Published in final edited form as:

Hepatology. 2009 November ; 50(5): 1412–1420. doi:10.1002/hep.23148.

Immature myeloid cells induced by a high fat diet contribute to liver inflammation

Zhong-bin Deng¹, Yuelong Liu¹, Cunren Liu¹, Xiaoyu Xiang¹, Jianhua Wang¹, Ziqiang Cheng¹, Spandan V Shah¹, Shuangyin Zhang¹, Liming Zhang¹, Xiaoying Zhuang¹, Sue Michalek³, William E. Grizzle², and Huang-Ge Zhang^{1,4,+}

¹Division of Clinical Immunology and Rheumatology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

²Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294

³Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294

⁴Birmingham Veterans Administration Medical Center, Birmingham, AL 35233

Abstract

Chronic inflammation plays a critical role in promoting obesity-related disorders, such as fatty liver disease. The inflammatory cells that mediate these effects remain unknown. This study investigated the accumulation of immature myeloid cells in the liver and their role in liver inflammation. We found that the accumulation of immature myeloid cells, i.e., CD11b⁺Ly6C^{hi}Ly6G[−] cells, in the liver of B6 mice fed a high-fat diet contribute to liver inflammation. Adoptive transfer of CD11b⁺Ly6C^{hi}Ly6G[−] cells isolated from the liver of obese B6 mice, but not from lean B6 mice, resulted in liver damage that was evident by an increase in the activity of liver transferases in serum. CD11b⁺Ly6C^{hi}Ly6G[−] cells isolated from the liver of obese mice are more easily activated via toll-like receptor (TLR) stimulation resulting in interleukin 12 and other inflammatory cytokine expression in a MyD88 dependent fashion. TLR7 activated CD11b⁺Ly6C^{hi}Ly6G[−] cells also enhance liver NKT cell death in a Fas dependent manner. Experiments using mice depleted of Gr-1⁺ immature myeloid cells demonstrated the important role of CD11b⁺Ly6C^{hi}Ly6G[−] in liver inflammation. Repeated injection of exosome-like particles causes CD11b⁺ cell activation and subsequent homing to and accumulation of the cells in the liver. In conclusion, consumption of a high-fat diet by B6 mice triggers an accumulation of immature myeloid cells in the liver. The immature myeloid cells release proinflammatory cytokines and induce NKT cell apoptosis. Activation induced NKT apoptosis further promotes excessive production of Th-1 cytokines. This diet-induced accumulation of immature myeloid cells may contribute to obesity-related liver disease.

Introduction

Obesity is a high risk factor for nonalcoholic fatty liver disease. Studies in an animal model of obesity-related liver disease revealed the involvement of dysfunctioning hepatic immune cells (1). Recent research has implicated the innate immune system in the pathophysiology of obesity-related liver damage (2,3). Toll-like receptors (TLRs), which are present on all resident cells in the liver, act as innate immune sensors of foreign or abnormal structures (4). A number of obesity related factors have been proposed as stimuli that activate the TLR pathway (5). Signaling through these receptors can promote nonalcoholic fatty liver disease

⁺Address correspondence and reprint requests to: Dr. Huang-Ge Zhang, University of Alabama at Birmingham, 1825 University Boulevard, SHEL 306 Birmingham, AL 35294-0007 Huang-Ge.Zhang@ccc.uab.edu.

by inducing expression of a host of proinflammatory mediators (4,6). However, the immune cells releasing these proinflammatory cytokines have not been identified.

Immature myeloid cells ($CD11b^{+}Gr-1^{+}$) play a role in the induction of inflammatory cytokines (7) through activation of innate immune pathways. The role that immature myeloid cell populations play in obesity-related liver disease is unknown. The present study examined the role of immature myeloid cells in obese conditioned nonalcoholic fatty liver disease. We hypothesize that accumulation of immature myeloid cells in the liver may be an important component in the development of inflammatory responses in liver tissue that are triggered by obesity, which in turn contributes to metabolic consequences, such as steatohepatitis.

Material and Methods

Hepatic leukocytes, including myeloid cells and NKT cells, were isolated and sorted using a fluorescent activated cell sorter (FACSVantage, BD PharmMingen). The sorted immature myeloid cells were then used to determine their ability to induce proinflammatory cytokines and NO. In addition the biological effects of the immature myeloid cells was determined employing a number of techniques: 1) NKT cell apoptosis using a FACS based method, 2) inhibition of NKT proliferation by measuring 3H -thymidine incorporation, 3) liver damage by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, and 4) leukocytes infiltration of the liver and liver triglyceride were also evaluated after immature myeloid cells were adoptively transferred into mice. Details of each method and the mice used in this study are described in the supplemental experimental procedures.

Results

A high fat diet induces the accumulation of immature myeloid cells in the liver

To determine whether immature myeloid cells are crucial in mediating inflammatory hepatic steatosis, activation and accumulation of immature myeloid cells in the liver was determined. After six months of feeding mice a high fat diet (HFD), an increased number of leukocytes (myeloperoxidase $^{+}$) and an accompanying increased liver triglyceride were observed in the liver of B6 fed a high fat diet, (Figure 1A). FACS analysis further indicated that $20\pm 2.2\%$ of total leukocytes isolated from liver were $CD11b^{+}Gr-1^{+}$ immature myeloid cells, which was a two times greater number of this cell type than detected in mice fed a regular chow diet (RCD, low fat). A more pronounced increase in the $CD11b^{+}$ with an intermediate intensity stained Gr-1 subpopulation ($CD11b^{+}Gr-1^{int}$, $10\pm 0.8\%$) was detected in the liver of mice fed a HFD when compared with mice fed a RCD, which had only $2.5\pm 0.6\%$ $CD11b^{+}Gr-1^{int}$ cells in the liver (Figure 1B). Similar results were obtained when the livers of three-month old ob/ob mice (obese mouse model) were analyzed for the accumulation of $CD11b^{+}Gr-1^{int}$ cells (Figure 1B). The accumulation of immature myeloid cells was liver specific as $CD11b^{+}Gr-1^{int}$ cell numbers and percentages were unchanged in the spleen, and lung (Data not shown). However, there was a significant increased in the percentage of $CD11b^{+}Gr-1^{int}$ cells circulating in the peripheral blood of B6 mice fed a HFD or ob/ob mice after the six month experimental period (Figure 1C). Analysis of activation associated cell surface markers including CD115, MHCII, F4/80, CD1d, CD36, and CD204 on the liver $CD11b^{+}Gr-1^{int}$ cell population increased significantly in mice fed a HFD and in ob/ob mice compared with mice fed a RCD over the same 6 month period (Figure 1D). These data indicate that the percentages of $CD11b^{+}Gr-1^{int}$ cells are increased in the liver and peripheral blood of HFD fed mice and ob/ob mice and that the cells are activated.

TLR plays a role in the activation of immature myeloid cells

The CD11b⁺ population was further characterized using cells isolated from liver with anti-Ly-6C and anti-Ly-6G Abs. The CD11b⁺ with an high intensity stained Ly6C (CD11b⁺Ly6C^{hi}Ly6G⁻) subset was increased in the liver of HFD fed mice as well as ob/ob mice (Figure 2A) when compared to B6 mice fed a RCD fed mice. The same subset of immature myeloid cells increased in a similar manner when peripheral blood from the HFD and RCD fed mice was analyzed and the results compared (Figure 2A). The TLR pathway is recognized as an important pathway in hepatic steatosis and inflammation associated with obesity (3, 4, 8, 9). TLR4 and TLR7 analogues are potent inducers for activating liver monocytes (10). The CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from HFD fed B6 mice receiving TLR7 analogue stimulation had significantly increased production of TNF- α , IL-12, and IL-6 when compared to the same cell type isolated from RCD fed B6 mice (Figure 2B). When TLR4 analogue was used, similar results were obtained (Data not shown). Induction of an array of inflammatory cytokines is CD11b⁺Ly6C^{hi}Ly6G⁻ specific as no significant induction was observed when CD11b⁺Ly6C^{low}Ly6G⁻ cells were stimulated in an identical manner (Figure 2B). Since activation of the TLR7 in myeloid cells was particularly potent in stimulating NKT cells (10), we focused on the TLR7 for the rest of study.

Accumulation of CD11b⁺Ly6C^{hi} Ly6G⁻ cells inhibits NKT cell proliferation in a NO dependent manner

Activated NKT cells play a role in liver damage (11,12). To determine the effects of liver CD11b⁺Gr-1^{int} cells on the number of NKT cells, the percentage of NKT cells in the liver were determined at specific time points over an 6 month period. Percentages of NK1.1⁺TCR- β ⁺ NKT cells were decreased significantly in the liver of B6 mice fed the HFD (Figure 3A). The decrease of NK1.1⁺TCR- β ⁺ NKT cells in the liver is inversely proportional to CD11b⁺Gr-1^{int} cell numbers (Figure 3B). To further determine whether CD11b⁺Ly6C^{hi}Ly6G⁻ accumulation causes a decrease in NKT cell proliferation, FACS sorted CD11b⁺Ly6C^{hi}Ly6G⁻ cells were co-cultured with liver NKT cells. Following anti-CD3 and anti-CD28 stimulation, NKT cells co-cultured with CD11b⁺Ly6C^{hi}Ly6G⁻ cells demonstrated a reduced proliferation capacity in comparison with NKT cells cultured alone. Co-culturing of NKT cells with CD11b⁺Ly6C^{low}Ly6G⁻ myeloid cells did not cause an inhibition of NKT cell proliferation (Figure 3C). The role of myeloid cell mediated inhibition of T cell proliferation through induction of arginase I and NO induction has been documented (13). As shown in figure 3D, the level of NO in tissue culture fluids was markedly increased when NKT cells were co-cultured with CD11b⁺Ly6C^{hi}Ly6G⁻ cells. When the cells were co-cultured in the presence of a NO inhibitor, but not an arginase I inhibitor, NKT cell proliferation was rescued (Supplementary Figure 1), suggesting that the induction of NO plays a role in the inhibition of NKT cell proliferation. Furthermore, the inhibition of NKT cell proliferation mediated by CD11b⁺Ly6C^{hi}Ly6G⁻ cells decreased as the number of CD11b⁺Ly6C^{hi}Ly6G⁻ cells was reduced (Supplementary Figure 1), suggesting that the inhibition is CD11b⁺Ly6C^{hi}Ly6G⁻ cell dose dependent.

Accumulation of CD11b⁺Ly6C^{hi} Ly6G⁻ cells enhances apoptosis of activated NKT cells in an IL-12 dependent manner

TLR7 stimulated CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from the liver of HFD fed mice co-cultured with isolated NKT cells led to the production of IFN- γ (Figure 4A) and up-regulation of Fas and FasL in NKT cells (Figure 4B). In addition NKT cell apoptosis was induced (Supplementary Figure 2A) suggesting that the NKT cells are being activated. Results of trans-well assays where CD11b⁺Ly6C^{hi}Ly6G⁻ cells and NKT cells were separated by a filter membrane suggested that direct contact of NKT cells with CD11b⁺Ly6C^{hi}Ly6G⁻ is required for IFN- γ production (Supplementary Figure 2B) and NKT cell apoptosis (Supplementary Figure 2C). IL-12 has been known to play a role in

NKT cell activation and our data show that when anti-IL-12 neutralizing antibody, but not a control goat IgG antibody, was added to co-cultures there was a reduction in IFN- γ production (Supplementary Figure 2D). In our experiments a reduction of IL-12 production and Fas expression correlated with NKT apoptosis (Supplementary Figure 2E), suggesting that IL-12 plays a role in CD11b⁺Ly6C^{hi}Ly6G⁻ mediated induction of NKT activation and subsequent activation induced NKT cell death.

Induction of NO and inflammatory cytokines is MyD88 dependent

Next, we examined whether there is a common branching point of the TLR pathway that can regulate induction of both NO and inflammatory cytokines. Our results indicate that NO and inflammatory cytokines contribute to the reduction in NKT cell numbers. Reduced induction of inflammatory cytokines, i.e., TNF- α , IL-6, and IL-12 (Figure 5A), and NO (Figure 5B) was observed when CD11b⁺Ly6C^{hi}Ly6G⁻ cells from the liver of MyD88 knockout (MyD88 KO) B6 mice fed a HFD over 6 months were stimulated with the TLR7 analogue. The stimulated CD11b⁺Ly6C^{hi}Ly6G⁻ cells also have a diminished capacity for inducing NKT cell death when they are co-cultured with liver NKT cells (Figure 5C). Knockout of MyD88 in CD11b⁺Ly6C^{hi}Ly6G⁻ cells also resulted in these cells losing their capability to inhibit NKT cell proliferation (Figure 5D). In addition there was not as much of a decrease in liver NKT cell numbers (Figure 5E), and there was a greater reduction of serum ALT and AST in MyD88 KO mice when compared to wild-type B6 mice even when both groups of mice were fed a HFD over 6 months (Figure 5F). These results suggest that MyD88 plays a role in immature myeloid cell mediated liver damage.

Exosome-like particles isolated from peripheral blood induce the accumulation of immature myeloid cells

Our previous work suggests that myeloid cells can take up tumor exosomes (14). The ability of peripheral blood isolated exosome-like particles to activate CD11b⁺Ly6C⁺Ly6G⁻ cells was examined by determining whether CD204 and CD36 molecules were up-regulated on monocytes. CD11b⁺ monocytes ingested more PKH26 dye labeled exosomes isolated from peripheral blood of HFD fed B6 mice than labeled exosomes from RCD fed B6 mice (Supplementary figure 3A). Exosomes injected twice weekly for 4 weeks led to a significant accumulation of CD11b⁺Gr-1⁺ cells in the peripheral blood (Supplementary figure 3B, left panel) and to the accumulation of CD11b⁺Ly6C^{hi}Ly6G⁻ cells in the liver of B6 mice fed RCD (Supplementary figure 3B, right panel). When using exosomes isolated from HFD fed mice, the percentages of CD11b⁺Ly6C^{hi}Ly6G⁻ cells with upregulated CD204 and CD36 were also markedly increased in the peripheral blood (Supplementary Figure 3C). Serum IL-6 (Supplementary Figure 3D), ALT and AST (Supplementary Figure 3E) were also increased 6 h after the final injection. In contrast, there was no accumulation of CD11b⁺Ly6C^{hi}Ly6G⁻ cells in the liver or increase of serum IL-6, ALT or AST when B6 mice were injected with exosomes isolated from peripheral blood of RCD fed B6 mice (Supplementary Figure 3B, C, D, E).

Adoptive transfer of CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from HFD fed B6 mice causes liver damage and apoptosis of NKT cells

We further tested whether the depletion of CD11bGr-1 cells prevents liver damage; and whether adoptively transferred CD11b⁺Ly6C^{hi}Ly6G⁻ cells home to the liver and caused liver damage. Depletion of Gr-1⁺ cells in B6 mice fed a HFD resulted in a significant decrease in serum AST and ALT levels (Figure 6A). The role of immature myeloid cells in causing liver damage was substantiated by adoptive transfer experiments. Immature CD11b⁺Ly6C^{hi}Ly6G⁻ myeloid cells purified from the liver of HFD fed or RCD fed mice were adoptively transferred into HFD fed B6 recipients having monocytes including CD11bGr-1 cells and macrophages pre-depleted. An increased number of PKH26⁺ labeled

CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from liver of HFD fed B6 mice migrated into the liver of B6 recipients 12 h after adoptive transfer (Figure 6B) when compared to RCD fed mice. Notably, FACS analysis result also is supported by the evidence that an increased number of leukocytes infiltrated in the liver (Figure 6C, left panel). Adoptive transferring of CD11b⁺Ly6C^{hi}Ly6G⁻ myeloid liver cells led to an increased quantity of triglyceride in the liver (Figure 6C, right panel), which is one of the pathological markers of fatty liver shared by all the patients and mouse models. AST and ALT serum levels increased in B6 mice that were recipients of the CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from the liver of HFD fed B6 mice (Figure 6D). In contrast, when CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from the liver of RCD B6 mice or MyD88 KO mice were adoptively transferred to B6 mice, there was not a significant increase of serum AST or ALT (Figure 6D). To exclude the possibility that endogenous MyD88 activation of other cell types caused the liver damage rather than the adoptively transferred CD11b⁺Ly6C^{hi}Ly6G⁻ cells, MyD88 KO mice were recipients of a CD11b⁺Ly6C^{hi}Ly6G⁻ subset of myeloid cells isolated from HFD fed wild-type B6 mice or isolated from MyD88 KO mice fed a HFD. Higher levels of serum ALT and AST were detected in MyD88 KO mice receiving CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from wild-type B6 mice fed a HFD when compared with MyD88 KO mice receiving cells isolated from MyD88 KO mice fed a HFD (Supplementary Figure 4A). The increase in liver specific enzymes in the serum was also correlated with an increase in liver NKT cell apoptosis (Supplementary Figure 4B). These data suggest that MyD88 plays a role in liver damage through myeloid cells and in the induction of liver NKT cell apoptosis.

Discussion

Our studies indicate a previously unidentified role of a subset of immature myeloid cells (CD11b⁺Ly6C^{hi}Ly6G⁻) in obesity related induction of liver damage. The CD11b⁺Ly6C^{hi}Ly6G⁻ possess inducible expression of IL-6, IL-12, and TNF- α . It has been documented that these cytokines, i.e., IL-6, IL-12, and TNF- α (15), and NO (16,17) in the liver microenvironment contribute to chronic inflammation and liver damage. Up-regulation of CD115, CD204, and MHCII on the cell surface is associated with activation of immature myeloid cells. An increased expression of CD1d on the CD11b⁺Ly6C^{hi}Ly6G⁻ cells suggests that these cells could act as antigen-presenting molecules to NKT cells. These results along with several other published reports (10,18), illustrate a potential fundamental mechanism whereby a HFD could trigger activation of CD11b⁺Ly6C^{hi}Ly6G⁻ cells that initiate an inflammatory response. In addition the CD11b⁺Ly6C^{hi}Ly6G⁻ cells could communicate or interact with liver NKT cells in such a way that would lead to the depletion of NKT cells through induction of apoptosis. Interestingly, unlike α -galactosylceramide pulsed CD11b⁺Ly6C^{hi}Ly6G⁻ cells that can stimulate NKT cells to release both IFN- γ and IL-4, TLR7 stimulated CD11b⁺Ly6C^{hi}Ly6G⁻ induce the production of IFN- γ but not IL-4. Similar results have also been reported in TLR9 stimulated myeloid dendritic cells (10).

Although we demonstrated that the TLR7 receptor-mediated pathway plays a role in the induction of CD11b⁺Ly6C^{hi}Ly6G⁻ in a MyD88 dependent manner, using analogues of TLR4, and TLR2 as stimuli, CD11b⁺Ly6C^{hi}Ly6G⁻ cells are also induced (data not shown). TLR4 is unique in that it activates both the MyD88 and TRIF signaling cascades. Therefore, the role of TRIF mediated induction of CD11b⁺Ly6C^{hi}Ly6G⁻ cells can not be ruled out. In addition, although our data reveal a preference for TLR7 and TLR4 in CD11b⁺Ly6C^{hi}Ly6G⁻ cell induction, we however cannot fully eliminate the possibility that other TLRs expressed by CD11b⁺Ly6C^{hi}Ly6G⁻ cells could also be involved in induction of CD11b⁺Ly6C^{hi}Ly6G⁻ cells during obesity development.

Our data correlate with data published by others (19), which shows that some mycobacterial lipids can be incorporated into host cell derived macrophage exosomes and elicit an

inflammatory response. It is speculated that HFD-derived metabolic products such as lipids could be incorporated into exosomes, released from local tissues into the blood and act as TLR ligands with subsequent activation of recipient cells. Exosome function is dependant on the cell type from which they were derived. Our work and others indicate that tumor-derived exosomes may serve as a vehicle for suppressive signals and have negative effects on dendritic cell differentiation and antitumor immune responses (14,20). Interestingly, in both a tumor model and the model we presented in this study, inflammatory cytokines including IL-6 and TNF- α play a role in the disease process. Inflammation has long been recognized as a causative factor for both obesity and cancer. The linkage between immature myeloid cells mediated liver damage in an obese mouse model and their potential in liver cancer needs to be addressed in future studies.

In this study, our data also show that the liver of obese mice is one of the major organs where CD11b⁺Ly6C⁺Ly6G⁻ immature myeloid cells accumulate. It is not clear why these cells are preferentially recruited into the liver. Chemotactic cytokines, chemokines, are small-protein mediators that direct the migration of immune cells including myeloid cells may be responsible for the cell accumulation. Several hepatic cell populations, including hepatocytes, Kupffer cells, sinusoidal endothelial cells and hepatic stellate cells, can secrete chemokines upon activation. High fat diet derived products could activate one of these cells in the liver, resulting in the recruitment of these circulating activated immature myeloid cells into the liver. Since IL-6 is over expressed in the NAFLD patient (21), and IL-6 has been shown to block immature myeloid cell differentiation (14). As a result, these activated cells are accumulated in the liver. The specific chemokines or other factors that play a role in the recruitment of these cells to the liver warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health Tetramer Facility for providing CD1d tetramers, and Dr. Jerald Ainsworth for editorial assistance.

References

1. Sahai A, Malladi P, Pan X, Paul R, Melin-Aldana H, Green RM, Whittington PF. Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. *Am J Physiol Gastrointest Liver Physiol*. 2004; 287:G1035–1043. [PubMed: 15256362]
2. Maher JJ, Leon P, Ryan JC. Beyond insulin resistance: Innate immunity in nonalcoholic steatohepatitis. *Hepatology*. 2008; 48:670–678. [PubMed: 18666225]
3. Szabo G, Mandrekar P, Dolganiuc A. Innate immune response and hepatic inflammation. *Semin Liver Dis*. 2007; 27:339–350. [PubMed: 17979071]
4. Hritz I, Velayudham A, Dolganiuc A, Kodys K, Mandrekar P, Kurt-Jones E, Szabo G. Bone marrow-derived immune cells mediate sensitization to liver injury in a myeloid differentiation factor 88-dependent fashion. *Hepatology*. 2008; 48:1342–1347. [PubMed: 18798338]
5. Vitseva OI, Tanriverdi K, Tchkonja TT, Kirkland JL, McDonnell ME, Apovian CM, Freedman J, et al. Inducible Toll-like receptor and NF-kappaB regulatory pathway expression in human adipose tissue. *Obesity (Silver Spring)*. 2008; 16:932–937. [PubMed: 18292749]
6. Szabo G, Velayudham A, Romics L Jr, Mandrekar P. Modulation of non-alcoholic steatohepatitis by pattern recognition receptors in mice: the role of toll-like receptors 2 and 4. *Alcohol Clin Exp Res*. 2005; 29:140S–145S. [PubMed: 16344599]

7. Delano MJ, Scumpia PO, Weinstein JS, Coco D, Nagaraj S, Kelly-Scumpia KM, O'Malley KA, et al. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med*. 2007; 204:1463–1474. [PubMed: 17548519]
8. Hritz I, Mandrekar P, Velayudham A, Catalano D, Dolganiuc A, Kodys K, Kurt-Jones E, et al. The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adaptor MyD88. *Hepatology*. 2008; 48:1224–1231. [PubMed: 18792393]
9. Velayudham A, Hritz I, Dolganiuc A, Mandrekar P, Kurt-Jones E, Szabo G. Critical role of toll-like receptors and the common TLR adaptor, MyD88, in induction of granulomas and liver injury. *J Hepatol*. 2006; 45:813–824. [PubMed: 16935388]
10. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, Sheehan KC, Capron M, et al. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity*. 2007; 27:597–609. [PubMed: 17950005]
11. Ma X, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol*. 2008
12. Li Z, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology*. 2005; 42:880–885. [PubMed: 16175608]
13. Weigert A, Brune B. Nitric oxide, apoptosis and macrophage polarization during tumor progression. *Nitric Oxide*. 2008; 19:95–102. [PubMed: 18486631]
14. Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, Li C, et al. Tumor exosomes inhibit differentiation of bone marrow dendritic cells. *J Immunol*. 2007; 178:6867–6875. [PubMed: 17513735]
15. Matsushita T, Ando K, Kimura K, Ohnishi H, Imawari M, Muto Y, Moriwaki H. IL-12 induces specific cytotoxicity against regenerating hepatocytes in vivo. *Int Immunol*. 1999; 11:657–665. [PubMed: 10330271]
16. Liu J, Waalkes MP. Nitric oxide and chemically induced hepatotoxicity: beneficial effects of the liver-selective nitric oxide donor, V-PYRRO/NO. *Toxicology*. 2005; 208:289–297. [PubMed: 15691592]
17. Stadler K, Bonini MG, Dallas S, Duma D, Mason RP, Kadiiska MB. Direct evidence of iNOS-mediated in vivo free radical production and protein oxidation in acetone-induced ketosis. *Am J Physiol Endocrinol Metab*. 2008; 295:E456–462. [PubMed: 18559982]
18. Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. CD1d ligation on human monocytes directly signals rapid NF-kappaB activation and production of bioactive IL-12. *Proc Natl Acad Sci U S A*. 2005; 102:11811–11816. [PubMed: 16091469]
19. Giri PK, Schorey JS. Exosomes derived from M. Bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo. *PLoS ONE*. 2008; 3:e2461. [PubMed: 18560543]
20. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res*. 2007; 67:2912–2915. [PubMed: 17409393]
21. van der Poorten D, Milner KL, Hui J, Hodge A, Trenell MI, Kench JG, London R, et al. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. *Hepatology*. 2008; 48:449–457. [PubMed: 18627003]

Figure 1. Immature myeloid precursor cells (CD11b⁺Gr-1^{int}) that accumulate in the liver of obese mice are activated

(A) Shown are representative hematoxylin and eosin (left and middle columns), and anti-myeloperoxidase (right column) stained sections of C57BL/6j mice liver and mean hepatic triacylglycerol content for the two groups. Values are mean $\pm$ SD with 8 C57BL/6j mice per group. (B) Flow cytometry analysis of leukocytes isolated from the liver (upper panels) and peripheral blood (lower panels) of C57BL/6j mice fed a HFD (B, middle column of panels) or RCD (B, left column of panels), or 3-month old ob/ob mice fed with a regular diet (B, right column of panels). A representative FACS analysis (bar graph) of CD11b⁺Gr-1^{int} is shown in B. Numbers adjacent to the outlined areas indicate the percent CD11b⁺Gr-1^{int} cells. Data represent two independent experiments performed in triplicate (**, $p < 0.01$) for liver leukocytes (B, the right panel) or peripheral blood analysis for HFD fed B6 mice (C, left panel) and ob/ob mice analyzed at age of 1, 3, and 6 months (C, right panel). (D) Liver CD11b⁺Gr-1^{int} cells were gated as shown in Figure 1B and screened for expression of CD115, MHCII, F4/80, CD1d, CD36, CD204 and CD31. One representative experiment out of three is shown (D, left panels). Data represent two independent experiments performed in triplicate (**, $p < 0.01$).

Figure 2. TLR7-stimulated CD11b⁺Ly6C^{hi}Ly6G⁻ cells increase the production of inflammatory cytokines

Ly6C^{hi} and Ly6G⁻ cells in gated CD11b⁺ cells isolated from the liver and peripheral blood of B6 fed RCD or HFD over 6 months were analyzed by FACS. The percentages of liver and peripheral blood Ly6C^{hi} and Ly6G⁻ cells in gated CD11b⁺ in 6-month old ob/ob mice were also analyzed. A representative FACS analysis is shown in A (left panel), and the percent of CD11b⁺Ly6C^{hi}Ly6G⁻ cells is shown (A, right panel). Data represent the mean $\pm$ SEM of four samples per group. (B) CD11b⁺Ly6C^{hi}Ly6G⁻ and CD11b⁺Ly6C^{low}Ly6G⁻ cells were sorted from liver leukocytes of B6 mice fed a HFD or RCD, and stimulated in culture for 24 h with a TLR7 analogue (CL097, 1 μ g/ml). Quantification of cytokines using a standard ELISA was done on culture supernatants. Data are given as the mean $\pm$ SEM obtained for five samples in two independent experiments **, $p < 0.001$.

Figure 3. A subset of CD11b⁺ cells (CD11b⁺Ly6C^{hi}Ly6G⁻) inhibits the proliferation of NKT cells in a NO dependent manner

Hepatic leukocytes were isolated from B6 mice at 0, 2, 4, and 6 months after being fed a HFD. The cells were stained and analyzed for TCRβ/NK1.1 (NKT cells) and CD11b/Gr-1 (immature myeloid cells). A representative FACS analysis of TCRβ⁺NK1.1⁺ (A, top panels) and CD11b⁺Gr-1^{int} (A, bottom panels) is shown. The percentage of TCRβ⁺NK1.1⁺ cells in total liver leukocytes is shown (A, histogram graph). The correlation between the percent of TCRβ⁺NK1.1⁺ and CD11b⁺Gr-1^{int} cells was calculated using a linear regression analysis (B). TCRβ⁺NK1.1⁺ cells sorted from the liver of B6 mice and co-cultured at varying ratios with CD11b⁺Ly6C^{hi}Ly6G⁻ cells also sorted from the liver of B6 mice fed a HFD. Co-cultures included anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml). After 48 h in co-culture, 1 μCi of [³H]thymidine was added to each well, the wells incubated overnight, the cell cultures harvested and the proliferation of the NKT cells determined by ³H-thymidine incorporation (C). Supernatants were collected 24 hours after initiating co-cultures and NO was quantified (D). Results represent the mean ± SEM of triplicate cultures, **p < 0.01.

Figure 4. TLR7-stimulated CD11b⁺Ly6C^{hi}Ly6G⁻ cells lead to activation induced NKT cell death

CD11b⁺Ly6C^{hi}Ly6G⁻ and CD11b⁺Ly6C^{low}Ly6G⁻ cells describe in figure 2A were co-cultured for 48 h with sorted liver TCR β ⁺NK1.1⁺ cells (1:1) in the presence of CL097 (1 μ g/ml). The supernatants were collected for quantifying cytokines (A), the cells were stained for analysis by FACS for the expression of CD95 and CD95L (B). Data represent the mean $\pm$ SEM of five independent experiments performed in triplicate.

Figure 5. Effects of MyD88 on the induction of CD11b⁺Ly6C^{hi}Ly6G⁻ derived inflammatory cytokines, reduction of liver NKT cell numbers and HFD-induced liver damage in obese mice (A) 1.5×10^5 CD11b⁺Ly6C^{hi}Ly6G⁻ cells from the liver of wild-type or MyD88 KO B6 mice fed a HFD were FACS sorted and stimulated for 24 h with TLR7 analogue (CL097, 1 μ g/ml). Secreted inflammatory cytokines, including IL-6, IL-12, and TNF- α , were quantified by ELISA (A), and NO release was quantified using the Griess reaction (B). Data are representative of two independent experiments. For NKT cell proliferation, sorted CD11b⁺Ly6C^{hi}Ly6G⁻ cells as described in figure 2A were co-cultured with liver leukocytes at the ratio of 1:1 for 48 h in the presence of CL097 (1 μ g/ml). Induction of apoptosis in TCR β ⁺NK1.1⁺ was determined by FACS analysis (C) and proliferation of NKT cells (D) was determined by a ³H-thymidine-incorporation assay, described in the method section. Data represent the mean $\pm$ SEM of three independent experiments. TCR β ⁺NK1.1⁺ cells from HFD fed wild-type and MyD88 KO B6 mice were analyzed by FACS to determine the percentages of liver NKT cells. One representative graph is shown (E, left panel). Results were pooled from 2 independent experiments (n=5 mice/experiment) and are presented as the mean $\pm$ SEM, ** p < 0.01 (E, right panel). Serum levels of ALT and AST (F) of both groups of mice fed a HFD were also determined at 3 h and 6 h after intravenous injection of CL097 (25 μ g/mouse). Data represent the mean $\pm$ SEM of five mice per group of two separate experiments.

Figure 6. HFD induced accumulation of liver CD11b⁺Ly6C^{hi}Ly6G⁻ cells lead to liver damage in a MyD88 dependent manner

B6 mice fed a HFD over 6 months were depleted of Gr-1 cells by an i.p. injection of 200 μ l of rat anti-mouse Gr-1 antibody (0.4 mg/200 μ l) prepared as described in the method section or injected with a rat IgG as a control. 24 h after the final injection, mice were injected with CL097 (25 μ g/mouse). Serum levels of AST and ALT were then analyzed at 3 and 6 h post injection. Data are mean area $\pm$ SEM; $n = 5$ animals per group (A); * $p < 0.05$ and ** $p < 0.01$ compared with a control group. 4×10^6 PKH26 stained CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from B6 mice fed a HFD or RCD were injected i.v. into B6 mice pre-treated with clodronate to deplete monocytes. 12 h after the adoptive transfer, the percentage of injected PKH26-stained CD11b⁺Ly6C^{hi}Ly6G⁻ cells migrating to the liver was determined by FACS. Data represent the mean $\pm$ SEM of five mice within each group (B); **, $p < 0.01$. Liver tissue sections were HE stained (original magnification $\times 60$) and the content of liver triglyceride was quantified (C). Wild-type B6 mice that received CD11b⁺Ly6C^{hi}Ly6G⁻ cells isolated from the liver of different donors, as labeled in figure 6D, were injected i.v. with CL097 (25 μ g/mouse). Serum levels of AST and ALT were then analyzed at 3 and 6 h post injection. Results obtained from two independent experiments ($n=5$) were pooled and are presented as the mean $\pm$ SEM (D).