Homeostatic regulation of T cell trafficking by a B cell–derived peptide is impaired in autoimmune and chronic inflammatory disease

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During an inflammatory response, lymphocyte recruitment into tissue must be tightly controlled because dysregulated trafficking contributes to the pathogenesis of chronic disease. Here we show that during inflammation and in response to adiponectin, B cells tonically inhibit T cell trafficking by secreting a peptide (PEPITEM) proteolytically derived from 14.3.3 zeta protein. PEPITEM binds cadherin-15 on endothelial cells, promoting synthesis and release of sphingosine-1 phosphate, which inhibits trafficking of T cells without affecting recruitment of other leukocytes. Expression of adiponectin receptors on B cells and adiponectin-induced PEPITEM secretion wanes with age, implying immune senescence of the pathway. Additionally, these changes are evident in individuals with type 1 diabetes or rheumatoid arthritis, and circulating PEPITEM in patient serum is reduced compared to that of healthy age-matched donors. In both diseases, tonic inhibition of T cell trafficking across inflamed endothelium is lost. Control of patient T cell trafficking is re-established by treatment with exogenous PEPITEM. Moreover, in animal models of peritonitis, hepatic ischemia-reperfusion injury, Salmonella infection, uveitis and Sjögren’s syndrome, PEPITEM reduced T cell recruitment into inflamed tissues.

In vertebrates, a lymphocyte (T cell and B cell)-based adaptive immune system has evolved to augment innate immunity. Adaptive responses require lymphocyte trafficking between the bone marrow, lymphoid organs and peripheral tissues using blood as a vehicle for dispersal1. Our knowledge of the trafficking process is still incomplete. However, unregulated T cell recruitment during inflammation is pathogenic and contributes to chronic disease2,3. Here we reveal the function of a homeostatic pathway, which imposes a tonic inhibition on T cell trafficking during inflammation. Identification of this pathway arose through studies on the circulating adipokine adiponectin. Adiponectin affects both metabolic and immune pathways4–7, including the recruitment of leukocytes during an inflammatory response8, and plasma concentrations of adiponectin are low in a number of chronic diseases, including diabetes4. We tested the hypothesis that adiponectin might regulate lymphocyte trafficking and that changes in adiponectin function might contribute to pathogenic lymphocyte recruitment in chronic inflammatory and autoimmune diseases.

We started by observing lymphocyte trafficking in vitro across isolated human endothelial cells, which are the gatekeepers to the tissues for circulating leukocytes. To enter inflamed tissue, T cells migrate through endothelial cells lining the post-capillary venules8,9, and this has been modeled both in vitro and in vivo10–15. Thus, memory T cells moving rapidly in the flowing blood are preferentially recruited by endothelial cells activated by cytokines (for example, tumor necrosis factor α (TNF-α) and/or interferon γ (IFN-γ)). Tethering from flow and rolling adhesion are supported by E-selectin and vascular cell adhesion molecule 1 (VCAM-1)16, whereas integrin-mediated stable adhesion and migration are supported by sequential signals from chemokines and prostaglandin D2 (PGD2; refs. 17–23). Here we show that in the presence of adiponectin, B cells recruited to the endothelial cell surface during inflammation reduce the efficiency of memory T cell

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migration by imposing a tonic inhibition on this process. Thus, we believe that the adaptive immune system has evolved a robust strategy for regulating inappropriate or excessive activity, thereby limiting the possibility of establishing a chronic inflammatory response that might contribute to disease.

RESULTS

Adiponectin regulates T cell migration

In vitro, adiponectin dose-dependently inhibited the TNF-α- and IFN-γ-induced trans-endothelial migration of human peripheral blood lymphocytes (PBLs) with a half-maximal effective concentration (EC50) of 2.6 nM (0.94 μg/ml) (Fig. 1a and Supplementary Fig. 1a), with the most marked effects seen at physiological circulating levels observed in healthy humans (5–15 μg/ml). Although migration was reduced compared to the untreated control so that more cells were firmly adherent to the apical surface of the endothelium (Supplementary Fig. 1b), the number of lymphocytes recruited was unaffected by adiponectin (Supplementary Fig. 1c). The effects of adiponectin on PBL migration were seen in both a static system (Fig. 1a, b) and under conditions of flow (Fig. 1b), and they were evident on human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs) (Fig. 1c). The majority of transmigrating PBLs were CD3+CD45RO-memory T cells, as expected for this model (ref. 17 and data not shown). Adiponectin did not alter the expression and/or function of lymphocyte integrins (α4β1 and α5β2), the chemokine receptor CXCR3, or the PDGβ receptor DP-2 on PBLs (Supplementary Fig. 1d). Moreover, chemotactic responses to CXCL12, CXCL10, or PGD 2 were unaltered by adiponectin (Supplementary Fig. 1e). Fewer than 5% of T cells (CD3+ cells), including memory and naïve subsets, expressed adiponectin receptors (adipoR1 and adipoR2) (Fig. 1d–f). However, circulating B cells (CD19+ cells) expressed both receptors abundantly (Fig. 1d–f). We also found that endothelial cells expressed both adiponectin receptors (Supplementary Fig. 2). However, adiponectin did not directly target endothelial cells in our system, as treated PBLs are washed to remove any adiponectin before their addition to the endothelial cells. To ensure that any residual carryover of this agent did not influence lymphocyte recruitment, we verified that adiponectin did not modulate the gene expression of adhesion molecules and chemokines in TNF-α- and IFN-γ-stimulated endothelial cells (Supplementary Table 1). As T cells lack adiponectin receptors but show altered patterns of migration in response to adiponectin, we postulated that another lymphocyte population mediated the inhibition of T cell trafficking. Upon depleting B cells from the PBL mixture, T cells were released from the inhibitory effects of adiponectin (Fig. 1g). Adding back purified B cells to isolated T cells could reconstitute the adiponectin-dependent inhibition of T cell migration, and using supernatants from adiponectin-stimulated B cells was as effective as adding B cells themselves (Fig. 1g). The ability of B cell supernatants to impair T cell migration was lost when B cells were activated with adiponectin in the presence of an inhibitor of protein secretion, brefeldin-A (Fig. 1g). These experiments suggest that B cells release a soluble factor in response to stimulation by adiponectin that regulates migration of T cells.

Adiponectin induces PEPITEM secretion by B cells

We used an unbiased proteomic screen to identify the agent(s) secreted by purified B cells after stimulation with or without adiponectin. A comparative analysis identified a 14-amino acid peptide, SVTEGGAELSNR, specific to the supernatants of adiponectin-stimulated B cells (Fig. 2a). By comparing this to an in silico library of published and predicted sequences, we found that the peptide demonstrated 100% sequence homology to a single human protein, and that it represents amino acids 28–41 of the 14.3.3.ζ protein, which is a 245-amino acid product of the YWHAZ gene (Fig. 2b).
Proteolytic release of the peptide from the parent protein was confirmed after a tryptic digestion of recombinant 14.3.3.C8 generated the same 14-amino acid product (Supplementary Table 2). A time course of the 14.3.3.C8-derived peptide secretion from adiponectin-stimulated B cells showed rapid and sustained release (Supplementary Fig. 3a). A synthetic peptide exhibited an identical mass-charge (m/z) ratio to that of the native peptide (m/z = 774.88), suggesting that the B cell–derived product was not subject to post-translational modification before secretion (Supplementary Fig. 3b, c). Synthetic peptide showed a dose-dependent inhibitory effect on the trafficking of PBLs across TNF-α- and IFN-γ-stimulated endothelial cells in vitro (Fig. 2c), with a 19 pM EC₅₀ (28 pg/ml) (Supplementary Fig. 4a). A similar response was observed in the absence of bovine serum albumin, which removed any source of arachidonic acid that might be used to generate bioactive eicosanoids such as PGD₂, which are known to regulate T cell trafficking (Supplementary Fig. 4b). Similarly to adiponectin, the peptide had no effect on the number of lymphocytes adherent to the endothelial cells (Supplementary Fig. 4c), but it increased the number of surface adherent cells, as migration through the monolayer was inhibited (Supplementary Fig. 4d). Neither a scrambled peptide, containing the same amino acids in random order, nor other peptides of unrelated sequence but with known biological activity (proinsulin chain A peptide and tetanus toxoid peptide) had an effect on T cell migration (Fig. 2c).

As the peptide inhibited T cell migration, we named it PEPtide Inhibitor of Trans-Endothelial Migration or PEPITEM. PEPITEM specifically inhibited the migration of CD4+ and CD8+ memory T cells, without affecting either the transmigration of neutrophils or monocytes (Fig. 2d) or the total adhesion of any leukocyte subset (Supplementary Tables 3 and 4 for adiponectin). In addition, PEPITEM inhibited T cell transmigration on both HDMECs and HUVECs (Supplementary Fig. 4e). PEPITEM was ineffective if PBLs were pre-treated, and it only had inhibitory effects when pre-incubated with endothelial cells, implying that PEPITEM operated by stimulating a receptor on these cells (Supplementary Fig. 4f).

When we fractionated B cells into their subsets, we found that plasma cells (CD38+++, IgD−, IgM−) were able to secrete higher quantities of PEPITEM compared to naïve (CD38++, IgD+, IgM+) or memory cells on cytokine-stimulated endothelial cells (Supplementary Fig. 4f). These data imply a dominant role of circulating plasma cells in the regulation of memory T cell trafficking.

The endothelial cell receptor for PEPITEM is cadherin-15

To identify a PEPITEM receptor on endothelial cells, we used PEPITEM with a biotin conjugate on its N terminus as ‘bait’ to ‘fish’ for binding partners on the endothelial cell surface. This peptide...
Figure 3 PEPITEM induces the S1P release from endothelial cells, which inhibits T cell migration. (a, b) The effects of an S1PR antagonist (10 µM) on T cell migration in the presence or absence of adiponectin (n = 3–5) (a) or PEPITEM (n = 3–7) (b). (c) The effects on T cell transmigration of adding S1P to B cell–depleted PBLs, n = 3–6. (d, e) The effects of SPHK1-specific inhibitor (5 µM) (d) or SPHK1/2 inhibitor (5 µM) (e) on T cell transmigration in the presence of PEPITEM, n = 3. (f) The expression of SPHK1 and SPHK2 mRNA in endothelial cells, n = 7–8. (g) The effect of SPNS2-specific siRNA on T cell transmigration in the presence of PEPITEM, n = 4. (h, i) The expression of S1PR1 on memory T cells (CD3+CD45RO+) on stimulated endothelial cells (n = 3) (h) and on plated ICAM-1-stimulated with CXCL10 (n = 6) (i). (j) The effects of S1P on the expression of the LFA-1 activation epitope (KIM127) on ICAM-1-adherent memory T cells (CD3+CD45RO+) stimulated with CXCL10, n = 4. Data are means ± s.e.m. and normalized to control (g–j). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to untreated control by analysis of variance (ANOVA) (a–g) and Dunnett (a, b, d, e, g) or B cell depletion no-adiponectin control (c) or to unstimulated (0) control (f) or Bonferroni post-test (j) or paired t-test on raw data (h) or Wilcoxon signed-rank test (i).

Endothelial cell sphingosine-1-phosphate impairs migration

Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid generated in the cytosol from sphingosine, which is phosphorylated by the sphingosine kinases (SPHKs) SPHK1 or SPHK2 (ref. 19). The endothelial cell transporter spinster homolog 2 (SPNS2) is also necessary for translocation of S1P into extracellular fluid24–26. S1P has an important regulatory role in the movement of T cells from inflamed tissue to afferent lymphatics27 and in T cell egress from secondary lymphoid organs28–31. Here we found the S1P receptor antagonist W146 (trifluoroacetate salt) released T cells from the inhibitory effects of both adiponectin and PEPITEM (Fig. 3a, b). Moreover, the effects of adiponectin and PEPITEM on T cell migration could be mimicked dose-dependently when exogenous S1P was added to purified T cells (Fig. 3c). We confirmed that S1P was of endothelial origin by pre-treating endothelial cells with the SPHK1 inhibitor (2,2-dimethyl-4S-(1-oxo-2-hexadecyn-1-yl))-1,1-dimethyl-3-oxazolidinecarboxylic acid) or the SPHK1 and SPHK2 (SPHK1/2) inhibitor (N,N-dimethylsphingosine), which in both cases abolished the ability of PEPITEM to inhibit T cell migration (Fig. 3d, e). In agreement with the patterns of activity of these inhibitors, we found that SPHK1, but not SPHK2, was highly expressed in endothelial cells but not in B cells (Supplementary Fig. 6a), and that mRNA levels for SPHK1, but not SPHK2, were increased upon stimulation of endothelial cells with TNF-α and IFN-γ compared to controls (Fig. 3f). Knockdown of endothelial SPNS2 mRNA also released T cells from the
Figure 4 PEPITEM inhibits T cell migration in vivo. (a) T cells recruited into the peritoneum of BALB/c B cell–deficient mice after induction of peritonitis using zymosan and treatment with PEPITEM or scrambled peptide. All data were normalized to the number of T cells in BALB/c WT mice treated with zymosan, n = 3–8. (b) Mean CD3+ T cells per infectious foci in the liver of Salmonella–infected C57BL/6 B cell–deficient mice treated with PEPITEM or PBS (control), n > 4. (c) Adherent CD3+ T cells per mm² in the liver sinusoid following reperfusion in C57BL/6 WT mice treated with PEPITEM or scrambled peptide by intravital microscopy. (d) Representative pictures of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labeled T cells in the sinusoids in scrambled (top) and PEPITEM-treated mice (bottom), n = 4 per group. Scale bars, 100 µm. (e) CD3+ T cells in the ocular infiltrate of C57BL/6 WT mice treated with PBS (control) or PEPITEM after induction of uveitis by intravitreal injection of LPS, n = 9–10. (f) CD3+ T cells in the salivary glands of C57BL/6 WT mice treated with scrambled peptide (control) or PEPITEM after induction of Sjögren’s syndrome by cannulation of salivary glands and injection of luciferase-encoding replication-defective adenovirus (AdV5), n = 7–8. (g) Representative pictures showing CD3+ T cells and CD19+ B cells in the salivary glands after scrambled peptide (left) or PEPITEM (right) treatment. Scale bars, 100 µm. Data are means ± s.e.m. *P ≤ 0.05, **P ≤ 0.01 compared to zymosan-treated WT mice by Dunnett post-test (a) or compared to scrambled (a,f) or PBS-treated B cell–deficient mice by unpaired t-test (b,e) or two-way ANOVA (c).

Inhibitory effects of PEPITEM (Fig. 3g and Supplementary Fig. 6b–d). The addition of S1P to endothelial cells at a concentration equivalent to the total circulating concentration in plasma (~0.2 µM) (ref. 32) inhibited T cell transmigration efficiently (Fig. 3c). However, in the circulation S1P is bound to plasma proteins such as albumin and lipoproteins2,33, which limit its availability. Published data estimates that biologically active S1P circulates at approximately 5 nM (refs. 33,34).

In the presence of this concentration of S1P, the effects of PEPITEM...
on migration were readily observable (Supplementary Fig. 6e–g). Moreover, PEPITEM inhibition of T cell trafficking in the presence of 5 nM S1P could be reversed by treating endothelial cells with SPHK inhibitors or by SPNS2 knockdown in these cells, showing that additional and functional S1P was released in response to PEPITEM stimulation (Supplementary Fig. 6e–g).

The process of T cell egress from lymph nodes into blood results in rapid S1P-mediated internalization of S1P receptors (S1PR1 and S1PR4) from the surface of circulating T cells30,31. Thus, for S1P to be an effective mediator during T cell migration across endothelial cells, S1P receptors on T cells require upregulation. We observed a robust increase in the expression of S1PR1 on the cell surface of memory T cells that had been recruited by cytokine stimulated endothelial cells (Fig. 3h). Moreover, we observed rapid upregulation of surface S1PR1 when memory T cells adherent to immobilized, recombinant ICAM-1 were stimulated by CXCL10 (Fig. 3i and Supplementary Fig. 7a). This mode of T cell activation could override the desensitization pathways for S1PR1, as high expression was maintained even in the presence of 10 μM exogenous S1P (Supplementary Fig. 7a). Moreover, this was not due to the recruitment of a subset of cells with high S1PR1 expression, as these were not evident in the isolated T cells before addition (Supplementary Fig. 7a). Thus, during the process of recruitment, chemokines presented by endothelial cells rapidly and sustainably induce expression of S1PR1 on memory T cells.

S1P downregulates the affinity of the T cell integrin adhesion receptor αβ2 (LFA-1), reducing binding to ICAM-1 after stimulation with the chemokine CXCL10 (Fig. 3) and Supplementary Fig. 7b). This change in integrin function did not alter the levels of T cell recruitment, which is dependent on E-selectin and VLA-4, in this model of in vitro transmigration (ref. 17 and data not shown). However, in the presence of adiponectin (and therefore S1P) there was a modest increase in the number of cells rolling on the endothelial cell surface compared to the untreated control (Supplementary Fig. 7c).

Taken together, these data imply that S1P-mediated changes in the function of LFA-1 are able to interrupt the process of LFA-1 mediated trans-endothelial migration.

PEPITEM is functional in vivo

As PEPITEM is derived from B cells, we conducted a series of studies in the BALB/c B cell–deficient mouse35. In a mouse model of zymosan-induced peritonitis, more T cells trafficked into the peritoneal cavity in B cell–deficient mice compared to controls (Fig. 4a). Injection of these mice with PEPITEM during challenge with zymosan inhibited trafficking of T cells into the peritoneum (Fig. 4a). Additionally, we tested the efficacy of PEPITEM in a model of systemic bacteremia upon Salmonella typhimurium infection in the C57BL/6 B cell–deficient mouse. Salmonella colonizes the spleen and liver during primary infection46, where it promotes an inflammatory infiltrate into infectious foci. PEPITEM reduces the number of T cells resident in these infectious foci in Salmonella-infected B cell–deficient mice compared to controls (Fig. 4b), with a trend toward reduced T cells in PEPITEM-treated wild-type (WT) mice (Supplementary Fig. 8a,b). PEPITEM also abolished T cell recruitment in the hepatic sinusoids, with a concomitant increase in the number of free-flowing T cells, after acute liver ischemia and reperfusion injury in C57BL/6 WT mice, as assessed by real-time intravital microscopy (Fig. 4c,d and Supplementary Fig. 8c). This observation is consistent with integrin-mediated processes that support leukocyte tethering in the low-shear environment of the hepatic sinusoids37,38.

In a model of endotoxin-induced (lipopolysaccharide; LPS) autoimmune uveitis (ocular inflammation) in C57BL/6 WT mice, administration of PEPITEM with LPS into the eye reduced the number of T cells in the ocular infiltrate (Fig. 4e). PEPITEM also reduced T cell trafficking into the salivary glands of C57BL/6 WT mice challenged with a virally induced model of tertiary lymphoid organ formation, which mimics changes observed in the autoimmune rheumatic
The PEPITEM pathway is impaired in disease and the elderly

Efficacy in in vivo models of inflammation prompted us to investigate whether the PEPITEM pathway was compromised in individuals with the T cell–driven autoimmune disease type 1 diabetes (T1D) or in individuals with rheumatoid arthritis. First, we measured the expression of adipor1 and adipor2 on the circulating B cells of individuals with T1D or rheumatoid arthritis, comparing these to healthy age- and gender-matched control donors (cohort statistics are shown in Supplementary Tables 6–8). The expression of both adipor1 and adipor2 was reduced on B cells from individuals with T1D (Fig. 5a for the percentage of positive B cells and Supplementary Fig. 9a for representative intensity histograms) and with rheumatoid arthritis (Fig. 5b for the percentage of positive B cells and Supplementary Fig. 9a for representative intensity histograms) compared to healthy controls. We observed no difference in the expression of CD19 on B cells and in B cell number between healthy controls and individuals with T1D or rheumatoid arthritis, suggesting that these differences reflect changes in adipor1 and adipor2 expression (Supplementary Fig. 9b,c). There was a positive correlation between the expression of adipor1 and adipor2 on B cells with the quantity of PEPITEM released by B cells upon stimulation with adiponectin (Fig. 5c,d). This was the case for both individuals with T1D and healthy controls (adipor2 only), although in individuals with T1D only, low levels of PEPITEM could be detected, which reflected the paucity of expression of adiponectin receptors. In addition, we were able to detect low concentrations of PEPITEM in serum from healthy controls, and we found that this was reduced in individuals with T1D (Fig. 5e).

Decreased PEPITEM secretion by B cells released T cells from the inhibitory effects of adiponectin, so that there was no longer an inhibition of T cell migration in individuals with T1D (Fig. 5f) or those with rheumatoid arthritis (Fig. 5g). The effects of adiponectin could be mimicked by the addition of exogenous PEPITEM in the ex vivo migration assay, using lymphocytes from individuals with T1D or rheumatoid arthritis (Fig. 5f,g), meaning that the loss of tonic inhibition of T cell migration in these individuals could be readily replaced with appropriate PEPITEM treatment.

One of the major risk factors for developing chronic inflammatory or autoimmune diseases is age. Thus, we analyzed the expression of adipor1 and adipor2 in healthy donors of different ages. There was a negative correlation between the expression of adipor1 and adipor2 and age (Fig. 5h,i).

DISCUSSION

The processing of an immune-regulatory 14-amino acid peptide from an intracellular protein (14.3.3ζδ) with no known association to the inflammatory response has not yet been described in, nor could it have been predicted from, any of the known pathways that regulate leukocyte trafficking. In fact, the functions of the seven members of the 14.3.3 protein family are diverse. For example, they are involved in regulating the function of cytosolic proteins that support metabolic, cell cycle, apoptotic and protein trafficking pathways. Their importance in such homeostatic pathways is highlighted by their association with diseases as varied as cancer, hyper-proliferative skin disorders and Alzheimer’s disease, when their function is disrupted. The effects of losing the function of the PEPITEM pathway that we document here now allows us to add T1D and rheumatoid arthritis to the list of diseases in which the dysregulated function of 14.3.3 proteins has a role.

Here we show that extracellular PEPITEM impairs lymphocyte trafficking by indirectly regulating the function of the β3-integrin, LFA-1. Interestingly, both intracellular 14.3.3ζδ have been implicated in the regulation of adhesion dependent cellular functions in other contexts. For example, morphology and adhesion in dendrites, embryonic kidney cell lines and rat fibroblasts are associated with the 14.3.3ζδ-dependent regulation of β3-integrins. Moreover, 14.3.3ζδ protein in platelets regulates the function of the adhesion complex GPIb/IX/V, and it is required for efficient recruitment of platelets to von Willebrand factor (VWF). It is also notable that S1P, the terminal mediator in the PEPITEM pathway, can also regulate the function of endothelial cell–borne adhesion receptors (for example, VE-cadherin) that are involved in the regulated trafficking of leukocytes. Thus, although a role in lymphocyte trafficking is novel for 14.3.3 proteins, the regulation of adhesion molecules in other cells and contexts does provide a generic link between their known biological functions and the new role described here.

In the lymph node, a process of reciprocal cross-talk between B cells and T cells is known to be important in establishing an antigenspecific immune response. Cross-talk between B cells and T cells also has a role in the initiation of T cell–mediated autoimmune events in T1D and rheumatoid arthritis. Indeed, depletion of B cells by the monoclonal antibody rituximab has beneficial effects in these diseases. It might be assumed that such a B cell depletion strategy might compromise the inhibitory effects of PEPITEM on T cell trafficking by removing the tonic ‘brake’ on inflammation provided by this pathway (described in detail in Fig. 6). However, our data implies that this pathway is no longer functional in individuals with established disease. Thus, it is unlikely that the benefit of removing pathogenic B cells is being achieved at the expense of the protective functions of PEPITEM-secreting B cells. However, our observation that exogenous peptide can regain control of the trafficking of T cells raises the possibility that the PEPITEM pathway may present a tractable target for the development of novel therapeutic agents with which to treat chronic inflammatory and autoimmune diseases. In T1D or rheumatoid arthritis this might be achieved using the peptide itself. However, we predict that in other disease states, alternative aspects of this pathway may be compromised. For example, the expression and proteolysis processing of 14.3.3ζδ to yield mature peptide could be altered, as could the secretory pathways required for PEPITEM release in response to adiponectin. In endothelial cells, changes in the expression of cadherin-15 or the release of S1P in response to PEPITEM signaling could lead to inappropriate T cell traffic. Finally, T cells themselves may lose the capacity to respond to S1P by failing to up regulate S1PRs in response to inflammatory chemokines, or through alterations in the intrinsic signaling pathways lying downstream of these molecules. Many of these processes have yet to be defined mechanistically, such as the identity and localization of the protease(s) that cleave PEPITEM from 14.3.3ζδ. However, with a fuller understanding of the biology of the PEPITEM pathway it will be important to determine whether alterations in these other steps are detectable in disease. If they are, they will offer unique opportunities to develop new therapeutic agents.
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Author Contributions

M.C. conceived and performed experiments, analyzed and interpreted the data, and co-wrote the manuscript. H.M.M. conceived, performed experiments, analyzed and interpreted the data. B.A., S.J.K., C.M.Y., A.K., A.O., M.A., M.H., S.N., J.R.H., D.A.C. and J.R. performed experiments and analyzed the data. A.M., F.B., A.F., C.R., A.F., A.D.C., A.D.D., N.K., L.S.K.W., C.D.B. and G.B.N. organized and conducted the study, including analysis, data interpretation and critique of the manuscript. K.R. and A.F. recruited and diagnosed patients in early arthritis clinics, and conducted the study, including analysis, data interpretation and critique of the manuscript. G.E.R. conceived, designed, and organized and conducted the study, including analysis and interpretation of data, and co-wrote the manuscript.

Competing Financial interests

The authors declare competing financial interests: details are available in the online version of the paper.

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Isolation of leukocytes. We obtained blood samples from healthy donors with written informed consent and approval from the University of Birmingham Local Ethical Review Committee (ERN_07-058). We isolated peripheral blood mononuclear cells (PBMCs) and neutrophils from blood using a two-step density gradient of Histopaque 1077 and 1119 (Sigma-Aldrich, Poole, UK). Lymphocytes were purified by panning PBMCs on culture plastic for 30 min at 37 °C to remove monocytes as previously described18. PBLs were then counted, resuspended in M199 (Life Technologies Invitrogen Compounds, Paisley, UK) containing 0.15% bovine serum albumin (BSA; Sigma-Aldrich) at 1 × 10^6 cells per ml for transmigration assays, or in PBS containing 0.5% BSA and 2 mM EDTA (Sigma-Aldrich) for cell sorting. B cells were depleted from PBLs by positive selection using anti-CD19 beads (Miltenyi Biotec, Surrey, UK). When B cells were reconstituted into PBLs or used to generate supernatants, B cells were sorted by negative selection to yield untouched cells (StemCell, Grenoble, France). Memory and naive CD4+ and CD8+ T cells were isolated using negative selection kits (StemCell). Monocytes and their subsets were isolated by positive selection using CD14 and CD16 beads (Miltenyi Biotec).

In vitro transmigration assay. Human umbilical cords were obtained from the Human Biomaterials Resource Centre (HBRC, University of Birmingham) (09/1010/075) which holds ethical approval and collected fully consented tissue from the Birmingham Women’s Hospital NHS Trust. HUVECs were isolated from umbilical cords as previously described20 and cultured in M199 supplemented with 20% FCS (fetal calf serum), 10 ng/ml epidermal growth factor, 35 µg/ml gentamycin, 1 µg/ml hydrocortisone (all from Sigma-Aldrich), and 2.5 µg/ml amphotericin B (Life Technologies Invitrogen Compounds). Primary HUVECs were dissociated using trypsin/EDTA (Sigma-Aldrich) and seeded on 12-well tissue culture plates (Falcon; Becton Dickinson Labware). Both fractions were labeled and washed after 30 min, so the treatments did not modulate HUVEC function, to the PBLs or different leukocyte subsets at room temperature under agitation and washed after 30 min, so the treatments did not modulate HUVEC function, prior incubation of cells on endothelial cells.

Firmly adhered PBL were collected from the endothelial cell surface using cold EDTA for 3 washes. Transmigrated PBL were collected by treatment of endothelial cells with Accutase (Sigma-Aldrich). Both fractions were labeled and analyzed by flow cytometry as described below in the 'Flow cytometry' section.

Flow cytometry. PBMC were stained with the relevant antibodies for 30 min at 4 °C. Subsequently, samples were labeled with the relevant secondary conjugated antibodies for 30 min at 4 °C. Isotype controls and secondary-only conditions were used as negative controls. Rabbit anti-human adipoR1 (357–375) and adipoR2 (374–386) antibodies (Phoenix pharmaceuticals, Karlsruhe, Germany) were used at 5 µg/ml and detected using a goat-anti rabbit Alexa Fluor 488 secondary antibody used at 8 µg/ml (Life Technologies Invitrogen Compounds). Gating to measure the expression of adipoR1 and adipoR2 on PBL and B cells was based on the isotype control. Isotype control frequencies were subtracted from the adipoR1 and adipoR2 frequencies for each subject. The following antibodies were used to stain human PBMCs: CD4-FITC (1:50) (OKT-4), CD3-PerCp-Cy5.5 (1:50) (OKT3), CD19-PECy7 (1:50) (HIB19), CD8-Pacific Blue (1:50) (OKT8), CD56-PE (1:50) (MEM188) (all from eBioscience, Hatfield, UK), CD4–Pacific orange (1:10) (clone S5.5) (Life Technologies Invitrogen Compounds) and CD45RO-APC (1:20) (UCHL1) (BD Bioscience, Oxford, UK), CD4–Pacific orange (1:10) (clone S5.5) (Life Technologies Invitrogen Compounds) and CD45RO-APC (1:20) (UCHL1) (BD Bioscience, Oxford, UK), α4β1-PE (1:100) (PSD2), α4β1-FTTC (1:100) (212701), DP-2-FITC (1:10) (301108) (R&D Systems, UK), CX3CR1-PE (1:50) (2A11) (US Biological). B cell subsets were labeled using CD19-PerCP-Cy5.5 (1:100) (3LT9), IgM-PE (1:100) (PJ2-22H3), IgD-APC (1:60) (IgD26), CD38-FTIC (1:150) (IB6). CD27-APC (1:20) (M-T271) (all from Miltenyi Biotec).

The following antibodies were used to stain mouse PBMC from blood and peritoneal lavage: CD3–FITC (1:50) (145-2C11), CD3–PECy7 (1:50) (145-2C11), CD4–PB (1:100) (GK1.5), CD8–TR (1:200) (5H10), CD11c–PECy7 (1:50) (N418), CD19–APC–FITC (1:50) (1D34), CD45–PerCP-Cy5.5 (1:200) (30-F11), CD26–PE–FITC (1:500) (MEL-14), B220–APCCy7 (1:100) (RA3–6B2), gp38–PE–FITC (1:200) (8.1.1) (all from eBioscience) and F4/80–APC (1:20) (CL3–A3) (AbD Serotec, Kidlington, UK). Samples were assayed using a CyaN (Dako) with Summit software and then analyzed using FlowJo software (TreeStar, Ashland, OR). Between 10,000 and 50,000 events per sample were assayed and plotted and counted as the frequency of positive cells or mean fluorescence intensity (MFI). All samples analyzed were included in the study unless insufficient records were collected for flow cytometry analysis.

Confocal microscopy. Cells were grown to confluence onto glass chamber slides (Becton Dickinson Falcon) at 37 °C. They were then fixed with 2% formaldehyde and 4% sucrose for 15 min, washed in PBS and stained with 10 µg/ml of either a sheep anti-human CD11b antibody (Val22-Ala606) (R&D systems) or sheep IgG (Southern Biotech, UK) overnight at 4 °C. Goat anti-sheep IgG1 conjugated to Alexa Fluor 488 (1:1000) (Abcam, UK) was then applied and the slides were visualized using a Zeiss LSM 510 inverted laser-scanning confocal microscope using a 40x water-immersion objective with excitation at 488 nm and 543 nm (Zeiss, Gottingen, Germany). Constant acquisition parameters and calculated. In some experiments, data was normalized to the control by dividing the percentage transmigration of the treated sample by the percentage of transmigration of the control and multiplied by 100.

For the B cell reconstitution experiments, B cells were negatively selected using StemSep magnetic kit and 100,000 cells were incubated with 15 µg/ml adiponectin for 1 h at room temperature. Cells were centrifuged 1500 g for 7 min and 1 ml of supernatant was added to 1×10^6 B cell–depleted PBLs (1 B cell to 10 B cell-depleted PBLs). Negatively sorted B cells (100,000) were incubated with brefeldin-A (10 µg/ml) for 4 h and adiponectin (15 µg/ml) for the last hour. Cells were then washed and supernatant added back to B cell-depleted PBLs and transmigration was measured.

In some experiments, lymphocytes were pre-treated with 10 µM S1PR antagonist (W146) or SIP (0.0001 to 100 µM) at room temperature under agitation and washed after 30 min, so the treatments did not modulate HUVEC function. Alternatively, HUVECs or HDMECs were pre-treated with 5 µM SPHK1 or SPHK1/2 antagonists at 37 °C and washed after 30 min. PEPITEM was added to the PBLs or different leukocyte subsets at room temperature under agitation and washed after 30 min, so the treatments did not modulate HUVEC function, prior incubation of cells on endothelial cells.

Firmly adhered PBL were collected from the endothelial cell surface using cold EDTA for 3 washes. Transmigrated PBL were collected by treatment of endothelial cells with Accutase (Sigma-Aldrich). Both fractions were labeled and analyzed by flow cytometry as described below in the 'Flow cytometry' section.
laser power were maintained throughout individual experiments for analysis and images were processed using Zeiss LSM Image Examiner software (Zeiss). Digital images were recorded in two separately scanned channels with no overlap in detection of emissions from the respective fluorochromes. Confocal micrographs were stored as digital arrays of 1024 × 1024 pixels with 8-bit sensitivity.

**Immunoprecipitation and western blotting.** Whole cell lysates were extracted by suspending cells in lysis buffer with 50mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, 1% (wt/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, and protease inhibitor cocktail (Invitrogen). After incubation for 15 min at 4 °C, this preparation was centrifuged at 600g for 10 min. The supernatant was subjected to immunoprecipitation by incubating for 30 min at 4 °C with protein G–Dynabeads (Invitrogen) and polyclonal sheep anti-CDH15 antibody (10 µg/ml) (Val22-Ala606) (R&D systems). Blots were then probed with appropriate horseradish peroxidase-conjugated anti-sheep secondary antibody (1:3,000) (Cell Signaling Technology, UK). Immunodetection was carried out using the ECL plus Kit (Amersham, GE Healthcare Life Sciences, and UK) followed by exposure to X-ray film for 15 min. Controls were run in parallel with application of the recombining CDH15 (R&D systems).

**siRNA transfection.** HUVECs were plated in 12-well plates (87,500 cells per well) for 24 h or about 80% confluence. The relevant siRNAs were added at a final concentration of 50 nM to 83.75 µl of RNAi transfection (Amersham, GE Healthcare Life Sciences, and UK) followed by exposure to 10% glycerol, 1% (wt/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, and protease inhibitor cocktail (Invitrogen). After incubation for 15 min at 4 °C, this preparation was centrifuged at 600g for 10 min. The supernatant was subjected to immunoprecipitation by incubating for 30 min at 4 °C with protein G–Dynabeads (Invitrogen) and polyclonal sheep anti-CDH15 antibody (10 µg/ml) (Val22-Ala606) (R&D systems). Blots were then probed with appropriate horseradish peroxidase-conjugated anti-sheep secondary antibody (1:3,000) (Cell Signaling Technology, UK). Immunodetection was carried out using the ECL plus Kit (Amersham, GE Healthcare Life Sciences, and UK) followed by exposure to X-ray film for 15 min. Controls were run in parallel with application of the recombining CDH15 (R&D systems).

**Real-time PCR.** Total mRNA was extracted using the RNAeasy Minikit (Qiagen, Crawley, UK) according to the manufacturer’s protocol. Briefly, PBMC were first lysed, then added to a column, after three washes, mRNA was eluted from the column with water. mRNA concentration was measured using NanoDrop spectrophotometer (LabTech) and mRNA was stored at −80 °C. To convert mRNA to cDNA, random primers (Promega, USA) were annealed to 1 µg of mRNA for 5 min at 70 °C, after which the following mastermix was added to give a final volume of 30 µl: 10 µl U Superscript II Reverse Transcriptase (RT), 10 U RNAsafe RNase inhibitor, 1× Superscript Buffer (all from Invitrogen) and 10 mM dNTPs (Promega). The reaction was run at 37 °C for 1 h, followed by 5 min at 95 °C. To analyze mRNA, FAM-labeled SPHK1, SPHK2 and SPNS2 primers and VIC-labeled 18S primers were bought as Assay on Demand kits from Applied Biosystems. Samples were amplified in duplicates using the 7500HT real-time PCR machine (Applied Biosystems) and analyzed using the software package SDS 2.2 (Applied Biosystems). Data were expressed as relative expression units relative to 18S or as fold change (ΔΔCt method).

**Identification of PEPITEM.** B cells (2 x 10^5–5 x 10^5) were incubated in presence or absence of adiponectin at 15 µg/ml. Adiponectin (15 µg/ml) was added in M199 and used as a negative control. The peptides from the three samples were purified using C18 solid-phase extraction columns from Supelco (DSC-18, Sigma-Aldrich). The columns were conditioned by adding 1 ml of 0.1% trifluoroacetic acid (TFA, Thermo Scientific) in acetonitrile (ACN, Thermo Scientific), which like all additions was allowed to drip through the column under gravity. The column was then equilibrated with 0.1% TFA/water and the sample, adjusted to 0.1% TFA, was added to the column. The column was washed with 1 ml of 0.1% TFA/water and the peptides eluted using 0.1% TFA/acetonitrile (1 ml) which was dried under vacuum and the samples resuspended in 20 µl in 0.1% formic acid in 2% acetonitrile. 10 µl of the purified samples was subjected to a LC-MS/MS analysis using a gradient of 2–36% ACN in 0.1% formic acid over 30 min at 350 nl/min using a Dionex Ultimate 3000 HPLC system. The HPLC column was connected to a Bruker ETD Amazon ion-trap mass spectrometer with an online nanospray source. A mass spectrometry survey scan from 350 to 1,600 m/z was performed and the five most intense ions in each survey scan were selected for collision-induced dissociation (CID) fragmentation. After ions were fragmented twice they were placed on an exclusion list for 0.5 min. The raw data was processed using the Bruker Data Analysis peak detection program to select peaks which were then searched using the Mascot search engine (version 2.1) using the SwissProt protein database. The minimum mass accuracy for both the mass spectrometry and tandem mass spectrometry scans were set to 0.5 Da and no protease selection was used. The peptides were filtered using a minimum Mascot score of 30. The data output was analyzed via the Bruker ProteinScape software package. To identify a potential candidate in the B cell supernatant with adiponectin treatment, we applied a subtractive data analysis method. Hits from the adiponectin-stimulated sample were compared to the B cell supernatant without adiponectin and recombining adiponectin controls and common analytes were discarded. The recombining 14.3.3-ζ protein (100 µg) (Fitzgerald Industries International) was digested using trypsin at 40 µg/ml (in 10% ACN, 90% HPLC D3O, 0.1% TFA) for 1 h at 37 °C and samples were purified on C18 columns with 90% ACN and analyzed using mass spectrometry and database searching as described above.

**Identification of PEPITEM receptor.** Endothelial cells were incubated with an N terminus biotinylated version of PEPITEM or biotinylated scrambled control for 4 h at 4 °C. Cells were then wash twice in cold PBS and lysed with a Triton X-100, Protease inhibitor, all from Sigma-Aldrich). After 30 min, lysates were collected and centrifuged for 20 min at 600g at 4 °C. Supernatants were collected and dried under vacuum and the samples resuspended in 20 µl 5 µM urea/2% SDS and loading buffer (Sigma-Aldrich and Life Technologies Invitrogen Compounds). Samples were loaded onto a 4–12% SDS-PAGE gel (Life Technologies Invitrogen Compounds) and stained overnight with colloidal Coomassie staining buffer (0.08% Coomassie Brilliant Blue G250, 1.6% orthophosphoric acid, 8% ammonium sulfate, 20% methanol, all from Sigma-Aldrich). Gels were detained in 1% Acetic acid in distilled water (several changes) until the background was clear. Protein bands were cut-out of the gel and washed twice in 50% acetonitrile (ACN)/50 mM ammonium bicarbonate (AB, Sigma-Aldrich) for 45 min at 37 °C with agitation. The gel fragments were then incubated at 56 °C for 1 h in 50 mM DTT in 10% ACN/50 mM AB and left overnight at 37 °C. Supernatant was then collected and bands were washed twice in 10% ACN/40 mM AB for 15 min and dried under vacuum until completely dry. Trypsin (Promega, Southampton, UK) was then added on the bands at 200 µg/ml in 10% ACN/40 mM AB and left overnight at 37 °C. Supernatant was then collected and bands were washed twice in 3% formic acid for 1 h at room temperature under agitation. Supernatants were collected and pooled together after all washes and samples were dried under vacuum, resuspended in 20 µl in 0.1% formic acid in 2% ACN. 10 µl of the purified samples was subjected to an LC-MS/MS analysis using a gradient of 2–36% ACN in 0.1% formic acid over 30 min at 350 nl/min using a Dionex Ultimate 3000 HPLC system, as already described.

**Biaco re assay.** All Biaco assays were performed with the help of Dr. Catherine McDonnel (GE Healthcare) using a Biaco T200 system (GE Healthcare). To test peptide binding to CDH15, recombiant CDH15-Fc (50 µg/ml) (R&D Systems) was immobilized on protein A bound to a chip using a standard protocol (900 s, 5 µl/min). N-terminals biotinylated PEPITEM (24 µg/ml to 770 µg/ml) in HBS-P buffer (GE Healthcare), 5 mM CaCl2 and 0.05% P20 was flowed over the chip.
at a flow rate of 30 µl/min for 60 s injection and 600 s dissociation. For this experiment, buffer alone and random peptides were used as controls in case of any non-specific binding, as well as scrambled biotinylated PEPITEM. Binding kinetics were measured in response units (RU) and BiaEvaluation software (GE Healthcare) was used to analyze the data traces.

**Quantification of PEPITEM.** 500,000 negatively selected B cells were incubated in the presence or absence of adiponectin 15 µg/ml for 1 h at room temperature. After centrifugation at 250g for 7 min, supernatants were spiked with 10 ng of 3H (tritium)-radiolabeled PEPITEM as an internal mass standard for relative intensity quantification. Peptides were purified on DSC-18 solid-phase extraction columns as described above and analyzed by liquid chromatography–tandem mass spectrometry as described above. Owing to their identical chromatographic properties, endogenous and synthetic radiolabeled versions of PEPITEM elute at the same point on the gradient, allowing comparison in the same mass spectrum. However, owing to their different physical properties, these versions are readily resolved as separate peaks within this mass spectrum. Therefore, extracted ion chromatograms (EIC) comparing intensity of the 10 ng radiolabeled standard (m/z 780.88±0.05) to that of endogenous PEPITEM (m/z 774.88±0.05) allowed quantification of the native peptide. This method of relative intensity quantification is time-honored within the field of analytical science.

Alternatively, B cell subsets were isolated by automated cell sorting using a MoFlo Astrios EQ (Beckman Coulter) and labeled as previously described in the ‘In vitro transmigration assay, section. The B cell subsets were then incubated with 15 µg/ml of adiponectin for 1 h. Supernatants were collected and analyzed by mass spectrometry to quantify PEPITEM using the radiolabeled quantification method.

**Identifying changes in the affinity of α1β2 on lymphocytes.** 96well plates were coated with 50 µg/ml recombinant ICAM-1/Fc (R&D Systems) overnight at 4 °C. The plate was blocked using PBS 2% BSA for 1 h at room temperature and PBL treated with CXCCL10 (80 ng/ml) and/or S1P (10 µM) were added for 6 min. Excess unbound PBLs were washed away. Bound PBLs were collected using cold PBS by rough pipetting and PBLs were labeled at 4 °C for the lymphocyte integrin α1β2 (LFA-1) using the mouse anti-human KIM127 (10 µg/ml) antibody recognizing the intermediate affinity epitope (N. Hogg, London) or the S1PR1 antibody (5 µg/ml; Cayman Chemicals; amino acids 241–253 (ISKASRSSKESSL)). KIM127 was detected using a goat anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen) at 8 µg/ml and S1PR1 with a donkey anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen) at 8 µg/ml. The expression of the affinity site was measured on memory T cells (CD45RO CD43+ T cells) by flow cytometry as described in the ‘Flow cytometry’ section. To measure the expression of S1PRs on firmly adhered and transmigrated memory T cells, we washed the endothelial cells after 6 min of PBL transmigration using cold EDTA (Sigma-Aldrich). The EDTA wash was repeated until all firmly adhered cells were recovered. Transmigrated cells were collected with endothelial cells using Accutase for one minute at 37 °C and firmly adhered and transmigrated cells were labeled with CD45RO-APC (1:20) (UCHHL1) (BD Bioscience) and CD3-PerCp-Cy5.5 (1:50) (OKT3) (eBioscience) and S1PR1 and analyzed by flow cytometry.

**Acute peritoneal inflammation.** BALB/c WT or BALB/c B cell–deficient (lgl–/–lml–/– N2+N2; these mice carry a deletion of the J segments of the Ig heavy chain locus) (Taconic, New York, USA) were housed in the Biomedical Services Unit at the University of Birmingham. Mice were used between 6 and 8 weeks of age and were matched for sex, as both male and females were used. Peritonitis was induced by the intraperitoneal injection (i.p.) of 1 mg of type A zymosan (Sigma-Aldrich) as previously described. In some animals zymosan was delivered with 300 µg of PEPITEM or scrambled peptide. Cells washed from the peritoneal cavity were collected in PBS at 48 h after injection. Erythrocytes in the peritoneal exudates were lysed and leukocytes stained for analysis by flow cytometry. Tubes containing the cells from the peritoneal exudates were fully acquired on the flow cytometer to accurately count the cells. Gates were set up on all cells in the peritoneum and the number of T cells was determined based on CD3 expression. Blood was drawn by cardiac puncture under the anesit and processed as for peritoneal exudate cells. All data were normalized to the number of T cells in WT mice treated with zymosan.

**Systemic bacterialia upon Salmonella infection in the liver.** For Salmonella infections, attenuated Salmonella Typhimurium (strain SL3261, originally obtained from R.A. Kingsley (Wellcome Trust Sanger Institute, Cambridge) was grown overnight and bacteria were harvested from log-phase cultures as described previously. C57BL/6 WT or C57BL/6 B cell–deficient (B cell–deficient) mice were generated in house from breeding out the QM (quasi-monoclonal) IgH transgene from QM mice, which have the other IgH locus inactivated. Mice were infected by i.p. injection with 5 × 10⁶ S. Typhimurium in PBS containing 100 µg PEPITEM. Mice were used between 6 and 8 weeks of age and were matched for sex, as both male and females were used. Control mice received 5 × 10⁵ S. Typhimurium in PBS only. Mice received further PEPITEM (or PBS) injections (100 µg; i.p.) daily for the next 4 d and all samples were collected at day 5 or 7 after infection. Livers were immediately frozen and were subsequently examined by immunohistochemistry (IHC) as has been described elsewhere. All IHC analysis was performed in Tris buffer (pH7.6) at room temperature. Primary antibodies specific to CD3 (1:300) (145-2C11, BD Pharmingen) and F4/80 (1:500) (CLA3.1, AbD Serotec), and secondary antibodies (Dako Cytomation) (horse-radish peroxidase-1:300) or biotin-conjugated (1:600) were added for 60 and 45 min respectively. Slides were developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) or alkaline phosphatase (ABCComplex, Vector Laboratories) and levamisole with naphthol AS-MX phosphate and Fast Blue BB salt (all from Sigma-Aldrich) respectively. Slides were mounted in glycerol (Sigma-Aldrich) and images acquired using a Leica CTR6000 microscope (Leica, Milton Keynes, UK) with Image J and QCapture software. Mean CD3+ cells per foci were quantified for a minimum of 50 foci per tissue section at 25× magnification.

**Acute liver ischemia and reperfusion injury.** Splenic T cells were isolated from 8–10-week-old male C57BL/6 WT mice (Harlan, Oxford, UK) through negative-selection magnetic activated cell sorting (MACS) using the Pan T cell isolation kit II (Miltenyi Biotec, Surrey, UK) according to the manufacturer’s instructions. C57BL/6 mice were anesthetized by i.p. injection of ketamine (100 mg/kg, Vetar, V, Pfizer, Kent, UK) and xylazine hydrochloride (10 mg/kg, Xylacare, Animalcare, York, UK) delivered in 0.9% saline solution. The trachea and right common carotid artery were cannulated and the liver was exposed. Prior to ischemia, 250 µl of PEPITEM or scrambled peptide was injected via the carotid artery and allowed to circulate for 5 min. Ischemia of the left and median lobes of the liver was induced through application of a traumatic vascular clamp to the hepatic artery and portal vein supplying these lobes for 90 min. After 90 min of ischemia the clamp was removed and intravital observations carried out. Using an Olympus IX81 inverted microscope (Olympus, UK) the microvasculature of the liver was viewed through a 10× objective. 1 million fluorescently labeled T cells (CFDA-SE, 10 µM, Life Technologies, Paisley, UK) with 20 µl of PEPITEM/scrambled peptide were injected into the carotid artery at the point of clamp removal. A random field was selected every 10 min and imaged for 20 s. Five additional fields of view in a pre-defined pattern were then imaged for 20 s each. Adherent cells were defined as cells that were static for at least 20 s. Cells were counted on each field.
Animal Services Unit at Bristol University (Bristol, UK). Mice were housed in specific pathogen-free conditions with continuously available water and food. Female mice immunized for disease induction were aged between 6 and 8 weeks. All mice were kept in the animal house facilities of the University of Bristol. Treatment of animals conformed to United Kingdom legislation and to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Local administration of LPS from Salmonella Typhimurium (50 ng per eye) (Sigma-Aldrich) was performed by intravitreal injection in anesthetized Balb/c mice as previously described. Animals were anesthetized by i.p injection of 150 µl of Vetalar (ketamine hydrochloride 100 mg/ml; Pfizer, Sandwich, UK) and Rompun (xylazine hydrochloride 20 mg/ml; Bayer, Newburg, UK) mixed with sterile water in a ratio of 0.6:1:84. The pupils of all animals were dilated using topical tropicamide 1% (Minims from Chauvin Pharmaceuticals Ltd., UK).

Intravitreal injections were performed under the direct control of a surgical microscope with the tip of a 12-µm 33-gauge hypodermic needle mounted on a 5-µl syringe (Hamilton AG, Bonaduz, Switzerland). For treatment groups either PEPITEM (6 µg) or PBS were combined with 50 ng per eye of LPS as a single injection, administered in a total volume of 4 µl per injection. The injection site was cleaned with chloramphenicol ointment.

At 15 h after LPS/treatment administration, eyes were enucleated and carefully cleaned to remove all extraneous connective and vascular tissue. The aqueous humour and vitreous humor, iris, ciliary body and retina were microscopically dissected in HBSS (Life Technologies, Paisley, UK). These ocular components were homogenized and forced through a 70-µm cell strainer with a syringe plunger, to obtain a single-cell suspension, and stained for flow cytometry analysis.

Cells were incubated with 24G2 cell supernatant for 10 min at 4 °C before incubation with fluorochrome-conjugated monoclonal antibodies (all from BD Bioscience, Oxford, UK) against cell surface markers CD45 (1:1,000) and CD8 (1:100) (53-6.7) at 4 °C for 20 min. Cells were resuspended in 7-aminoactinomycin D (7AAD) (Molecular Probes), and dead cells were excluded from analysis by gating on 7AAD-negative cells. Measurement of cell suspensions were acquired using a three-laser BD LSR-II flow cytometer (BD Cytometry Systems, Oxford, UK) and analyzed with FlowJo software version 7.6.5 (TreeStar, Ashland, OR).

Cell numbers were calculated by reference to a known cell standard, as previously performed using FlowJo software version 7.6.5 (TreeStar, Ashland, OR). Cell numbers were calculated by reference to a known cell standard, as previously performed using FlowJo software version 7.6.5 (TreeStar, Ashland, OR).

At 15 h after LPS treatment, eyes were enucleated and carefully cleaned to remove all extraneous connective and vascular tissue. The aqueous humour and vitreous humor, iris, ciliary body and retina were microscopically dissected in HBSS (Life Technologies, Paisley, UK). These ocular components were homogenized and forced through a 70-µm cell strainer with a syringe plunger, to obtain a single-cell suspension, and stained for flow cytometry analysis.

Cells were resuspended in 7-aminoactinomycin D (7AAD) (Molecular Probes), and dead cells were excluded from analysis by gating on 7AAD-negative cells. Measurement of cell suspensions were acquired using a three-laser BD LSR-II flow cytometer (BD Cytometry Systems, Oxford, UK) and analyzed with FlowJo software version 7.6.5 (TreeStar, Ashland, OR). Cell numbers were calculated by reference to a known cell standard, as previously performed using FlowJo software version 7.6.5 (TreeStar, Ashland, OR).

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Virally induced Sjögren’s syndrome. C57BL/6 mice were from Harlan. Under ketamine-domitor (Pfizer, Sandwich, UK) anesthesia, the submandibular glands were cannulated with a 100-µm needle, to obtain a single-cell suspension, and stained for flow cytometry analysis.

Salivary glands from cannulated mice treated with PEPITEM and scrambled peptides were harvested, snap frozen in OCT (Sakura, UK) over liquid nitrogen. Frozen sections of 7 µm thick were cut, fixed to dry overnight at room temperature, next day they were stored in −80 °C until use. For immunofluorescence analysis, slides were allowed to come to room temperature and then fixed for 20 min in ice-cold acetone, left to dry and then were hydrated in PBS. For immunofluorescence staining, all dilutions of reagents and antibodies were made in PBS with 1% BSA. First, to block endogenous biotin sections were made in PBS with 1% BSA. Single cells suspensions were incubated for 30 min in PBS (with 0.5% BSA and 2 mM EDTA), washed for 5 min with PBS in between the two incubations. This was followed by blocking with 10% horse serum (Sigma-Aldrich) for 10 min. Slides were then incubated for 60 min with ‘cocktails’ containing the following primary antibodies in PBS (1% BSA): CD19–Alexa Fluor 647 (1:50) (eBio1D3), CD3e–biotin (1:50) (eBio500A2) (both were from ebioscience). Biotinylated CD3e was detected using streptavidin–Alexa Fluor 555 (1:500) ( Molecular Probes). Hoechst (1:1,000) ( Molecular Probes) was used for nuclear stain. All secondary antibodies were incubated for 30 min. Slides were mounted with Prolong Gold Antifade reagent (Life Technologies).

Images were acquired on a Zeiss 780 upright confocal head with a Zeiss Axio Imager Z1 microscope and viewed through a 10x objective. Digital images were recorded in three separately scanned channels with no overlap in detection of emissions from the respective fluorochromes. Confoical micrographs were stored as digital arrays of 1024 x 1024 pixels with 8-bit sensitivity; detectors were routinely set so that intensities in each channel spanned the 0–255 scale optimally.

The Zen 2010 software (Zeiss) was used to process these images.

Cannulated salivary glands were harvested and chopped into small pieces and digested for 20 min at 37 °C with gentle stirring in 2 ml of RPMI 1640 medium (Sigma-Aldrich) containing collagenase and dispase (250 µg/ml; from Roche, Welwyn, UK), DNase I (25 µg/ml; from Sigma-Aldrich) and 2% (vol/vol) FCS. The suspension was gently pipetted to break up aggregates. During the final pipetting, EDTA (Sigma-Aldrich) was added to a final concentration of 10 mM to further reduce cell aggregates. Cells were then passed through a 70-µm mesh with a syringe, washed twice and resuspended in PBS (with 0.5% BSA and 2 mM EDTA).

Single cells suspensions were stored for 30 min in PBS (with 0.5% BSA and 2 mM EDTA) with cocktails of the following antibodies CD3e–PEcY7 (1:200) (145-2C11), CD19–APC-Cy7 (1:100) (eBio1D3) (from ebiosciences). Cells were then washed twice in PBS (with 0.5% BSA and 2 mM EDTA), resuspended in PBS (with 0.5% BSA and 2 mM EDTA) and then analyzed using a CyaN-ADP (Dako) with forward/side scatter gates set to exclude nonviable cells. Data were analyzed with FlowJo software (TreeStar, Oregon, USA).

Patient studies. Sample size for the patient studies were guided by the variance from a previous study in our laboratory in which we determined the frequency of adiponectin receptors on monocytes from individuals with T1D versus matched healthy controls. Randomization was not required for this study. All patients and controls were analyzed in parallel on the same flow cytometer over a period of weeks, as they became available from clinic. The expression of the adiponectin receptors was always determined compared to the isotype control for each sample. The same batch numbers of flow cytometry reagents was used throughout to ensure standardization and reduce inter-experimental variation.

Type 1 diabetes. Individuals with T1D were recruited from University Hospital Birmingham Department of Diabetes outpatient clinics. Individuals had received a diagnosis of T1D fulfilling the 1999 World Health Organization (WHO) framework. Blood samples were obtained with written informed consent and approval from the Birmingham, East, North and Solihull Research Ethics Committee (06/Q2703/47).

Healthy volunteers were matched to the T1D cohort on gender, age and BMI (clinical parameters in Supplementary Tables 6 and 7). AdipoR1/2 expression on B cells was measured in 19 healthy controls (58% males) and 29 individuals with T1D (65% male).

Quantification of PEPITEM secretion by B cells under adiponectin stimulation was measured in 10 healthy controls (60% males) and 10 individuals with (60% males). Lymphocyte transmigration was measured for 15 healthy controls (55% males) and 9 (for the PEPITEM cohort) or 22 (for the adiponectin cohort) individuals with T1D (60% males).

Rheumatoid arthritis. Individuals with rheumatoid arthritis were recruited from the Birmingham Early Arthritis Cohort (BEACON). Rheumatoid arthritis was classified according to 1987 American College of Rheumatology criteria. Blood samples were obtained with written informed consent and approval from the National Research Ethics Service (NRES) committee West Midlands, the Black Country (2/WM/0258). Healthy volunteers were matched to the rheumatoid arthritis cohort on gender and age (clinical parameters in Supplementary Table 8). AdipoR1/2 expression on B cells was measured in ten healthy controls (30% males) and 12 individuals with rheumatoid arthritis (41% males). Lymphocyte transmigration was measured for seven healthy controls (57% males) and eight individuals with rheumatoid arthritis (12.5% males).

Aging study. AdipoR1/2 expression on B cells was measured in 40 healthy volunteers ranging in age from 21 to 66 years old.

Statistics. In vitro data are from at least three experiments including three separate donors for PBLs, HUVECs or HDMECs and are the mean of these different experiments ± s.e.m. or s.d. as stated. Numbers of animals in each model are stated in the figure legends. The numbers of individuals with T1D or rheumatoid
arthritis, or healthy controls compared are stated in the figure legends. Differences were analyzed using GraphPad Prism software (GraphPad software Inc., La Jolla, CA, USA) by paired or unpaired t-test or by one way analysis of variance (ANOVA) followed by post hoc analysis for multiple group comparison (Dunnett or Bonferroni). A Dunnett post hoc analysis was used to compare all the data sets on the graph to a common control. A Bonferroni post hoc test was used to compare all data sets in a graph with each other. Normality was checked using the Kolmogorov–Smirnov test. A nonparametric test (Mann–Whitney test) was used when data did not pass the normality test. The Wilcoxon signed-rank test was used to compare a data set to a normalized control, where the data was presented as a percentage of that control (i.e., where control values were the same, e.g., 100%). P values of ≤0.05 were considered significant.

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