B-1a lymphocytes attenuate insulin resistance

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ABSTRACT

Obesity associated insulin resistance, a common precursor of type 2 diabetes, is characterized by chronic inflammation of tissues including visceral adipose tissue (VAT). Here we show that B-1a cells, a subpopulation of B lymphocytes, are novel and important regulators of this process. B-1a cells are reduced in frequency in obese high fat diet (HFD) fed mice, and eGFP IL-10 reporter mice show marked reductions in anti-inflammatory IL-10 production by B cells in vivo during obesity. In VAT, B-1a cells are the dominant producers of B cell derived IL-10, contributing nearly half of the expressed IL-10 in vivo. Adoptive transfer of B-1a cells into HFD B cell-deficient mice rapidly improves insulin resistance and glucose tolerance through IL-10 and polyclonal IgM dependent mechanisms, whereas transfer of B-2 cells worsens metabolic disease. Genetic knockdown of B cell activating factor (BAFF) in HFD fed mice, or treatment with a B-2 cell depleting, B-1a cell sparing anti-BAFF antibody attenuates insulin resistance. These findings establish B-1a cells as a new class of immune regulators that maintain metabolic homeostasis, and suggest manipulation of these cells as a potential therapy for insulin resistance.
INTRODUCTION

Type 2 diabetes mellitus currently afflicts 257 million people worldwide and this number is expected to almost double by 2030 (1). Obesity-associated insulin resistance (IR) is considered to be the primary defect in the natural history of type 2 diabetes (2). While many factors appear to govern the pathogenesis of IR, chronic low grade inflammation in insulin sensitive tissues, such as the liver and visceral adipose tissue (VAT), appears to play a central role (3). Multiple studies have shown links between increased levels of pro-inflammatory cytokines (IL-6, TNF-α, IFN-γ) and worsened insulin resistance (4–6). Conversely, anti-inflammatory cytokine expression (IL-10 and IL-4) is associated with better glucose control (7–9). Similarly, immune cells with anti-inflammatory phenotypes (alternatively activated M2 macrophages, Th2, Treg) are resident in the adipose tissue of lean mice and individuals, while pro-inflammatory cells (classically activated M1 macrophages, Th1) become enriched and expanded in the adipose tissue of obese subjects (3, 6, 10, 11). Lastly, adipose cells are themselves capable of producing immune-related cytokines such as IL-6, IL-18 and BAFF (12–15). Thus, the complex interactions between innate and adaptive immune cells and adipocytes play a major role in insulin resistance.

We have previously shown in diet-induced obese mice that total CD19+ B cells and HFD-associated IgG antibodies are pathogenic in insulin resistance, and that B cell depleting therapy can alleviate disease (16). B cells also promote systemic and T cell mediated inflammation in obese mice and humans (9). B cells can be divided into 2 broad classes, B-1 or B-2 cells; B-1 cells can be further classified as B-1a and B-1b cells (17). B-2 cells are the conventional adaptive B cells that produce antibodies to T cell-dependent antigens and are enriched in secondary
lymphoid organs. B-1 cells are enriched in mucosal tissues, pleural and peritoneal cavities (PerC), and produce natural antibodies, which are a first line of defense against pathogens (17, 18). B-1a cells contribute 80% of the natural circulating IgM in the blood of mice (19) and make up the bulk of IL-10 expressing leukocytes in the peritoneal cavity (20). Recently, human B-1 cells have been identified in umbilical cord and adult peripheral blood based on functional criteria that they share with mouse B-1 cells (21).

Coupled with the fact that B cells are a nonredundant source of IL-10 (20, 22) and that B cells from diabetic patients and obese mice demonstrate an impaired anti-inflammatory cytokine profile (9), we hypothesized that B-1a cells might play an important role in glucose metabolism. Here we show that in opposition to B-2 cells, B-1a cells are novel immune regulators that protect against insulin resistance. The protective effects of these cells are mediated by IL-10 and polyclonal IgM, and these functions are impaired in obese mice. Depletion of B-2 cells in BAFF knockout mice and BAFF antibody treatment ameliorated insulin resistance in these mice. These discoveries suggest that B-2 depleting, B-1a sparing therapies could prove useful in type 2 diabetes.
RESEARCH DESIGN AND METHODS

Mice. C57BL/6, B cell deficient μMT (B6.129S2-Igh-6tm1Cgn/J), IL-10 eGFP (B6(Cg)-Il10 tm1.1Karp /J) and IL-10-deficient (B6.129P2-Ili10tm1Cgn/J) mice were purchased from Jackson Laboratory. sIgMnull mice (B6;129S4-Ighm tm1Che /J) were a gift from Troy Randall, University of Rochester. BAFF deficient mice (B6.129S2-Tnfsf13btm1Msc/J) were a gift from Mark Krasnow, Stanford University. The mice were maintained in a pathogen-free, temperature-controlled environment on a 12-h light and dark cycle. The mice received either NCD (LabDiet, 15 kcal% fat) or HFD (Research Diets, 60 kcal% fat) beginning at 6 weeks of age. Mice fed a HFD for at least 6 weeks were considered obese. All mice used in comparative studies were males and were age-matched within individual experiments. All protocols were approved by the Institutional Animal Care and Use Committee of Stanford University.

B cell transfer. To obtain B-2 cells, spleens were mechanically dissociated on 40-μm nylon cell strainers; this was followed by negative selection with EasySep™ Mouse B Cell Enrichment Kit which depleted CD43+ cells (Stemcell Technologies). B-2 cell purity was >90% as determined by flow cytometry. To obtain B-1a cells, peritoneal cells were harvested by injecting 7 mL of RPMI, no phenol (Lonza), plus 3% newborn calf serum into PerC. CD19+ CD22+ CD5+ B-1a cells were sorted as previously described to about 95% purity (23). For B-2 vs B-1a experiments, 5 x 10^6 B-2 and B-1a cells were injected i.p. For other transfer experiments, 3 x 10^6 cells were used.

Metabolic studies. Glucose tolerance test (GTT), insulin tolerance test (ITT), and serum insulin were measured as previously described (6). For intraperitoneal GTTs, mice were fasted for 14 hours with access to drinking water and then injected i.p. with 2g of glucose per kg body weight.
Blood glucose from the tail tip was measured using a blood glucose meter just before glucose injection and every fifteen minutes thereafter. For ITT, mice were fasted for 5 hours and then given 1 U per kg body weight human regular insulin (Eli Lilly). Serum insulin was measured using the Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem).

**Isolation of VAT-associated immune cells and VAT lysates.** VAT-associated immune cells were isolated from epididymal fat pads as previously described (6). VAT lysates were prepared as previously described (16).

**Cell cultures.** Unless otherwise indicated, 300,000 cells were cultured in 200 uL complete RPMI in a 96 well round bottom plate for 24 hours at 37°C, 5% CO₂. Where noted, cells were stimulated with 1 ug/mL LPS (Sigma-Aldrich). For macrophage-B-1a co-cultures, macrophages (CD19⁻ CD11b⁺ F4/80⁺) and B-1a cells (CD19⁺ F4/80⁻ CD5⁻) were sorted by FACS and co-cultured at a 1:3 ratio for 60 hours.

**Cytokine and antibody measurement.** Cytokines and antibodies were measured by ELISA (eBioscience and Bethyl, respectively) and cytometric bead array (BD Biosciences) according to vendors’ instructions. The anti-PC IgM antibody ELISA was adapted from a previous protocol (24). Antigens were diluted to 5 ug/ml in assay buffer (PBS containing 0.27 mM EDTA and 1% BSA) and applied to a polystyrene EIA plate (Costar) overnight at 4°C. Wells were washed with PBS containing 0.27 mM EDTA and blocked with assay buffer. Detection antibody for the mouse anti-PC IgM ELISA was HRP-conjugated goat anti-mouse IgM and for the human anti-PC ELISA was HRP-conjugated goat anti-human IgM (Bethyl). For the mouse anti-PC IgM ELISA, E06 IgM (Avanti Polar Lipids) was used as standard. For the human anti-PC IgM ELISA, results were reported as optical density (OD). Antigens probed for the dosage curve were
BSA (Sigma-Aldrich), PC-BSA (Biosearch Technologies), human LDL and human high ox-LDL (Kalen Biomedical), and MDA-LDL (Cell Biolabs).

**Human subjects.** We obtained sera from 62 age and BMI matched overweight to obese male and female subjects (mean age IR: 54 ± 9, IS: 54 ± 7; mean BMI IR: 30.9 ± 2.4 kg per m2; IS: 30.3 ± 2.4 kg per m2). Insulin sensitivity was determined by a modified insulin suppression test and defined as either IR or IS based on steady-state plasma glucose (SSPG) levels falling in the top (IR) or bottom (IS) 40th percentile (25). Serum samples were obtained under approval by the Stanford Internal Review Board (IRB) for Human Subjects.

**Flow cytometry.** We used the following gating schemes: total leukocytes (CD45.2^+^), total B cells (CD19^+^ CD3^-^), B-2 (CD19^+^ CD3^-^ B220^hi^ CD5^-^ CD23^-^), B-1a (CD19^+^ CD3^-^ IgM^-^ IgD^-^ B220^lo^ CD5^-^ CD23^-^), B-1b (CD19^+^ CD3^-^ IgM^-^ IgD^-^ B220^lo^ CD5^-^ CD23^-^), Breg (CD19^+^ B220^-^ CD22^-^ CD5^-^ IgM^-^ IgD^-^), total T cells (CD19^-^ CD3^+^), Tregs (CD19^-^ CD3^+^ CD4^-^ CD25^-^) and macrophages (CD19^-^ CD3^-^ CD11b^-^ F4/80^-^). Dead cells were distinguished by Live/Dead Fixable Aqua staining (Life Technologies). Baselines for IL-10 eGFP mice were set using age and diet-matched C57BL/6J mice. For macrophage intracellular cytokine staining, cells were stimulated with LPS (1 ug/ml, Sigma-Aldrich) and Brefeldin A (5 ug/ml, Biolegend) overnight and stained using the Cytofix/Cytoperm Kit according to vendor’s instructions (BD Biosciences). Data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

CD16/32, CD3-PacBlue, CD5-PE-Cy5, CD19-PerCP-Cy5.5, CD22-PE, CD23-PE-Cy7, CD25-PE, CD45.2-APC, B220-APC-Cy7, F4/80-PE, F4/80-PerCP-Cy5.5, IgD-PE, and TNF-α-PE antibodies were from Biolegend. IgM-efluor650, CD4-efluor650, and CD11b-efluor605
antibodies were from eBioscience.

**IgM treatment.** We modified a previous protocol for IgM treatment of atherosclerosis (26). 13 week old HFD-fed B\textsuperscript{null} mice were given 400 ug of mouse polyclonal IgM (Rockland Immunochemicals) or 200 ug mouse monoclonal anti-PC IgM (Clone E06, Avanti Polar Lipids) i.p. on days 0, 4, 7 and 11. The polyclonal IgM dose was chosen based on the observation that RAG-/- mice reconstituted with 0.4 mg of IgM have similar serum IgM levels to WT mice (27) and the half-life of IgM is 2-3 days (26, 28). 200ug of monoclonal anti-PC IgM is 5X the expected amount of anti-ox-LDL IgM in 400 ug of polyclonal IgM (29). Control mice received PBS or 400 ug isotype control (Clone TEPC 183, Sigma-Aldrich). For macrophage-IgM cultures, polyclonal IgM and isotype control were used at 0.5 ug/mL.

**BAFF depletion with BAFF mAb.** 200 ug per mouse BAFF/BLys-specific mAb (10F4, GlaxoSmithKline) or isotype control (hamster IgG1) was administered i.p. to mice fed HFD for 6 weeks on days 0 and 4.

**Real-time PCR analysis.** Tissue was dissociated in Trizol (Life Technologies) and RNA was extracted. RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primer pairs and probes, including their specificity, orientation (forward, F; reverse, R), and sequence were as follows: HPRT1 (F- TGGATACAGCCAGACTTTGTT, R-CAGATTCACCTTGCCGTCATC), IL-10 (F- TTTGAATCTCCCTGGGTGAGA, R-AGACACCTTTGGTCTGGAGC), IL-6 (F- GATGGATGCTACCAAACCTGGA, R- TCTGAACGGACTCTGGCTTTT). Quantitative RT-PCR was performed using SYBR select (Applied Biosystems) on a QuantStudio 6 Flex machine (Applied Biosystems). Results were normalized to HPRT1 expression.
**Statistics.** The unpaired Student’s t-test was performed, * P<0.05, ** P<0.005, *** P<0.0005, **** P<0.0001. Area under the curve (AUC) analysis was performed with correction for starting glucose level.
RESULTS

B-1a cells protect against glucose intolerance

To examine the effects of diet induced obesity on B cells, we placed C57BL/6J mice on a normal control diet (NCD) or 60kcal% HFD for 9 weeks, which reliably results in obesity-associated IR and glucose intolerance (16). We subsequently compared the frequencies of CD19+ B cell subpopulations in the PerC, VAT and spleen and found that HFD induced a significant increase in the relative percentage of B-2 cells, and a reduction in the percentage of B-1a cells in the PerC and VAT, but not the spleen (Fig. 1a). Absolute cell counts of the B cell subpopulations indicated that HFD led to a significant increase of B cells in the VAT, particularly B-2 cells (Fig. 1b).

To assess the effects of distinct B cell subsets on glucose intolerance, we sorted B-1a cells from the PerC and B-2 cells from the spleen of HFD mice (Fig. S1) and transferred 5 x 10^6 of one or the other population into 15 week old HFD B cell deficient (B^null) mice by i.p. injection. Control mice received PBS. When we examined the mice one week post transfer, there were no differences in weight between all groups (Fig. 1c). Flow cytometry confirmed the presence of B-1a cells in the PerC and the VAT but not the spleen while B-2 cells were present in all 3 tissues (Fig. S2). Consistent with our previous findings (16), B-2 cells worsened glucose intolerance and increased serum fasting insulin compared to controls (Fig. 1d,f). Remarkably, B-1a cell transfer had the opposite effect: B-1a cells induced marked improvement in glucose tolerance, relative improvement in insulin tolerance and reduced fasting insulin (Fig. 1d-f). These results suggest that B-1a cells protect against glucose intolerance. Given that the half-life of IgM is 2 days (28), detection of serum IgM and also IL-10 in culture supernatants from PerC cells in B-1a recipient
mice confirmed that the transferred B-1a cells remained viable and capable of producing IL-10 and IgM in recipients for at least one week (Fig. 1g,h).

**The protective effect of B-1a cells on glucose control is IL-10 dependent**

Since B-1a cells are important producers of B cell derived IL-10, we next examined how diet induced obesity influences IL-10 production by these cells *in vivo*. IL-10 eGFP reporter mice on the C57BL/6 background were placed on NCD or HFD for 9 weeks. PerC, VAT, and spleen-resident leukocytes were analyzed for IL-10 expression *ex vivo*. The results revealed a significant reduction in the frequency of IL-10-GFP^+^ total B cells and B-1a cells in the PerC. B-1a cells were also reduced in the VAT and spleen of HFD mice (Fig 2a-c). These cells were distinct from a recently identified population of IL-10-producing CD19^+^ B220^+^ CD22^+^ CD5^−^ IgM^+^ IgD^+^ adipose natural regulatory B (Breg) cells (22). When we examined the CD19^+^ B220^+^ IL-10-GFP^+^ population, we found that in the PerC and VAT 40-50% of these cells were B220^lo^ CD5^+^ IgM^+^ IgD^−^ B-1a cells whereas 12-20% were of the Breg cell phenotype (Fig. S3a).

We isolated total PerC and VAT cells from wild type (WT) C57BL/6J mice fed NCD or HFD for 9 weeks and cultured them for 48 hours. Cells from HFD mice showed decreased IL-10 in PerC (Fig. 2d) and VAT (Fig. 2e) cultures compared to NCD-fed mice. In addition, VAT cell cultures showed increased TNF-α production. Given that B cells comprise a major proportion of CD45^+^ leukocytes in the PerC (Fig. S3b), the loss of IL-10 production in B cells, and particularly in the B-1a cell subset, may explain the reduction in total PerC culture IL-10 production in HFD animals.

We hypothesized that the protective effect of B-1a cells on insulin resistance might be mediated
by IL-10. To study this, we adoptively transferred $3 \times 10^6$ B-1a cells isolated from HFD WT or HFD IL-10$^{null}$ mice i.p. into HFD B$^{null}$ recipient mice and the mice were monitored for metabolic parameters. One week post transfer, there was no difference in weight in recipient groups (Fig. 2f). IL-10$^{null}$ B-1a recipients exhibited partial restoration of glucose sensitivity, which is consistent with a protective role for IL-10 (Fig. 2g,h). IgM production by IL-10$^{null}$ B-1a cells was not significantly different from IgM production by WT B-1a cells (Fig. 2i). Taken together, these results indicate that the protective effect of B-1a cells is partially dependent on IL-10.

Next, we explored the interplay between B cells and other known immune mediators of insulin resistance. Macrophages have been well established as effectors of insulin resistance (30). In lean mice, M2 macrophages, which produce anti-inflammatory cytokines such as IL-10, are enriched in adipose tissue (31). HFD causes accumulation of pro-inflammatory M1 macrophages in the adipose tissue of obese mice and humans (10, 31, 32). M1 macrophages are a major source of TNF-α, which alters insulin receptor signaling and induces insulin resistance (5). To determine if B-1a-derived IL-10 influences macrophage pro-inflammatory cytokine production, we stimulated VAT macrophages isolated from recipients of WT or IL-10$^{null}$ B-1a cell transfers. Intracellular cytokine staining showed that TNF-α production by VAT macrophages in mice that received WT B-1a cells was lower than production by VAT macrophages isolated from mice that received IL-10$^{null}$ B-1a cells (Fig. 2j).

To explore this notion further, we cultured WT macrophages isolated from either the VAT or PerC of HFD mice with WT or IL-10$^{null}$ B-1a cells. WT macrophage-WT B-1a co-cultures yielded much more IL-10 than the WT macrophage-IL-10$^{null}$ B-1a co-cultures (Fig. 2k). The amount of IL-10 in the WT macrophage-WT B-1a co-cultures was considerably greater than the
sum of IL-10 produced by WT macrophages alone and WT B-1a cells alone, suggesting a synergy between macrophages and B-1a cells that might explain why B-1a-derived IL-10 is effective in controlling glucose intolerance. A similar anti-inflammatory effect, measured by a reduction in TNF-α and IL-6, was seen in co-cultures of VAT or PerC macrophages with WT B-1a cells (Fig. 2l,m). This reduction was partially dependent on B-1a-derived IL-10 since IL-10null B-1a cells were significantly less effective at suppressing pro-inflammatory cytokine production by macrophages. These findings point to an important role for B-1a cells in regulating inflammatory and anti-inflammatory cytokine production by macrophages.

The protective effect of B1a cells is also dependent on polyclonal IgM

Given that B-1a derived natural IgM has anti-inflammatory properties in other inflammatory diseases such as atherosclerosis (24), we hypothesized that, in addition to IL-10, secretion of IgM by B-1a cells may play a role in insulin resistance. Indeed, B-1 cells from obese db/db mice show an impaired IgM response which may be due to high glucose levels (33). To explore this, we obtained secretory IgM-deficient (sIgMnull) mice whose B cells do not secrete IgM but still express surface IgM and IgD and undergo class switching to express other Ig isotypes (34), allowing us to study the effect of B cells on insulin resistance separately from any effect of IgM. We transferred 3 x 10⁶ B-1a cells or B-2 cells isolated from HFD WT or sIgMnull mice into HFD Bnull recipient mice. One week after transfer, WT B-2 and sIgMnull B-2 cells both worsened glucose tolerance in recipient mice, WT B-1a cells improved glucose tolerance, while transfer of sIgMnull B-1a cells resulted in an intermediate phenotype (Fig. 3a-c). IgM was only detectable in the serum and VAT lysate of WT B-1a recipients but not sIgMnull B-1a recipients (Fig. 3d, e).
These results suggest that IgM secretion contributes to the promotion of glucose tolerance by B-1a cells.

Anti-atherosclerosis IgM antibodies directed against ox-LDL recognize a lipid group called phosphorylcholine (PC) (24). As approximately 10% of natural IgM in mice binds to ox-LDL (29), we hypothesized that polyclonal IgM, as well as anti-PC monoclonal IgM antibody (E06) (24), would improve glucose tolerance. After 2 weeks of IgM treatment, there was no statistical difference in weight in all groups (Fig. 3f). Recipients of 400 ug of polyclonal IgM showed significantly better glucose tolerance than PBS or isotype treated controls as well as mice that received 200 ug of anti-PC IgM (Fig. 3g). The former also showed improved fasting insulin (Fig. 3h). Hence, polyclonal IgM improves glucose tolerance, but the relevant target antigen does not appear to be a major epitope commonly studied in atherosclerosis. Indeed, PC-BSA-specific IgM ELISA indicated that most of the polyclonal IgM did not bind to PC or ox-LDL (Fig. S4). We also investigated the levels of anti-PC IgM in NCD vs HFD mice, as well as in a cohort of 62 overweight to obese human male and female subjects who differed from one another only in their insulin resistance status, as determined by a modified insulin-suppression test (16). There was no difference in the levels of serum anti-PC IgM in NCD vs HFD mice (Fig. 3i) or between insulin resistant (IR) vs insulin sensitive (IS) humans (Fig. 3j). To determine if polyclonal IgM affected macrophages, we cultured PerC macrophages with polyclonal IgM or isotype control. Compared to isotype-treated macrophages, polyclonal IgM-treated macrophages produced significantly less TNF-α, MCP-1 and IL-6 but showed no changes in IL-10 production (Fig. 3k).

**BAFF deficiency and BAFF depleting therapy improve glucose tolerance**

Insulin resistance can be reversed by depleting mature B cells using an anti-mouse CD20
depleting antibody (16). However, this therapy partly depletes the B-1a population and is also immunosuppressive. Hence, a safer and more efficacious option might be to deplete the B-2 compartment selectively, leaving the B-1a cells intact. BAFF is required for the maintenance of mature B-2 cells in the periphery (35) and BAFF knockout mice have a deficiency of B-2 cells and a relative increase of B-1a and CD23- CD5- B cells (Fig. S5a). To study the effect of BAFF on glucose tolerance, we first administered a HFD to WT, BAFF knockout and Bnull mice for 9 weeks and performed GTT and ITT on these mice (Fig. 4a-c). BAFF knockout mice showed dramatic improvement in glucose tolerance and insulin resistance compared to both WT and B cell deficient control mice. This result suggested that B-1a cells could potentially account for the difference.

Next, to determine if B-1a cells could be therapeutically manipulated, we treated HFD WT mice with a monoclonal hamster anti-mouse BAFF antibody (clone 10F4) or isotype control hamster IgG, while maintaining the mice on HFD. BAFF antibody caused a relative depletion of B-2 cells in the peripheral blood, spleen, and PerC up to 7 weeks after the first dose and also a reduction in absolute cell counts of total B cells and most B cell subsets. (Fig. S5b-d). BAFF antibody-treated mice showed improved glucose tolerance compared to the controls (Fig. 4d-f) and depletion of IgG in serum and VAT lysate (Fig. 4g,h). BAFF-antibody-treated mice also displayed a marked anti-inflammatory cytokine profile in PerC and spleen cell cultures (Fig. 4i,j), consistent with a state of reduced systemic and local inflammation, in spite of HFD. VAT mRNA expression displayed the same trend although the differences were not statistically significant (Fig. 4k).
DISCUSSION

This study reveals a novel role for B-1a cells in modulating glucose intolerance in opposition to pathogenic B-2 cells. Consistent with a previous report (33), we observed that HFD resulted in decreased frequency of B-1a cells in the PerC and VAT, but not the spleen. At the same time, total B cells and B-2 cells increased in the VAT, in agreement with our previous findings (16). In our previous work, transfer of total CD19$^+$ cells from the spleen of HFD mice worsened insulin resistance in recipient mice (16). B-2 cells comprise the dominant B cell subset in the spleen and were likely responsible for the previously observed effect of total B cells. Indeed, in the current study when we transferred B-1a or B-2 cells, they played opposing roles in insulin resistance. These results are similar to those seen in atherosclerosis, a disease that is also characterized by chronic inflammation (36). Hence, obesity appears to simultaneously impair regulatory B-1a cells and cause the expansion of pathogenic B-2 cells.

We also observed that B-1a cells in obese mice produce less IL-10, in keeping with findings that B cells from obese mice and diabetic patients express a pro-inflammatory cytokine profile (9). B cell-derived IL-10 has been shown to be important in regulating autoimmune and inflammatory diseases, including EAE and arthritis (37, 38). The failure of IL-10-deficient B-1a cells to protect against glucose intolerance suggests that the same holds true for insulin resistance. We also found that IL-10-deficient B-1a cells were unable to stimulate macrophages to produce IL-10 or reduce IL-6 and TNF-α secretion. This finding is interesting as several studies have intimately linked classically activated M1 macrophages with insulin resistance (10, 31, 39). B-1a cells can polarize macrophages toward an alternatively activated phenotype (40) and can down regulate macrophage phagocytic activity (41) and pro-inflammatory cytokine production (42). In obese
mice lacking mature T and B lymphocytes and NK cells, macrophages are incapable of causing insulin resistance (43). Hence, the defect in B-1a production of IL-10 may exacerbate the pro-inflammatory state of macrophages in obese individuals.

One intriguing finding is that polyclonal IgM reduces glucose intolerance, but apparently without involvement of ox-LDL. Natural IgM antibodies are circulating IgMs that arise without known immune exposure or vaccination (44). A substantial portion of these antibodies recognize PC and malondialdehyde groups which are present on oxidation-specific antigens such as ox-LDL (29) and materials shed by apoptotic cells (45). Natural IgM is believed to prevent inflammation by recruiting macrophages and dendritic cells to clear apoptotic debris (24, 45). Obesity and insulin resistance are associated with hypoxic and apoptotic adipocytes, and IgM may help in the clearance of such debris (46). Moreover, deposition of anti-ox-LDL natural IgM in atherosclerotic lesions helps neutralize ox-LDL and attenuates disease (23, 24, 47). It was therefore surprising to find that treating HFD B\textsuperscript{null} mice with polyclonal IgM, but not monoclonal IgM against PC, improved glucose tolerance. Additionally, the level of anti-PC IgM was no different between obese and lean mice, or between IS and IR patients. These results do not exclude the possibility that IgM antibodies directed against other potential targets, might mediate the beneficial effect of the class.

In BAFF-deficient mice, which show normal levels and function of B-1a and B-1b cells but markedly reduced B-2 cell frequency (35), we found improved glucose tolerance above and beyond that seen in B\textsuperscript{null} mice. We also found that treatment of HFD mice with a monoclonal anti-mouse BAFF antibody improved glucose metabolism, though not to the same extent as genetic knockout of BAFF. This might have been because BAFF antibody was less efficient at
depleting splenic B-2 cells, although it is also possible that the remaining CD23+ B cells were transitional T2 and T3 B cells (48). Anti-BAFF therapy is used for the treatment of systemic lupus erythematosus where excess BAFF is associated with a loss of self-tolerance and production of autoantibodies. BAFF has also been shown to induce production of pro-inflammatory cytokines in adipocytes as well as monocytes (14, 49) resulting in insulin resistance. Hence, BAFF depleting therapy, through its direct anti-inflammatory actions and its impact on the ratio of B-1a to B2 cells, may be effective in treating glucose intolerance. In a recent study, short term treatment of 5 lupus patients with Belimumab resulted in a modest reduction in HOMA IR, although the effect was not statistically significant (15). Thus, larger clinical trials in different patient populations will be needed to determine the potential utility of this drug in the treatment of glucose intolerance.

In a recent report, Nishimura et al described a unique subset of regulatory B cells in the adipose tissue of mainly lean mice that produce IL-10 constitutively and contribute to the maintenance of glucose homeostasis via IL-10 (22). These CD19+ B220+ CD22+ CD5− IgM+ IgD+ cells are distinct from the B1a cells studied here, not only in their surface phenotype but also in their tissue distribution. Additionally, it is not known whether they produce IgM or are BAFF-dependent. Nonetheless, it is interesting that two distinct populations of B cells can regulate glucose metabolism through the secretion of IL-10. Since the cells described by Nishimura et al reside only in adipose tissue and apparently do not circulate, these cells likely function locally while the B1a cells described in our study serve to regulate glucose metabolism more broadly. Moreover, in our study IL-10 secreting B1a cells accounted for the majority of B cell derived IL-10 in VAT in vivo, and were at least 5 times more contributory than the cells described by
Nishimura et al. For these reasons, we believe that B1a cells may be the predominant B cell population involved in regulating glucose metabolism, at least under the conditions studied here.

Collectively, our data support a model in which B-1a cells oppose B-2 cells and promote insulin sensitivity through production of IL-10 and natural IgM to modulate macrophage and T cell mediated inflammation. These functions become impaired in obesity, leading to chronic low-grade systemic and local tissue inflammation, which fuels insulin resistance. Thus, B-1a cells represent a novel immune subset governing glucose tolerance, and provide an important link in the complex interplay of immunity and metabolism.
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FIGURE LEGENDS

Fig 1. B-1a cells play a protective role against glucose intolerance. (A) Frequency of CD19^+ B cell subpopulations in PerC (left), VAT (middle), and spleen (right) from C57BL/6J mice fed NCD or HFD for 9 weeks (n=5, representative of 3 experiments). (B) Absolute cell counts of B cell subpopulations fed NCD or HFD (n=5). (C) Body weights, (D) GTT with area under the curve analysis (AUC), (E) ITT, and (F) fasting insulin levels of HFD B^null recipients, 1 week after receiving PBS, B-1a or B-2 cells (n=5). (G) IL-10 concentration in 24h PerC culture supernatant (n=5). (H) Serum IgM concentration (n=5). Values are given as mean ± SEM.

Fig 2. IL-10 production is impaired in obese mice. (A-C) Frequency of GFP^+ cells within each subpopulation in PerC, VAT and spleen from IL-10 eGFP mice fed NCD or HFD for 9 weeks (n=5-6, representative of 3 experiments). (D, E) IL-10 and TNF-α concentration in 48h PerC and VAT culture supernatants (n=6). (F) Body weights, (G) GTT with AUC, and (H) fasting insulin of HFD B^null mice, 1 week after receiving either PBS, WT B-1a or IL10^null B-1a cells (n=5). (I) Serum IgM concentration 1 week after B-1a cell transfer (n=5). (J) TNF-α production in VAT macrophages. FACS plots are representative of 2 experiments. (K-M) Cytokine concentrations in 60h co-culture of WT PerC and VAT macrophages with WT or IL-10^null B-1a cells (n=3 per group, 2 replicates). Values are given as mean ± SEM.

Fig 3. Polyclonal IgM but not monoclonal anti-PC IgM ameliorates glucose intolerance.
(A) Body weights and (B) GTT with AUC 1 week after receiving PBS, WT B-1a, sIgM^null B-1a, WT B-2 or sIgM^null B-2 cells (n=5-8). (C) Fasting insulin (D) IgM concentration in serum and (E) VAT lysate 1 week after B-1a cell transfer (n=4). (F) Body weights, (G) GTT with AUC, and (H) fasting insulin of HFD B^null mice, 1 week after receiving either PBS, isotype control,
polyclonal IgM, or E06 monoclonal anti-PC IgM (n=5 for E06 treatment, n=15 for the rest). (I) Anti-PC IgM in serum from NCD and HFD mice (n=10). (J) Anti-PC IgM in serum from IR and IS obese human males (n=32 and 30). (K) Cytokine concentrations in 24h supernatants from PerC macrophages cultured with isotype control or polyclonal IgM (n=3). Values are given as mean ± SEM.

**Fig 4. BAFF deficient and anti-BAFF antibody-treated obese mice exhibit superior glucose metabolic control compared to WT and B\textsuperscript{null} mice.** (A) Body weights, (B) GTT with AUC, and (C) ITT of HFD WT, BAFF\textsuperscript{null} and B\textsuperscript{null} mice (n=5). (D) GTT with AUC, (E) Body weights, and (F) fasting insulin of HFD WT mice 4 weeks after they received anti-BAFF antibody or isotype control (n=5). (G) IgG concentration in serum and (H) VAT lysate 5 weeks after anti-BAFF antibody treatment (n=5). (I,J) Cytokine concentrations in 24h cultures of PerC or spleen cells stimulated with LPS from isotype control and BAFF Ab-treated mice (n=5). (K) mRNA expression in VAT from isotype control and BAFF Ab-treated mice (n=4). Values are given as mean ± SEM.
SUPPLEMENTARY FIGURE LEGENDS

**Figure S1: B-1a and B-2 cell sorting strategy.** (A) Singlet, live PerC cells are sorted for CD19⁺ CD22⁺ CD5⁺ cells to about 95% purity. (B) Singlet, live spleen cells sorted using Easysep negative selection kit to > 90% purity.

**Figure S2: B cell trafficking post transfer.** (A) Frequency of live singlet CD19⁺ cells in the PerC, Spleen and VAT of Bnull mice 1 week post B-1a cell transfer and (B) post B-2 cell transfer. FACS plots are representative of 2 experiments.

**Figure S3: IL-10⁺ B cells in the PerC and VAT are mostly B-1a cells.** (A) IL-10⁺ CD19⁺ B220⁺ B cell subsets in the PerC and VAT (n=5). (B) Frequency of subsets within the CD45⁺ leukocyte compartment in the PerC of IL-10 eGFP mice fed NCD or HFD (n=5).

**Figure S4: Antigen binding of anti-PC IgM and polyclonal IgM.** (A) Dosage curves of known natural IgM targets and control antigens probed with anti-PC IgM and polyclonal IgM.

**Figure S5: B-2 cells are decreased by BAFF deficiency and BAFF antibody treatment.** (A) Frequency of B-2 cells, B-1a cells and CD23- CD5- cells in PerC, spleen, and VAT of WT and BAFFnull mice fed HFD. (B) Frequency of B-2 cells in blood at 3 and 5 weeks after BAFF Ab treatment (n=3). (C) Frequency of B-2, B-1a and CD23- CD5- cells in PerC (left) and spleen (right) 7 weeks after BAFF Ab treatment (n=3). (D) Absolute cell counts of B-2, B-1a and CD23- CD5- cells in PerC (left) and spleen (right) (n=3).