{-} and subsequently two subset cDC1 as CD24^{+} CD11b^{-} or XCR1^{+} CD172a^{-} and cDC2 as CD24^{-} CD11b^{+} or XCR1^{-} CD172a^{+}. XCR1 and CD172a gating used for statistics.

(B) Population percentages of cDCs subsets from CD and HFD mice at 3 ,8 ,14 in spleen or 3, 8 weeks in eWAT, spleen XCR1^{+} cDC1 (top-left), CD172a^{+} cDC2 (bottom -left) and eWATcDC1 (top-right), CD172a^{+} cDC2 (bottom-right). (n = 4/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.

4. cDC2 are consistently increased at the expanse of cDC1 in spleen, adipose tissue and bone marrow starting 3 weeks of high fat diet.

Despite evidence of cDCs presence in ADT, no real effort has been made to further investigate cDC subsets. Besides, many studies neglected cDC1 and defined adipose tissue cDC mainly as cDC2 [139,144, 153]. Therefore, I set up to investigate the effect of obesity on specific cDCs subsets. In order to gate cDC subsets, I designed a gating strategy to define cDCs subsets across lymphoid and non-lymphoid organs using general markers $CD24^{+}XCR1^{+}$ to define cDC1, $CD172^{+}CD11b^{+}$ to define cDC2 (fig5.A). Remarkably in the spleen and eWAT, I observed a significant reduction at 3 weeks, and it continued until the chronic phase of DIO for up to 8 weeks (fig5.B). Interestingly, at the same time points I could not observe any alteration in total cDCs (fig5.B). In contrast, I found an increase in cDC2 numbers at 3 weeks with HFD in the spleen and eWAT (fig5.B). This indicates that both during early and late-stage DIO induced obesity, cDC2 development may be favored over cDC1 development by unknown mechanisms.

cDCs develop in the bone marrow. Thus, I next analyzed mature cDCs subsets in bone marrow [136, 154]. Remarkably, I found that HFD reduced cDC1 percentage as early as 7 days of HFD, and this reduction continued until 20 weeks, similar to spleen and eWAT(fig6.B). However, I found an increase in cDC2 numbers simultaneously at 7 days and 14 weeks of diet (fig6.B). To confirm these findings, I performed in situ hybridization and Immunohistochemistry of bone sections for cDC1 markers, XCR1 and CLEC9 that are mainly expressed in cDC1 [153]. In line with our flow cytometric results, I found a significant reduction in XCR1 and CLEC9 signal in bone marrow from the HFD group (fig7A, B). Together, these results suggest that cDC1 development is sensitive to HFD at the early stages and continues to the late diet stage. Importantly, I conclude that the reduction in total cDCs during HFD is due to decreased cDC1 numbers.

This effect could potentially be explained by changes in programmed cells death or development of cDC1. First, to examine whether the reduction in the cDC1 population was due to cell death, I analyzed cDC1 from BM at 8 weeks of diet using Annexin V (detects apoptotic cells) and Fix viability dye (detects dead cells). Flow cytometric analysis did not indicate significant increase in cell death or induction of apoptosis within cDC1 between different diets (fig6.C). Therefore, I exclude that reduced cDC1 numbers are due to increased cell death.

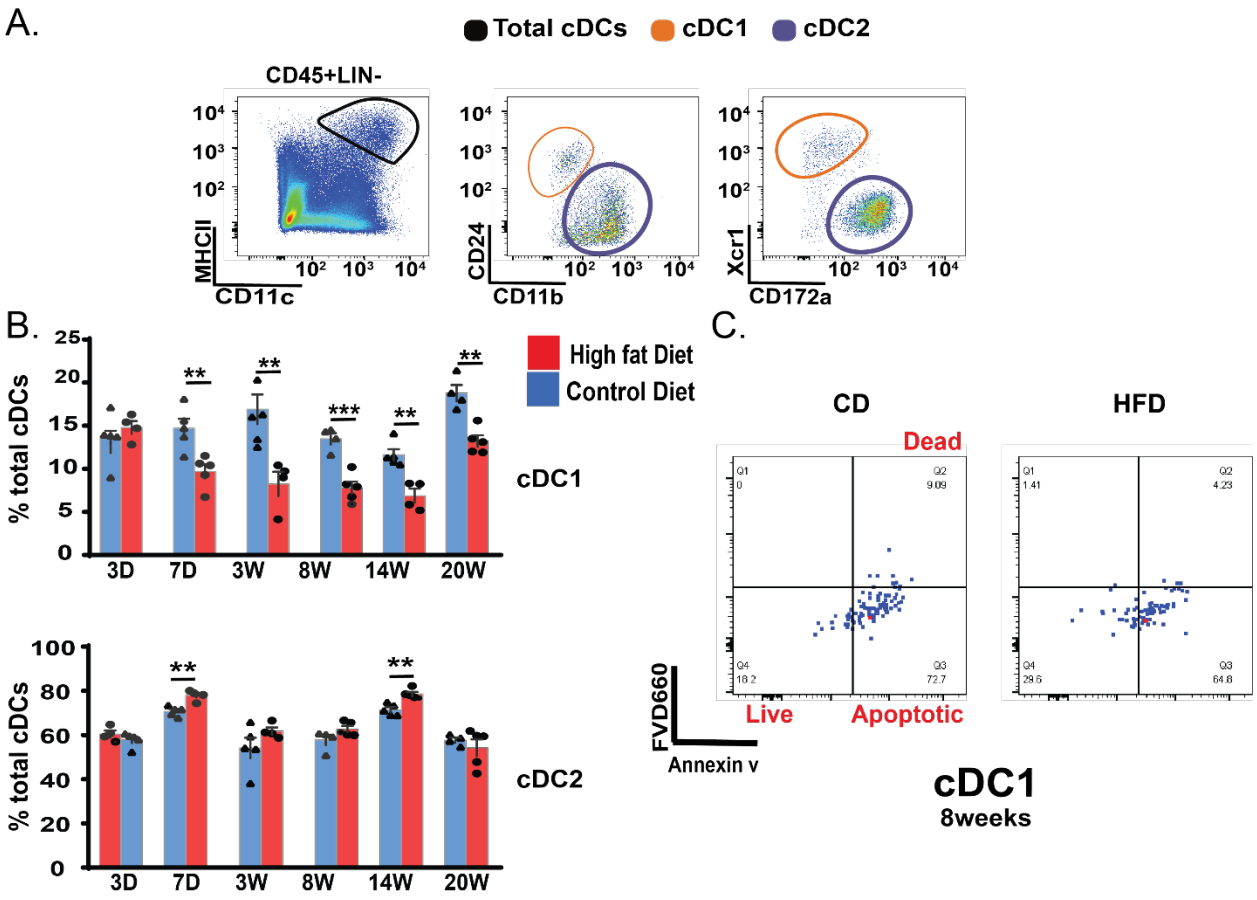


Figure 6. High fat diet remodels cDCs subset composition in the bone marrow by increasing cDC2 numbers at the expense of cDC1.

(A) Gating strategy for flow cytometry analysis cDCs subsets cDC1 and cDC2 in mouse bone marrow. All cells were gated as viable Lin (CD3, CD19, NK1.1, Ter119, B220) $^{-}$ and CD45^{+}, total cDCs were gated as MHCII^{+} CD11c^{-} and two subsets cDC1 as CD24^{+} CD11b^{-} or XCR1^{+} CD172a^{-} and cDC2 as CD24^{-} CD11b^{+} or XCR1^{-} CD172a^{+}, XCR1 and CD172a gating used for statistics.

(B) Population percentages of cDCs subsets from CD and HFD mice starting from 3 days until 20 weeks of diet for XCR1^{+} cDC1 (top), CD172a^{+} cDC2 (bottom) (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

(C) Representative percentages of cell viability and apoptosis following 8 weeks of diet in the XCR1^{+} cDC1 population from CD or HFD in the bone marrow.

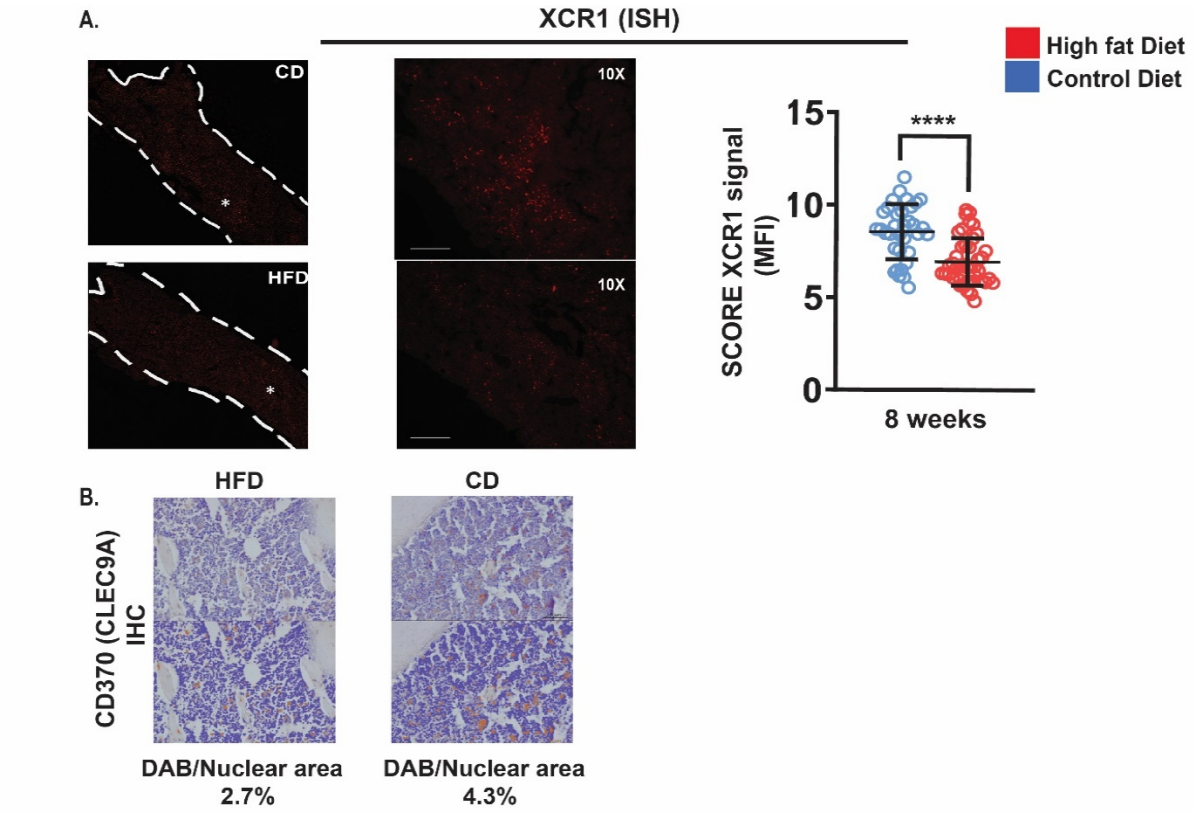


Figure 7. High fat diet leads to a tissue-wide reduction of cDC1 marker expression in the bone marrow (both on transcript and protein level).

(A) Representative immunofluorescence staining of XCR1 mRNA by in situ hybridization (ISH) in tissue sections of bone from CD and HFD group at 8 weeks of diet (left), quantification of the immunofluorescent signal of XCR1 using MFI

(mean fluorescent intensity) in both groups (n = 5/group) *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

(B) Repetitive immunohistochemistry (IHC) staining of CLEC9A/CD370 in bone sections from CD and HFD, CLEC9A signal is quantified by DAB/Nuclear area signal.

5. High fat diet reduces the proliferation and development of Pre-DC1 from bone marrow.

HFD is known to, directly and indirectly, affect many hematopoietic cells, leading to alteration in progenitors' development. Thus, I investigated if HFD leads to a reduced cDC1 and an induced cDC2 development by affecting their direct progenitors. Next, I analyzed the late stages of cDCs progenitors, including MDP, CDP, and Pre-DCs. In BM, I followed the changes in MDP, CDP, and Pre-DCs through different time point of diet starting of 3 days until 20 weeks of the diet, to study their development from acute to the chronic phase of DIO (fig8.A). I found that in mice fed with HFD, MDP percentage was increased 1 week, and CDP percentage 3 weeks post-HFD induction as compared to control diet. In contrast, I observed a reduction in total Pre-DCs due to HFD (fig8. B).

To investigate whether this reduction correlates with reduced mature cDC1, I focused on both cDC1 and cDC2 direct progenitors Pre-DC1 and Pre-DC2 using a previously published gating strategy [59]. I defined PreDC1 by expressing $CD117^{+}$ $SiglecH^{-}Ly6C^{-}$, which reported that it only gave rise to cDC1[65, 154] (fig8.A). Interestingly, I found that PreDC1 was significantly reduced in bone marrow at 8 weeks within the HFD group and continued up to 14 weeks. This reduction correlated with what I described for the mature population. Thus, I conclude that the decrease in cDC1 percentage is mainly due to a reduction in Pre-DC1 numbers (fig9.A).

Because HFD is known to induce progenitor mobilization[155], I next assessed whether the reduction I observed in Pre-DC1 within the BM was due to an increased migration of Pre-DC1. I quantified Pre-DC1 in blood after 8 weeks of HFD diet or control diet and found a significant reduction in the absolute numbers of Pre-DC1 in blood from the HFD group

(fig9.B). This indicated that both initial production and migration of Pre-DC1 is attenuated during high fat diet. Contrary to this, I could not find any change in Pre-DC2, with only a slight reduction observed at 8 weeks in the HFD group (fig9.B).

Next, I speculated that this reduction in Pre-DC1 could be due to a decrease in proliferation or differentiation or a combination of both. To confirm this, I analysed the proliferation capacity of Pre-DC1 under HFD, by Ki67 staining, which is known as a marker for proliferation[156]. In BM, I found that Pre-DC1 from HFD mice significantly displayed less KI67 expression compare to CD (fig9.C). This could indeed explain the reduction in their numbers and reduced mature cDC1 during HFD.

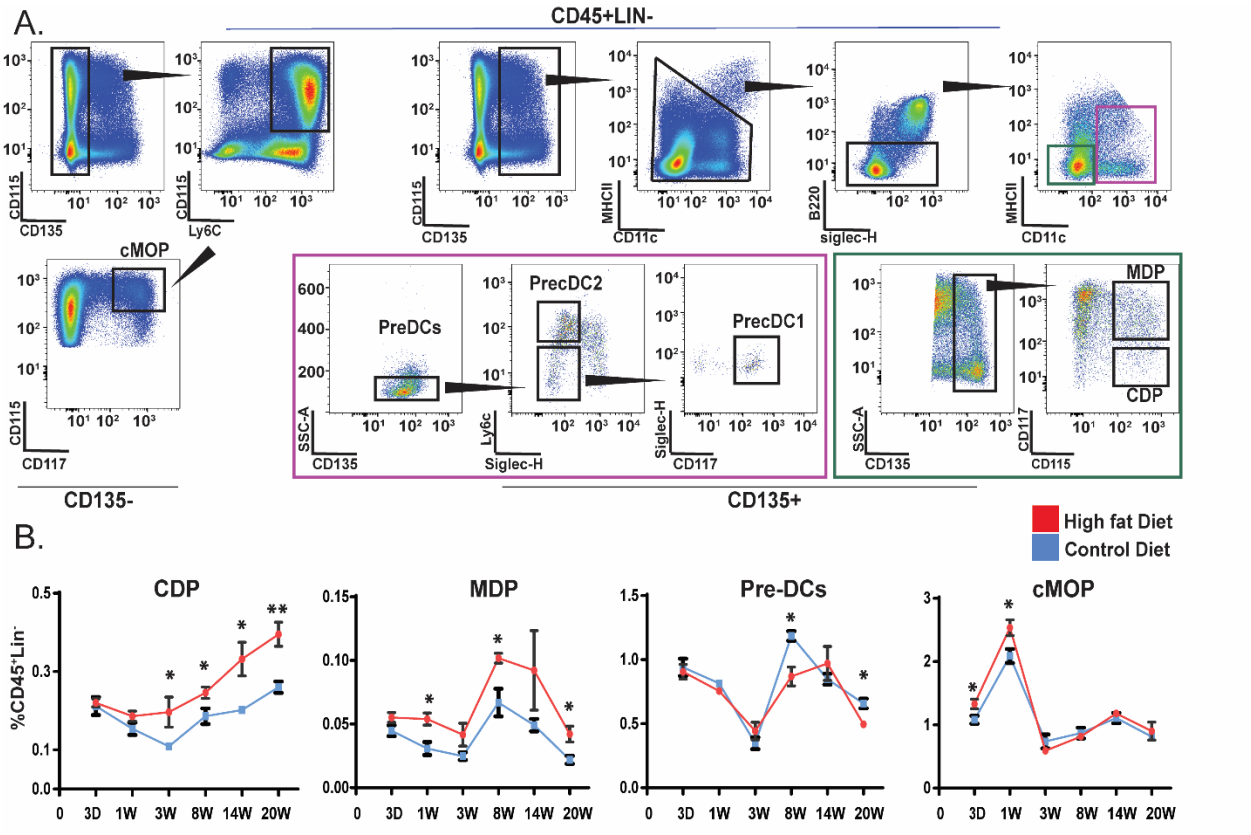


Figure 8. High fat diet alters dendritic cells progenitors in the bone marrow.

(A) Gating strategy for flow cytometry analysis of CDP, MDP, Pre-DCs, and cMOP as late Myeloid progenitors in mouse bone marrow. All cells were gated as viable lin

(CD3, CD19, NK1.1, Ter119) $^{-}$ and $CD45^{+}$; within this population from $CD135^{-}$ population cMOP were gated as $CD115^{+}$ $ly6C^{+}$ $CD117^{+}$. from $CD135^{+}$ $MHCII^{int/-}$ $CD11c^{int/-}$ $B220^{-}$ $Siglec\text{-}H^{nt/-}$ population Pre-DCs were gated as were gated as $CD11c^{+}$ $MHCII^{int}$ SSC-A ^{low} and two subsets Pre-DC1 as $Ly6C^{-}$ $Siglec\text{-}H^{-}$ $CD117^{+}$ or Pre-DC2as $Ly6C^{+}Siglec\text{-}H^{-}$.MDP was gated as $CD135^{+}CD11c^{-}$ $MHCII^{-}$ $CD117^{+}$ $CD115^{+}$..CDP was gated as $CD135^{+}CD11c^{-}$ $MHCII^{-}$ $CD117^{+}$ $CD115^{-}$.

(B) Population percentages of lin - and CD45+ from CD and HFD mice starting from 3 days until 20 weeks of diet for starting from left to right CDP, MDP, Pre-DCs and cMOP (n = 5/group)., *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m

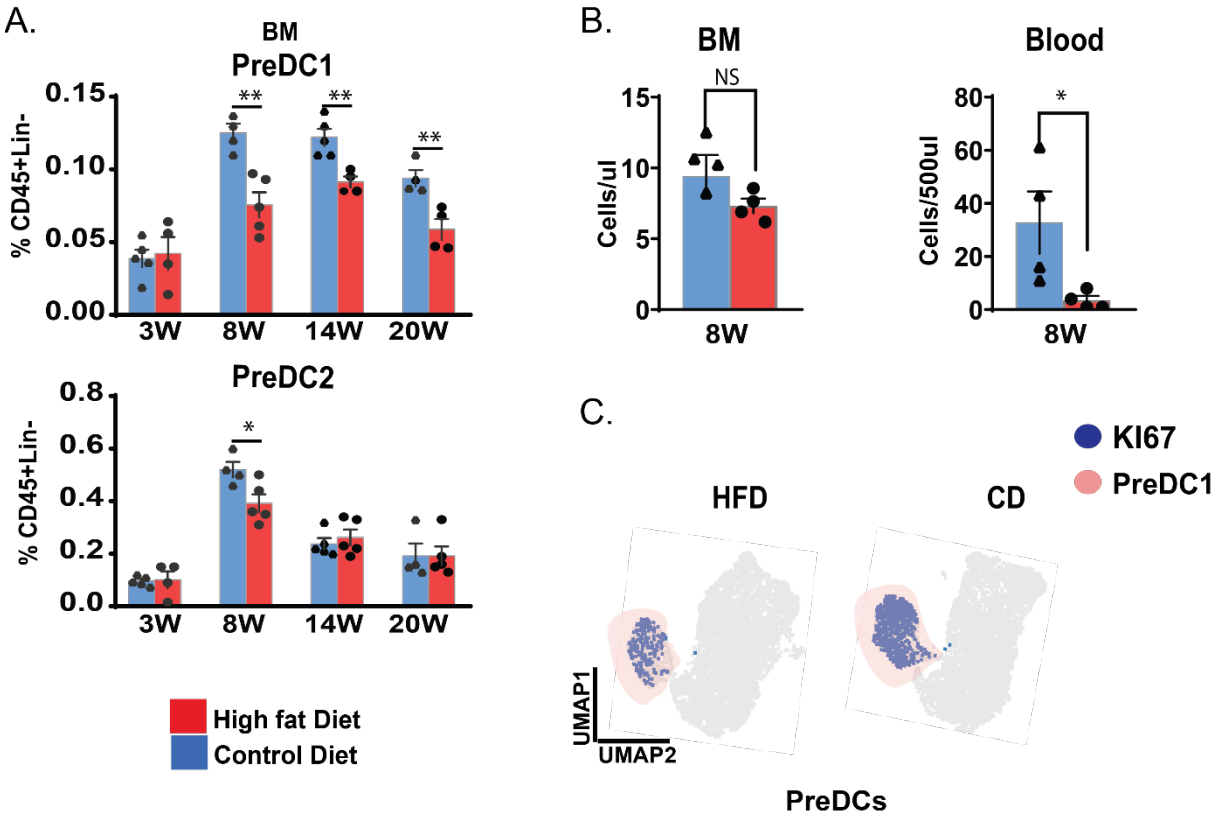


Figure 9. High fat diet reduces Pre-DC1 and not Pre-DC2 differentiation and proliferation.

(A) Population percentages of Lin-CD45^{+} for Pre-DC1 (Top) or Pre-DC2(bottom) from CD and HFD mice at 3,8,14,20 weeks of diet (n = 5/group). *P0.05, **P0.01, and ***P0.005., Student's t-test. Results are shown as mean±s.e.m.
(B) Absolute cell numbers of Pre-DC1 Cells at 8 weeks of diet in bone marrow (as Cells per µl) or blood (cells per 500ul). (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.
(C) Uniform manifold approximation and projection (UMAP) representation of analyzed total Pre-DCs from CD or HFD at 8 weeks of diet pre-DC1 area is colored in pink; ki67 expression overlayered with violet color. (n = 5/group).

6. High fat diet dampens the developmental capacity of Pre-cDC1 towards cDC1 in the BM by reduction of IRF8 expression.

Next, I wanted to investigate if this reduction was only due to a reduction in the proliferation capacity of Pre-DC1 or a reduction in their differentiation capacity. Obesity is known to control immune cell progenitors' development by modulating transcription factors at different stages by releasing adipokine and cytokine from ADT [105, 157]. Thus, to investigate if HFD affects cDCs development through IRFs, I tested the expression of IRF4 and IRF8 by intranuclear flow cytometry staining [43, 62, 65, 67] (fig10.A). First, at 7 days of diet in BM, when I previously saw the first reduction in cDC1 numbers, I evaluated the expression of IRF4 and IRF8 within immune cells. Interestingly at 7 days of the diet, HFD readily resulted in a significant reduction in IRF8 and a slight induction of IRF4 expression in total CD45^{+} cells (fig10.B), potentially explaining the decline in cDC1 numbers due to reduction in IRF8 expression in progenitors. Next, I wanted to assess IRF8 expression at different DC progenitors stages to see at which stage IRF8 expression is downregulated. Surprisingly, at 8 weeks of the diet, starting from CDP to mature cDC1, I observed a significant reduction of IRF8 expression in HFD compared to CD mice (fig10.C). This means that this reduction occurs in the early stages of progenitors development before the final commitment to cDC1.

Finally, I wanted to test whether the reduction in IRF8 is explicitly occurring in BM progenitors or DCs progenitors in other organs. By analyzing Pre-DC1 from the spleen at

8 weeks of the diet, I found a similar significant reduction in IRF8 expression within the Pre-DC1 population (fig10.C). Collectively, HFD reduced cDC1 numbers by reducing the expression of IRF8 in direct progenitors and mature cDC1.

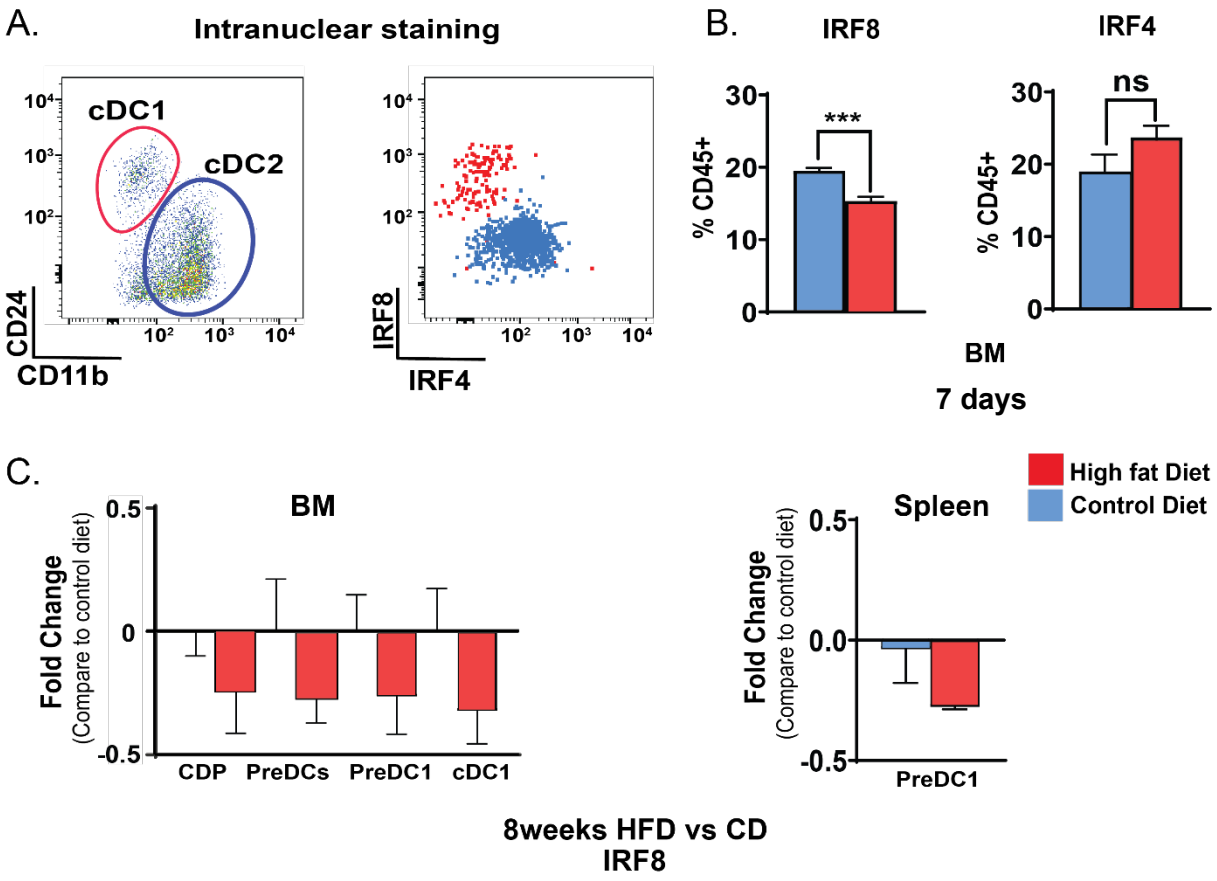


Figure 10. IRF8 expression is reduced under high fat in cDC1 and the their progenitors.

(A) Representative flow cytometry dot plots of intranuclear staining of transcription factories IRF8 and IRF4 in cDC1 and cDC2 from bone marrow.

(B) Population percentages of living CD45% for IRF8 positive cells (left) and IRF4 positive cells (right). (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

(C) Quantification of IRF8 expression (fold change of MFI in HFD relative to CD) in CD from BM (left) and precDC1 in spleen (right)) (n = 5/group).

7. High fat diet induces GM-CSF levels in the BM microenvironment reduces IRF8 expression by activating STAT5 in Pre-cDC1.

One important cytokine found to modulate DC development is GM-CSF, which was also upregulated during obesity in mice and humans [73, 77, 79, 158]. Thus, I hypothesized that GM-CSF plays a role in cDC1 suppression because there is a reverse correlation between IRF8 and GM-CSF as it was shown that IRF8 suppresses GM-CSF expression and vice versa[159, 160]. By analyzing bone marrow at 8 weeks of the diet, intracellular staining of non-stimulated cells showed the induction of GM-CSF production under HFD (fig11.A). Additionally, the production of GM-CSF was mainly found in non-immune cells. This result indicated that HFD also affects the non-immune bone marrow microenvironment. Next, to confirm if HFD induces GM-CSF release in the bone marrow microenvironment, I measured GM-CSF from BM supernatant using ELISA. I found significant GM-CSF released in BM supernatant under HFD, which means that HFD induced GM-CSF production in the bone marrow microenvironment (fig11.B).

GM-CSF modulates different IRFs expression through activation various STAT proteins[77, 82, 161]. It has been shown that stimulation of DCs progenitors stimulation by GM-CSF leads to increased activation of STAT5 by phosphorylation, which results in inhibition of IRF8 and induction of IRF4[81-83, 160, 161]. To confirm that, I measured STAT5 activation by phosphorylation. I stained phosphorylated STAT5 by intracellular FACS in Pre-DC1. Interestingly, in BM at 8 weeks, Pre-DC1 from HFD mice showed a significant increase in STAT5 activation by phosphorylation compared to CD, which goes in line with the induction of GM-CSF and IRF8 suppression (fig11. C). Collectively, these

results suggest a cascade of events induced by HFD. Increased GM-CSF-release leads to activation of STAT5 in Pre-DC1 that blocks IRF8, which is essential for proliferation and differentiation of progenitors toward mature cDC1.

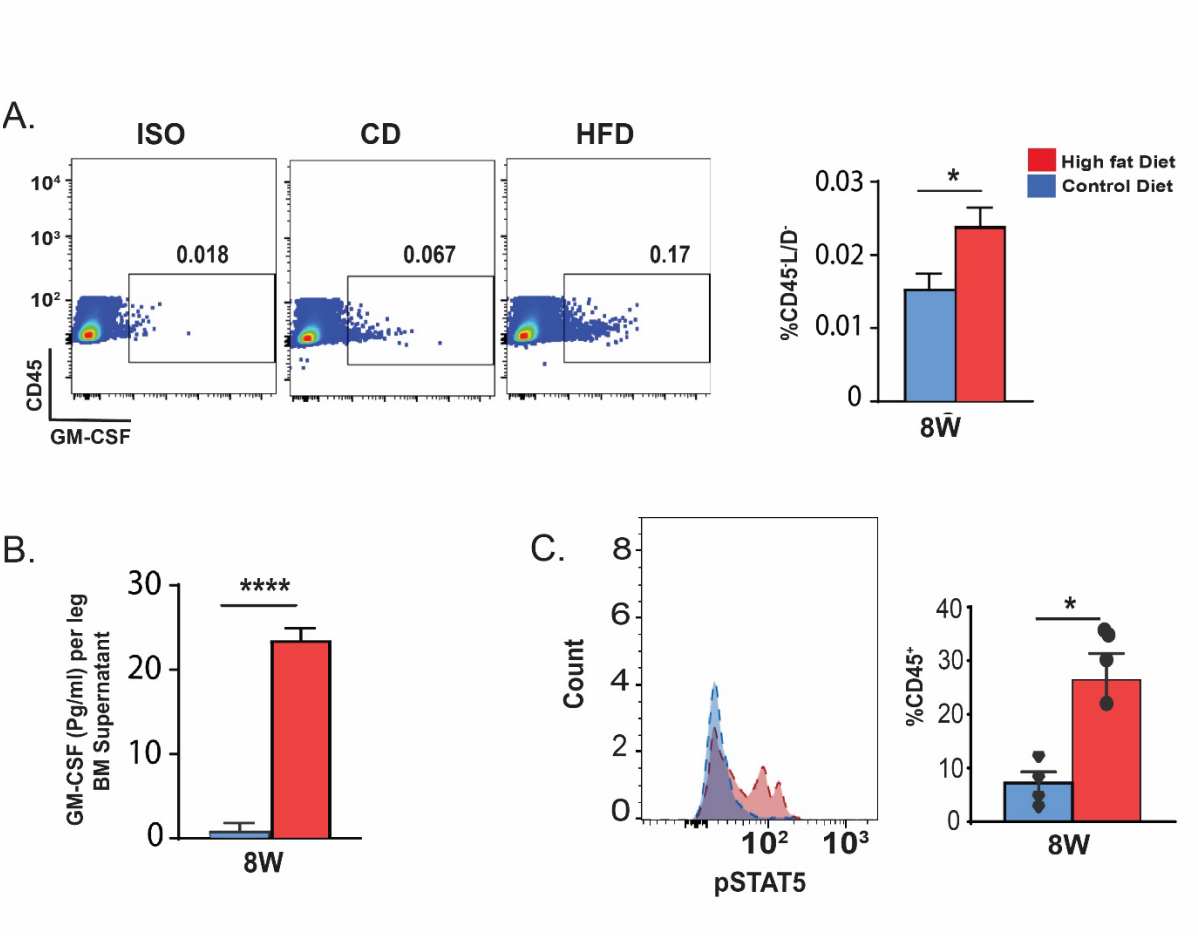


Figure 11. GM-CSF production and release induced during HFD leads to activation of STAT5 by phosphorylation.

(A) Representative flow cytometry dot plots of GM-CSF staining in viable CD45- cell at 8 weeks of diet in bone marrow (left), Percentage of GM-CSF+ cells within the viable CD45- cell fraction (right) (n = 4/group).*P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.

(B) Cytokine measurement by ELISA in BM supernatants at 8 weeks of diet from CD and HFD measured as (pg/ml) per leg. (n = 5/group). *P0.05, **P0.01, and ***P0.005, Student's t-test. Results are shown as mean±s.e.m.m.

(C) Representative histogram of flow cytometry intracellular staining of p-STAT5 in Pre-DC1 from CD and HFD at 8 weeks of diet (left), Percentage of positive P-STAT5 Pre-DC1 within the viable $CD45^{+}$ cell fraction (right) (n = 4/group).*Po0.05, **Po0.01, and ***Po0.005, Student's t-test. Results are shown as mean±s.e.m.

8. High fat diet impairs cDC1 function in ADT by the reduction of maturation and activation markers.

IRF8 does not only control cDC1 cell differentiation and terminal selection, but it is also essential for their maturation and activation. In mice upon IRF8 partial deletion ($Irf8^{+/-}$), cDC1 development and maturation is reduced [62, 65, 162]. Recently, obesity was found to cause DC dysfunction and to suppress the activity of DCs during infection and tumor clearance [163, 164]. Therefore, I wanted to address if HFD reduces cDC1 activity and maturation by reducing IRF8 expression. To this end, I isolated eWAT at 8 weeks of the diet, and I analyzed the expression markers that defined development and activation of cDC1 like XCR1, CD103a, and CD80[20, 30, 165]. As expected, I found that within cDC1 ($XCR1^{+}$), expression of CD103 and CD80 was significantly reduced under HFD in comparison to CD (fig12.A). One interesting observation was that the reduction in CD103 and CD80 was more prominent than that of XCR1 (fig12. B), which was very interesting because in most studies focusing on DC subsets within adipose tissue during obesity, CD103 was used to define cDC1[166]. This could also explain why cDC subset definition was difficult in these studies. That collectively means that HFD reduces cDC1 maturation, activation, and accumulation in ADT due to the reduction of IRF8, which is essential in cDC1 development and maturation.

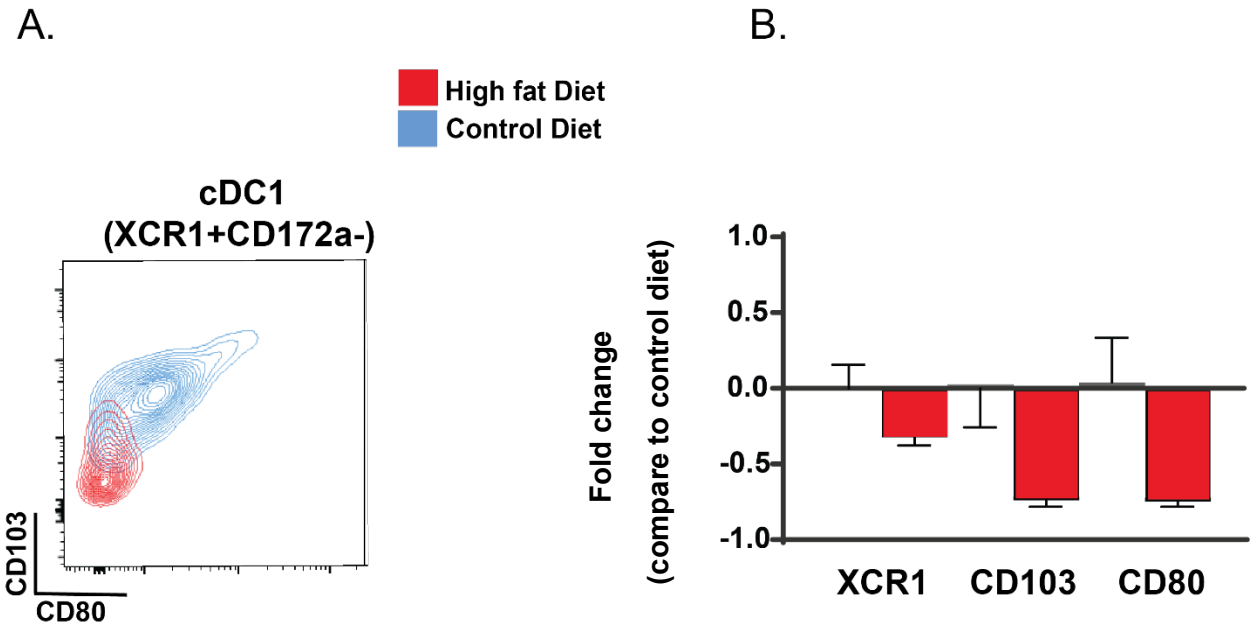


Figure 12. cDC1 in adipose tissue show a reduction in maturation and activation markers during HFD.

(A) Plots shows and overlay of CD103 and CD80 expression of cDC1 ($XCR1^{+}$) in eWAT from CD and HFD at 8 weeks of the diet, showing expression of CD103 vs. CD80.

(B) Fold change of XCR1, CD103, and CD80 expression (Mean fluorescent intensity) in cDC1 from the HFD group relative to CD (n = 5/group).

Discussion

In obesity, overnutrition triggers overloaded fat accumulation in the WAT depot and leads to chronic low-grade inflammation and insulin resistance (IR)[96]. Myeloid cells were among the first leukocytes identified as capable of altering adipose tissue and circulation[100, 132]. In recent years, accumulative evidence has found that cDC numbers correlate with obesity positively, and cDCs induce adipose tissue inflammation and insulin resistance in mice and humans[100, 166]. Even in those studies, very little was known about how different cDC subsets cause obesity and inflammation and how obesity affects their development and maturation. Using the DIO model in mice, I followed the development and maturation of cDCs through diet courses from the acute phase at 7 days to the chronic stage at 20 weeks. For the first time, my findings showed that HFD modulates cDC subsets differently. HFD reduces cDC1 percentage and induces or has no effect on cDC2 percentage in total cDCs within the spleen and non-lymphoid organs (eWAT, BM) from the early stage of diet (3 weeks) to the chronic stage (20 weeks). This reduction was mainly because of the reduction of cDC1 direct progenitors' numbers in BM and blood. HFD significantly reduces IRF8 expression in cDC1 progenitors, a transcription factor essential for cDC1 progenitor development, proliferation, and maturation. HFD induces the production of many inflammatory cytokines, including GM-CSF, that consecutively activates STAT5 to block IRF8 [81, 82, 160]. In HFD, I found increased production and release of GM-CSF, which eventually leads to activation of STAT5 by phosphorylation in Pre-DC1, leading to IRF8 suppression. Finally, due to a defect in their development, in eWAT fewer accumulated cDC1 showed reduced expression of maturation and activation markers. These findings reveal a new pathway by which obesity regulates cDC1 production from BM and maturation in eWAT by reducing IRF8 expression via inflammatory cytokine GM-CSF and STAT5.

The DIO model is used to mimic obesity in humans[167]. Obesity was induced in mice by feeding HFD a diet containing 60% fat compared to the 14% fat of CD diet[148]. First, body weight gain was significantly induced in HFD mice compared to CD. The significant increase in adipocyte size and crown-like shapes around adipocytes in eWAT indicated increased inflammatory macrophage infiltration under HFD. Besides, in BM, increased fat consumption leads to an increase in adipocytes' number and size. During HFD, excessive fat consumption leads to more fat globules and adipocytes, which causes WAT dysfunction[92, 96]. Dysfunctional WAT releases more inflammatory cytokines (such as MCP-1, RANTES, IL-6, interferon (IFN) gamma, or TNFα) that increase inflammatory immune cells' infiltration of macrophages, DCs, and T cells[113]. Inflammatory macrophages infiltrate WAT and form crown-like structures around apoptotic adipocytes. Increased fat consumption is known to induce fat accumulation outside adipose tissues in organs like the liver, BM, muscle, pancreas, and heart[168] [99, 168]. Bone marrow resident adipocytes (BMADT) reside in distinct niches within the bone marrow and interact with hematopoietic precursors to control lineage-committed [169]. They influence commitment choices and the cellular lineage selection of immune cells by secreting adipocyte-derived factors such as adiponectin, leptin , and IL-6. In adult mice and humans, BMAT mass increases with obesity, age, and osteoporosis [169]. The DIO module in mice induces body weight gain and fat accumulation in eWAT while increasing macrophage infiltration in eWAT and increasing the number of adipocytes in BM [93, 104, 132].

Myeloid cell production and activation are important in initiating metainflammation and are biomarkers of obesity-induced inflammation [129, 136]. Under HFD, the percentages of monocytes and neutrophils increased significantly in eWAT and blood starting at week three of the diet and continuing until week 20. While in the spleen, the neutrophil percentage decreased. Obesity-induced adipose tissue macrophage accumulation depends on the monocytes' recruitment to adipose tissues where they differentiate into macrophages and surround dead and dying adipocytes[152]. In line with previous studies, I observed an abundance of macrophages and monocytes within the eWAT under HFD compared to CD. In recent years, neutrophils were linked to inducing inflammation and insulin resistance during obesity[79, 112, 113]. Adipose tissue neutrophils (ATNs)

produce chemokines and cytokines, facilitating macrophage infiltration, which could contribute to the chronic low-grade inflammation that characterizes obesity. This explains the surging influx of neutrophils I observed in as early as 3 weeks in HFD mice[113, 170]. Collectively, my findings show that HFD induces remarkable remodeling in the development and possibly the function of myeloid cells in the affected adipose tissues and within the bone marrow. [113, 170].

Next, under HFD, I observed a significant reduction in total cDCs percentage within the spleen at 3 weeks and eWAT at 20 weeks, with no changes at 3 weeks. I could not detect any changes in the bone marrow. This is contrary to previous reports describing DC accumulation in ADT and induction of obesity, DCs accumulate in ADT and induce inflammatory immune cell recruitment to ADT [113, 137, 140, 171].In the inflammatory state during obesity, DCs accumulate in ADT and induce inflammatory immune cell recruitment to ADT[113, 137, 140, 171]. It has also been demonstrated that cDCs were essential for recruiting macrophages, which is a hallmark of obesity-associated inflammation [133]. In most of those studies, DCs were defined by the expression of CD11c, expressed with cDC and pDC and activated macrophages[110, 113, 140, 166]. That can explain the difference between our findings and the previous reports. Hence, I suspect that the previously reported increase of CD11c+ population during obesity was due to contamination with other immune cell populations, such as macrophages and pDC, which, as I have also observed, are affected by obesity [110, 121, 123]. It was recently shown that pDC recruitment that triggers a pro-inflammatory phenotype in macrophages macrophage switch to inflammatory phenotype [134, 135]. It was also found that deficiency in pDCs and type I interferon signaling prevents diet-induced obesity and insulin resistance in mice. This explains the improved production of pDC during HFD [134, 135]. Together, these findings pointed in the direction that cDC are affected by HFD feeding, and I therefore decided further to analyze cDC subsets distribution and activation during DIO.

cDC subpopulations are complex to define comprehensively, but with the right surface marker staining approach, it is possible. It is not easy to compare results from different studies due to the diversity of surface markers used to distinguish distinct cDC

subpopulations. A further problematic issue is that cDCs with similar functions had different phenotypes in lymphoid and nonlymphoid organs[22, 172, 173]. To overcome this problem, I I established and used a general universal combination of antibodies to define cDC1 and cDC2 in all organs. It was recently shown in many studies that murine cDCs could be universally classified based on the mutually exclusive expression of XCR1 and CD24 for cDC1 and CD172a and CB11b for cDC2[43, 174, 175]; however, this was not applied to HFD studies before. Using this gating strategy allowed a precise unified method for phenotyping of cDCs in various organs. Under HFD, I found a significant decrease in the percentage of cDC1 in lymphoid (spleen) and nonlymphoid organs (eWAT), starting at 3 weeks. This reduction continues through the late stage at 8 and 14 weeks of DIO. Next, for cDC2 in the spleen and eWAT, I observed a rise in numbers in the acute phase at three weeks but with no change in the late stages at eight and 14 weeks in the spleen and eWAT. These findings suggest that the development and maturation of cDCs under HFD are different between cDC subsets, and the development of each subset is affected differently. It also falls in line with previous reports describing that most infiltrating dendritic cells in ADT during HFD are cDC2 rather than cDC1[135, 136, 142]. Our findings can explain that HFD reduces cDC1 production and mainly induces cDC2 production and accumulation in different organs.

To better understand how HFD affects cDC development and abolishes cDC1 development, I focused on developing cDC progenitors within the bone marrow. cDC1 was significantly reduced at seven days of HFD, and this continued until the late chronic stage, 20 weeks, whereas cDC2 increased with similar dynamics. These results were similar to what I had found in the spleen and the adipose tissue, evincing that cDC1 generally displays reduced production under HFD. This could be interpreted in two ways. The first entails that HFD induces cellular apoptosis of cDC1. The second is that HFD modulates cDC1 progenitors, which leads to a reduction in the development of cDC1. First, I investigated whether HFD induces apoptosis of cDC1. No significant difference was observed between CD and HFD. These results showed that reduction in cDC1 was not due to the induction of cell death but could be caused by a changed developmental trajectory of cDC1 progenitors. To check whether HFD affects the direct cDCs progenitors, I analyzed late cDC progenitors from the bone marrow.

DC and monocyte lineages originate from a common progenitor, monocyte, and dendritic cell progenitors (MDPs). The two cell types split when MDPs give rise to monocytes and committed DC progenitors (CDPs) in the bone marrow. CDPs give rise to pre-DCs, which produce the two major subpopulations of cDCs [5, 39, 59]. At 3 weeks of a high-fat diet, I observed an upregulation of MDPs and CDPs under HFD, whereas no significant difference was observed in total pre-DCs between HFD and CD. At 20 weeks, both MDPs and CDPs continued to increase in HFD mice in the chronic phase compared to the control group.

In comparison, the pre-DC population plummeted under HFD. These results show that changes in cDC1 happened at the pre-DC stage, leading to the observed reduction in cDC1. For a more in-depth understanding, I investigated pre-DC1 and pre-DC2 subsets.

In the last stage of DCs development, different pre-DCs cells express gene profiles similar to those of mature cDC1, cDC2, and pDCs. In a paper by Schlitzer et al., these progenitors were shown to be fully committed and could not give rise to other subsets[59]. Additionally, Pre-DC1 and Pre-DC2 were defined among total pre-DCs by identifying surface markers (Siglec-H and Ly6C). In another study by Grajales-Reyes et al., only $CD117^{+}$ cells of pre-DCs were shown to develop into mature cDC1[64]. Combining these two studies, I defined Pre-DC1 as ($Siglec\text{-}H^{-}$ $Ly6C^{-}$ $CD117^{+}$) and Pre-DC2as ($Siglec\text{-}H^{-}$ $Ly6C^{+}$). First, I checked the two progenitor subsets in the bone marrow, and, as expected, pre-DC1 numbers decreased under HFD. Reduction in Pre-DC1 numbers started at the acute phase of DIO and continued until the chronic stage of DIO under HFD in BM. That explained the decline of mature cDC1 numbers I observed in different organs during HFD. Also, the migratory Pre-DC1 in blood was significantly lower in number in the HFD group. I thus concluded that HFD led to a total reduction in cDC1 and Pre-DC1 in both the bone marrow and the blood. Next, I wanted to see if HFD has the same effect on Pre-DC2. I could not detect any changes between HFD and CD apart from reducing the Pre-DC2 numbers at 8 weeks in the bone marrow. This reduction can be seen in progenitors but not in mature cDC2. These findings demonstrate that HFD mainly affected cDC1 development and that the exerted effects start at the early stage of 3 weeks of DIO and continue until the chronic stages of 20-weeks HFD. Given the drop in Pre-DC1 and cDC1

during HFD, I hypothesized that Pre-DC1 lost the ability to proliferate and differentiate toward cDC1 under HFD. Indeed, I found that Pre-DC1 cells have lower proliferative capacity resulting in the observed reduction of cDC1.

The process by which pre-DCs give rise to Pre-DC1 and Pre-DC2 and ultimately differentiate between cDC1 and cDC2 are controlled by lineage-determining transcription factors (TFs) [37, 62, 176]. The two significant TFs suggested for controlling this process are IRF8, essential for cDC1 development, and IRF4 for cDC2 development [16, 62, 65, 159]. IRF8 is a crucial TF required in many developmental stages throughout mononuclear cell development. In cDC1 development, IRF8 is essential as a terminal selector of the cDC1 lineage, and maintenance of IRF8 is required for cDC1 survival[62, 64, 65]. Many studies using homo- or hetero-IRF8 knockout mice showed defective cDC1 development and function[65]. This led us to assume that HFD could modulate IRF8 expression resulting in a changed cDC1 development. In the bone marrow, I observed that $CD45^{+}$ cells exhibited a significant reduction in IRF8 under HFD, while no changes in IRF4 were detected just after 7 days of HFD. When I examined the DC progenitors for IRF8 expression, I noted that a reduction in IRF8 occurs as early as the CDP stage and continues to decline towards a mature cDC1 stage. It also shows that HFD modulates IRF8 at a 7-day post-HFD feeding induction, indicating that IRF8 modulation is one of the first steps in diet-induced inflammation. The downregulation of IRF8 could thus explain the reduction I observed in Pre-DC1 and cDC1. These results showed for the first time that IRF8 modulation during DIO plays an important role in the development of immune cells and can affect other IRF8-dependent immune cells that need further investigation. To confirm if these phenomena are exclusive to BM progenitors or could be generalized to other organs, I analyzed Pre-DC1 IRF8 expression in the spleen. Similar to BM, Pre-DC1 in the spleen during HFD leads to a reduction in IRF8 expression. This indicates that HFD leads to a systemic decrease in IRF8 in cDC1 direct progenitors within the spleen and bone marrow, resulting in a reduction in cDC1 numbers within organs.

The transcription factor IRF8 is required for the development and maturation of immune cells, including dendritic cells, monocytes, macrophages, B cells, and T cells[177]. The IRF8 reduction in DCs progenitors during HFD I observed can be interesting to investigate

in other immune cells. One example of those immune cells is B cells. IRF8 was found to regulate their lineage specification, commitment, and differentiation in B cells from bone marrow[178]. Also, IRF8 knockout mice showed a significant reduction in pre-pro-B-cell numbers [178, 179]. During obesity, B cell development is decreased, and antibody production upon antigen challenge is impaired[180, 181], which can be explained by reduced IRF8 in their progenitors. These previous studies and our findings of reduced IRF8 expression during HFD indicate that obesity can regulate development and differentiation of different immune cells through IRF8 suppression. IRF8 plays a central role in regulating hematopoietic stem cells under HFD.

In the bone marrow microenvironment, cytokines secreted by immune and non-immune cells influence the transcriptional regulation and function of the immune cells[182, 183]. DC development and homeostasis depend on many cytokines. Flt3L and GM-CSF are crucial for cDC development[54, 71, 73]. DCs cultured *in vitro* with GM-CSF (GM-DCs) do not resemble steady-state cDCs but represent DCs that develop under inflammatory conditions[71, 73, 77]. GM-CSF is one of the proinflammatory cytokines secreted under conditions of HFD in humans and mice and modulates many myeloid cells that induce insulin resistance [124, 158]. Many studies have shown that GM-CSF modulates many myeloid cells that cause insulin resistance. During DC development, GM-CSF was found to induce the STAT5/IRF4 pathway and block the STAT3/IRF8 pathway modulated by Flt3[68, 81, 85, 160]. Combining this previous knowledge, I assumed that during HFD, GM-CSF/p-STAT5 suppress IRF8 expression in cDC1 progenitors. To assess that, I measured the concentration of GM-CSF from the BM supernatant using ELISA. I found that the amount of GM-CSF in the BM supernatant was significantly increased under HFD. To confirm these results, I stained intracellular GM-CSF in BM-derived cells, and similar to previous results, the GM-CSF content of immune and non-immune cells was upregulated under HFD.

It was previously described that during pDC development, GM-CSF and STAT5 supersede Flt3L-dependent signals in the early stages of the pDC developmental pathway to block pDC commitment and terminal selection[178]. Promoter-sequence analysis suggested IRF8 as the most likely direct target of GM-CSF-dependent STAT5

signaling. GM-CSF-mediated suppression, though, induces STAT5 recruitment to the IRF8 promoter[84, 178, 184]. Besides, $Stat5^{-/-}$ mice have higher amounts of cDC1 and reduced cDC2 compared to $STAT5^{+/+}$ chimeras in the spleen[161]. In light of these findings, I measured the phosphorylated STAT5 (active protein) in pre-DC1 using intranuclear staining of pSTAT5. I found that pre-DC1 from the HFD group manifests an upregulation of phosphorylated STAT5 compared to the control diet. This confirmed that in our module, GM-CSF produced in the BM environment leads to the alteration of IRF8 in cDC1 progenitors through the activating STAT5. Those observations go in line with previous studies showing that human and murine obesity lead to the upregulation of GM-CSF that induced inflammatory immune cells and insulin resistance in adipocytes. Upon weight loss, GM-CSF production was found to decrease [79, 185].

Similar activation of STAT5 by GM-CSF was described during stimulation with Lipopolysaccharide in monocytes. The activation of STAT5 was indirectly inhibited by anti-inflammatory cytokine IL-10 by suppressing GM-CSF production[186, 187]. Additionally, activation of STAT5 suppresses IL-10 production through blocked STAT3 activation[80]. Obese patients showed lower IL-10 circulating levels, and treatment with IL-10 prevents lipid-induced insulin resistance [188, 189]. Also, GM-CSF was found to drive monocyte transformation to inflammatory macrophages by activation of the STAT5 pathway[81, 190]. Collectively, the GM-CSF/STAT5 axis is essential for activating inflammatory immune cells and suppressing non-inflammatory cells by blocking anti-inflammatory cytokines. This indicates that during obesity, GM-CSF is critical for regulating cDCs development, maturation, and terminal selection, and this can also apply to other myeloid cells that need further investigation in the future.

IRF8 is not only essential for the development of cDC1 but also their functions. cDC1 from IRF8 knockout mice exhibited a significantly impaired phenotype compared with WT[64, 65] by expressing a reduced level of surface marker CD8alpha, costimulatory molecules (like ICAM1, CD40, CD80, CD86), and chemokine receptor CCR7. I found that cDC1 down-regulated XCR1, CD103, and CD80 in HFD mice. Also, I observed that the most significant loss was in CD103. This means that CD103 is not a useful surface marker to track cDC1 during HFD. As most studies focusing on DCs in ADT mainly used CD103

as a marker to study cDC1 in non-lymphoid organs which explain why, in most studies during HFD, cDC1s were neglected and only cDC2 were highlighted. As CD103 significantly decreases, it means smaller cell numbers could be detected. This data illustrated that the remaining cDC1 in ADT show a defect in maturation and function.

Notably, these results could explain the reports that link the increase of lung infection and tumors in obese individuals due to the failure of cDC1 to clear viruses and tumor cells [191-193]. In the lung, DCs play a vital role in initiating adaptive immune responses against infection, commensals, or tissue damage. During an influenza virus infection, cDC1 contribute significantly to the cytotoxic T-lymphocyte response to control and resolve conditions [194]. Patients lacking cDC1 due to IRF-8 genetic mutations are susceptible to infections[195]. Obesity weakens the adaptive immune response to the influenza virus[196].

Moreover, vaccinated obese adults have twice the risk of influenza or influenza-like illness than healthy individuals[197]. DIO mice infected with the influenza virus showed impairment in dendritic cell function and altered CD8+ T-cell responses[198]. Those previous studies, in addition to the results presented here showing that HFD impaired cDC1 development could reduce virus clearance in obese individuals.

Obesity promotes tumorigenesis, and obese patients show lower survival outcomes than lean individuals[199]. cDC1 are also critical for antitumor immunity. cDC1 induce activation of cytotoxic lymphocytes , including CD8+ T cells, NK cells, and NKT cells, which are all essential for antitumor immunity[200]. In the same direction, it was found that IRF8 is inversely correlated with tumor mass and directly related to survival outcome[201]. My results could explain the findings that obesity induces a general reduction in IRF8 expression and suppresses cDC1 development, resulting in tumors in obese patients.[199]. In the same direction, it was found that IRF8 is inversely correlated with tumor mass and directly related to survival outcome[201].

Dendritic cells (DCs) link innate and adaptive immunity communications. For this reason, there is an increased interest in the identification and characterization of DCs in adipose tissue and understanding their function in regulating tissue inflammation during obesity. Although many studies focused on understanding the role of DCs in adipose tissue or

during obesity, some of them are inaccurate, which leads to the misinterpretation of their real functions. One of the main reasons for this is that the identification of DCs is monitored by a high expression of the CD11c cell surface marker[113, 135]. Using only one surface marker, such as CD11c, leads to overlapping with some macrophages and monocytes. The contamination of the DCs population results in the lack of specific identification and poor characterization of DCs in ADT[110, 166]. Also, using only CD11c as a marker makes it impossible to separate between pDC and cDC. Furthermore, to our knowledge, no apparent attempts were made to determine cDC subsets and characterize their functions with obesity. Finally, how cDCs develop from progenitors during high fat intake has not yet been studied.

In summary, our findings reveal a novel mechanism by how obesity impairs cDC1 development in bone marrow, spleen, and adipose tissues. I show that HFD rewires the development of cDCs by downregulating IRF8 in progenitors as early as the CDP stage and continuing until the direct progenitor stage. I further show that HFD leads to a reduction of IRF8 and that this reduction coincides with the induction of STAT5 phosphorylation in pre-DC1 under GM-CSF stimulation. I found that modulation of cDC1 development during HFD results in decreased maturation and costimulatory-associated markers, such as CD80, XCR1, and CD103. cDC1 is essential for many immunological functions, and a functional defect can lead to impaired clearance of infections and tumors. Collectively, my findings provide evidence that HFD alters cDC subsets development and function and that this might contribute to the pathologies associated with obesity. Future studies also need to be made to reveal the role of cDC1 in maintaining ADT hemostasis and non-inflammatory state and how their disfunction within white adipose tissue during DIO leads to induce an inflammatory state. Furthermore, additional investigation of the effectiveness of using new cDC1 vaccination immunotherapy against tumors in obese individuals. Finally, depending on my findings, it might be beneficial to understand how HFD alters cDC subsets to find ways to ameliorate the harmful effects of DIO.

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