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(54) Title: PEPTIDE AND USES THEREOF

(57) Abstract: Provided is a method for treatment and/or prophylaxis of a condition associated with T cell mediated chronic inflammatory disease by administration, to a patient, of a peptide comprising N'-SVTEQGAELSNEER-C' {SEQ ID NO: 1) or an analogue thereof that inhibits T cell migration. Also provided is the peptide or its analogue for use in the methods of treatment: and/or prophylaxis of said condition.

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Peptide and Uses Thereof

The present invention relates to use of a peptide secreted from B cells that has an inhibitory effect on the migration of T cells (including auto-reactive T cells). This has applications in the treatment and/or prophylaxis of the conditions associated with such T cells, most notably type 1 diabetes mellitus.

Introduction

Pancreatic islet-reactive T cells play a central role in beta cell destruction and thus in the pathogenesis of type 1 diabetes (T1D). In evidence, T cells comprise a major part of the islet infiltrate in a T1D pancreas, and immunosuppressive drugs that target T cells preserve beta cell function. Understanding the mechanisms by which islet-reactive T cells are recruited from the blood, across inflamed endothelium, and into the pancreatic islet have been poorly examined in T1D.

This is particularly relevant because healthy humans can also have circulating islet reactive T cells that do no apparent harm. Therefore, we believe that in T1D, endogenous mechanisms that prohibit the trafficking of reactive T cells into the pancreas fail, and if such regulatory pathways could be re-established it may be possible to exclude auto-reactive T cells and preserve beta cell function. The adipocyte-derived cytokine, adiponectin, has a role to play in regulating T cell migration, but the picture is more complex than that as adiponectin's circulating levels do not seem to fluctuate in T1D.

Surprisingly, we have found that a certain peptide can inhibit T cell migration. Although this peptide is known, what we have shown is that adiponectin achieves its effects on T cell migration by the induction of a mediator released from B lymphocytes. This mediator, a peptide, appears to be an inhibitor of T cell trans-endothelial migration.

Summary of the Invention

The peptide has the sequence N'-SVTEQGAELSNEER-C' or is an analogue thereof that inhibits T lymphocyte migration.

Thus, in a first aspect, the present invention provides a method for treatment and/or prophylaxis of a condition associated with T cell mediated chronic inflammatory disease by

administration of a peptide comprising N'-SVTEQGAELSNEER-C' to a patient in need thereof. The peptide may also be an analogue or variant thereof that inhibits T lymphocyte migration.

The condition is, optionally, selected from the group consisting of T cell auto-reactivity, T cell mediated chronic inflammatory disease and autoimmune disease. Alternatively, the condition may be T cell auto-reactivity or T cell mediated chronic inflammatory disease or autoimmune disease.

It will be appreciated that the terms T cell and T lymphocyte can be interchanged herein. The migration of the T cells is, optionally, trans-endothelial. The endothelium is, optionally, that of the pancreatic microvasculature that separates the islet cells from the blood supply.

The peptide is, optionally, an isolated peptide. The peptide may be synthesized (i.e. chemically synthesized, for instance in the same way as a small molecule pharmaceutical) or it may be produced recombinantly, for instance in a separate cellular system (cell culture) or animal.

The amino acid sequence of the peptide that we have found to be useful is SVTEQGAELSNEER (SEQ ID NO: 1). This sequence may be comprised within a larger peptide or protein, or a chimaeric or fusion protein. Alternatively, the peptide may consist solely of SEQ ID NO: 1. All of these fall within the definition of the peptide as used herein. The peptide according to SEQ ID NO: 1 represents amino acids 28-41 of the 14.3.3 zeta/delta (14.3.3.ζδ) protein, which in turn is a 245 amino acid product of the *YWHAZ* gene.

It is also preferred that analogues or variants of the peptide can be used. Particularly preferred in this respect are analogues (or variants) based on conservative amino acid substitutions. The preferred peptide is 14 amino acids long, although the peptide can also be as few as 13, 12, 11 or 10 amino acids or as many as 15, 16, 17 18, 19 or 20 amino acids. Where amino acids are added or removed, these are preferably to or from the N and/or C terminus of the peptide. Other modifications to the chemical structure that protect the peptide from degradation or clearance *in vivo* are also preferred variants, for example but not restricted to, PEGylation which utilises a linker or spacer as is known in the art. Most preferably, any analogue should retain or improve upon the desired function, namely the inhibition of T cell migration, compared to SVTEQGAELSNEER. This may be through changes in affinity for cognate receptor(s) or changes that alter the pharmacokinetic profile of the peptide *in vivo*. It will be appreciated that it is now within the skill of the art to modify

peptide chemistry to increase the pharmacological 'profile' of peptides *in vivo*, and that these changes are not based solely on amino acid substitution.

Reference herein will be made to the peptide, but it will be understood that this also encompasses any analogues thereof, unless otherwise apparent.

The action of the peptide may be as an agonist of its cognate receptor(s).

The inhibition of the migration of the T cells may be the recruitment of said cells to the pancreas, for instance from the blood.

Optionally, the T cells are auto-reactive T cells. These may preferably target the pancreas, especially the islet cells of the pancreas. The T cells may be CD4+ or CD8+.

In a particularly preferred embodiment, the peptide serves to inhibit (i.e. reduce) the recruitment of auto-reactive T cells to the islets of the pancreas.

It will be appreciated that the peptide acts upon the individual to which it is administered. As such, the auto-reactivity of any T cells is reactivity against self (i.e. islet cells of the pancreas) from that individual. The individual is a mammal, optionally, a rodent such as a rat or mouse, or a primate, particularly an ape or human.

As the presence of the peptide serves to inhibit the migration of the T cells, increasing the amount of peptide that the individual is exposed to will serve to further inhibit said migration. Optionally, the level of inhibition of migration is such that migration is reduced by at least 50% (in terms of numbers of T cells that are recruited), but most preferably this reduction is at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 99% and most preferably reduced to negligible levels. Ideally, of course, no T cells will migrate but this may not be realistic and in fact, all that is required is that normal function of the target tissue, for instance the islet cells, is largely preserved and/or returned (or at east as close to normal levels as possible or desirable to alleviate the condition to be treated).

The present peptide is most useful, therefore, in treating a number of conditions. These include those in which T cells play a role in pathology or conditions associated with T cell auto-reactivity. These may include T cell mediated chronic inflammatory disease and autoimmune disease. Diabetes mellitus (type 1), is particularly preferred. Also envisaged are juvenile onset diabetes, rheumatoid arthritis and Crohn's disease, atherosclerosis, psoriasis, inflammatory and fibrotic liver disease(s) including steatohepatitis and cirrhosis and uveitis. The peptide therefore preferably functions to treat any of the above, but most

preferably type 1 diabetes (T1D). The peptide may be considered as serving to rescue or preserve residual pancreatic function. This may be lost function that has occurred due to attack by the auto-reactive T cells. The peptide may be considered as serving to improve diabetic outcomes, i.e. a reduction in the symptoms of T1D. The peptide may also be considered as serving to improve other morbidities associated with loss of pancreatic function which include renal (e.g. nephropathy; diabetic kidney disease), neurological (e.g. peripheral neuropathy) and cardiovascular complications (e.g. diabetic retinopathy and cardio-cerebral disease due to accelerated atherosclerosis), associated with the loss of pancreatic function (in turn) associated with T1D. Therefore, the peptide may be most useful in the treatment and/or prophylaxis of the above conditions, particularly T1D and its co-morbidities (as described).

The invention also provides a polynucleotide sequence coding for said peptide, which is also useful in the treatment and/or prophylaxis of any of the above conditions. The polynucleotide may be DNA, RNA or a DNA/RNA hybrid. This polynucleotide encodes the peptide or its analogue. Although there are a considerable number of possible combinations, we provide at least two examples of a polynucleotide that encode the amino acid sequence of SVTEQGAELSNEER (SEQ ID NO: 1). These are:

5'-AGU GUU ACU GAA CAA GGU GCU GAG UUA UCU AAU GAG GAG AGA-3'

(SEQ ID NO: 2); or

5'-AGC GUC ACC GAG CAG GGC GCC GAA UUG UCC AAC GAA GAG AGG-3'

(SEQ ID NO: 3).

The above sequences are only examples, and are given in RNA form, but the invention also provides for the DNA form (with T replacing U) and DNA/RNA hybrid form thereof, as well as the complementary sequences of both the RNA, DNA and RNA/DNA hybrid forms (the complementary sequences being in RNA, DNA or DNA/RNA). Variants having at least 80% sequence homology are preferred, the variant encoding a peptide that is at least 50%, for instance, as efficacious as SEQ ID NO: 1. Variants have at least functions 85% sequence homology, at least 90% sequence homology, at least 95% sequence homology, at least 99% sequence homology are also preferred (rounding to the nearest whole number). This may be determined by programs such as BLAST, for instance.

Also provided is a plasmid (i.e. a construct), comprising the polynucleotide which encodes the peptide (or its analogue or variant). The polynucleotide is preferably operably linked to a suitable promoter. The promoter may be a pancreas-specific promoter, for instance.

The polynucleotide encoding the peptide may be delivered by administration of a suitable vehicle containing the polynucleotide or to which it is bound. Examples include a so-called gene gun where the polynucleotide may be attached to a gold particle fired through the skin. Alternatively, and more preferably, the polynucleotide (for instance a plasmid comprising it) could be encapsulated within a viral vector or capsid. Preferred examples include adenoviral vectors. Those that target the pancreas are preferred.

Administration of the peptide may be by delivery of the peptide *per se*, for instance in the form of a pharmaceutically acceptable formulation, or by delivery and expression of the polynucleotide encoding the peptide, for instance in the forms described above. These may be delivered, for instance to the blood, by injection. This may be intramuscularly or subcutaneously. These may also be delivered via a mucosa, such as the oral, nasal or rectal mucosa. These may also be delivered in the form of a spray or tablet or in the form of a suppository. These may also be ingested orally into the stomach although in the case of the peptide this may require the provision of the peptide in a pro-drug form to alleviate or combat the effects of the GI digestion.

Although useful in one aspect, it will be appreciated that that it is not necessarily the case that that the peptide or the polynucleotide encoding it is, or needs to be, targeted at or to the pancreas (at least for T1D). Optionally, therefore, for peptide delivery, increasing systemic presentation in the blood plasma is all that is required. Delivery specifically to the pancreas is not required. Nevertheless, in an alternative embodiment, delivery specifically to the pancreas may be used as this could increase efficacy. The same applies for the polynucleotide.

Direct targeting to the pancreas is envisaged, as part of a targeted gene therapy including the polynucleotide encoding the peptide.

Also provided is a pharmaceutically-acceptable composition or preparation comprising the peptide, the polynucleotide, the plasmid or the viral vector described herein. Optionally, the pharmaceutically-acceptable composition comprises the peptide and is suitable for injection or ingestion.

As explained above, methods of treatment and/or prophylaxis of the conditions above are envisaged, particularly conditions associated with T cell mediated chronic inflammatory

disease, including T cell auto-reactivity, T cell mediated chronic inflammatory disease and autoimmune disease. Diabetes mellitus (type 1), is particularly preferred. Also envisaged are juvenile onset diabetes, rheumatoid arthritis and Crohn's disease, atherosclerosis, psoriasis, inflammatory and fibrotic liver disease(s) including steatohepatitis and cirrhosis and uveitis, as well as any of the above-mentioned morbidities. The methods may comprise administering to a patient in need thereof a therapeutic amount of the peptide or polynucleotide in any of the manners described herein.

Thus, provided is a method of treatment and/or prophylaxis of a condition associated with T cell mediated chronic inflammatory disease, including T cell auto-reactivity, T cell mediated chronic inflammatory disease and autoimmune disease. In particular, the condition is diabetes mellitus (type 1). However, the condition may also be selected from the group consisting of: juvenile onset diabetes; rheumatoid arthritis; Crohn's disease; atherosclerosis; psoriasis; inflammatory and fibrotic liver disease(s) including steatohepatitis and cirrhosis; and uveitis; or the condition may be selected from the group consisting of nephropathy; diabetic kidney disease; peripheral neuropathy; diabetic retinopathy; and cardio-cerebral disease.

The methods may be *for* the treatment of said conditions or *of* the treatment of said conditions. Alternatively, the methods may be *for* the prophylaxis of said conditions or may be *of* prophylaxis of said conditions. Alternatively, the methods may be any combination thereof.

Also provided is the peptide and/or the polynucleotide encoding it for use in the treatment and/or prophylaxis of the conditions descried herein. Reference herein to methods includes such use.

Brief Description of the Figures

The invention will now be described with reference to the Figures where:

Figure 1: Adiponectin inhibits the transendothelial cell migration of peripheral blood lymphocytes (PBL);

Figure 2: Inhibition of AMPK with compound C restores the migration of PBL;

Figure 3: PBL from T1D patients are released from the inhibitory effect of adiponectin on transendothelial cell migration;

Figure 4: The expression of adiponectin receptors on PBL is reduced in patients with T1D;

Figure 5: The expression of adiponectin receptors in T1D or healthy control subjects correlates with the inhibition of lymphocyte migration by Adiponectin;

Figure 6: The expression of adiponectin receptors on different leukocyte subsets;

Figure 7: B cells mediate the adiponectin-induced inhibition of T cell migration;

Figure 8: B cells modulate PBL transmigration through secretion of a peptide;

Figure 9 shows the sequence of the secreted peptide and different isoforms of the 14.3.3 proteins;

Figure 10: Comparison of MS/MS parent ion m/z 774.88 from B cell supernatants and a synthetic version of the peptide;

Figure 11: The peptide inhibits T cell migration across endothelial cells in vitro; and

Figure 12: Absolute number of T cells in the inflamed peritoneum of wild type or B cell knockout mice in the presence or absence of the peptide.

Figure 13: The effect of adiponectin (Aq) on the transendothelial cell migration of peripheral blood lymphocytes.

(A) Dose response with an EC50 of ≈ 40nM conducted in static adhesion assay.

(B) The effects of 15 µg/ml Aq on lymphocyte migration in a flow based assay (mimics the flow of blood).

(C) The inhibitory pathway is effective on endothelial cells isolated form different tissues (HUVEC = Umbilical cord; HSAVEC = saphenous vein; HSEC = liver sinusoidal endothelial cells; HDMEC = Dermal microvascular endothelium).

(D) The effects of an AMP-kinase inhibitor on the effects of adiponectin. AMPK is a signalling adapter that is required for adiponectin-receptor signalling.

Figure 14: B cells are required for the adiponectin mediated inhibition of T cell trafficking.

15µg/ml of Aq significantly reduced lymphocyte migration across endothelial cells. removing B cells from the peripheral blood lymphocyte preparation completely inhibited this response.

This could be reconstituted using supernatants from Aq stimulated B cells could also effectively inhibit lymphocyte migration, but this effect was lost when supernatants were prepared in the presence of Brefeldin-A, an inhibitor of B cell secretion. These data demonstrate that a soluble mediator released from B cells is required.

Figure 15: a 14 amino acid peptide released from B cells regulates T cell trafficking

Figure 16: PEPITEM inhibits T cells transmigration

A)The synthetic peptide was highly effective at inhibiting the transmigration of lymphocytes, while control peptides, including a scrambled version (randomized reorganisation of the native peptide sequence), were ineffective at inhibiting lymphocyte migration.

B)The peptide had an EC50 of ≈ 20pM. As it effectively inhibited lymphocyte migration across endothelial cells, we called the agent PEPtide Inhibitor of Trans Endothelial Migration; "PEPITEM."

Figure 17: PEPITEM inhibits T cell migration AND promotes the recruitment of anti-inflammatory regulatory T cells

PEPITEM inhibits T cell migration across EC with the same pattern as adiponectin (A); It is effective at inhibiting the migration of memory CD4+ and CD8+ T cells, but it has no effect neutrophils, or monocytes (including CD16- and CD16+ subsets. Naïve lymphocytes were not assessed in this analysis as they do not adhere to the endothelial cell monolayer (B). Interestingly, the efficiency of the migration of regulatory T cells (T-regs), which have anti-inflammatory functions, was increased by PEPITEM (C).

Figure 18: PEPITEM does not directly regulate T cell migration.

Again the most obvious mode of action of PEPITEM was by directly regulating the migratory functions of T cells. However, this was not the case. When PBL were treated with PEPITEM and the agent was washed away prior to assay on endothelium, the efficiency of lymphocyte migration was not effected. However, pre-treating the endothelial cells with PEPITEM resulted in inhibition of lymphocyte trafficking. Thus, PEPITEM operates by stimulating endothelial cells to release an agent that inhibits T cell trafficking.

Figure 19: The induction of sphingosine-1-phosphate (S1P) synthesis by endothelial cells inhibits T cell migration.

As PEPITEM did not directly inhibit lymphocyte migration we tested the hypothesis that a known regulator of lymphocyte trafficking in other tissues, sphingosine-1-phosphate (S1P), was the terminal step in this pathway.

A) A S1P-receptor antagonist (W146), releases lymphocytes from the inhibitory effects of adiponectin

B) A S1P-receptor antagonist (W146), releases lymphocytes from the inhibitory effects of PEPITEM

C) Addition of exogenous S1P dose dependently inhibits T cell migration

D) Endothelial cell express sphingosine kinase-1 (SPHK1) but not sphingosine kinase-2 (SPHK2)

E) An inhibitor of SPHK1 releases lymphocytes from the inhibitory effects of PEPITEM

Figure 20: S1P regulates the affinity of the lymphocyte integrin LFA-1 (CD11a/CD18; αLβ2) when the cells are immobilised on ICAM and activated with IP10 (CXCL10)

(A) KIM127 for the intermediate affinity site and

(B) antibody 24 for the high affinity epitope on memory T cells treated with S1P.

Figure 21: Absolute number of T cells in the inflamed peritoneum of wild type or B cell knockout mice in the presence or absence of the peptide.

A The recruitment of T cells into the peritoneum of Jh-/- (B-cell knockout animals) was greater at baseline (i.e. after challenge with PBS) than wild type animals. After challenge with intraperitoneal injection with zymosan T cells Numbers increased in the wild type animals. There was a dramatic and significant increase I T cell number in the B cell knockout mice and this was significantly reduced in the presence of PEPITEM, but not the scrambled peptide.

B The recruitment of T cells into the liver of B-cell knockout animals after challenge with none-typhoidal salmonella infection increased when compared to the wild type animals.

Figure 22: The adiponectin/PEPITEM pathway is altered in patients with type 1 diabetes

In type-1-diabetic patients the expression of adiponectin receptors is significantly reduced compared to healthy aged matched controls (A and B). The inhibition of lymphocyte

trafficking by adiponectin correlates significantly with the level of expression of adiponectin receptors on B cells (C), so that patient and healthy cohorts separate Into discrete clusters on the correlation graph. Importantly, although patient lymphocyte are refractory to stimulation by adiponectin (D), the inhibitory pathway can be recapitulated for these cells by addition of exogenous PEPITEM

Detailed Description of the Invention

WO2007127935 relates to the histone deacetylase, HDAC7. It sets out to identify the phosphatase that dephosphorylates HDAC7 and finds that a number of proteins bound to HDAC7, including the peptide described herein as SEQ ID NO NO: 1. The focus of the document is that a "target subunit" of the myosin phosphatase (MYPT1) also bound HDAC7 and as such the teaching is directed to the interaction between HDAC7 and Myosin Phosphatase via this subunit of myosin phosphatase. There is no mention that our peptide has any value, nor that it interferes with the HDAC7 - Myosin Phosphatase interaction. US2002164668 (A1) and US20030064411 (A1) disclose our peptide and pharmaceutical preparations/compositions comprising it in relation to the treatment of Alzheimer's disease. US20040053309 (A1) also discloses our peptide, but relates to the identification of proteins and protein isoforms that are associated with kidney response to toxic effectors. However, none of the prior art discloses the use of our peptide or analogues thereof.

We have been interested in the ability of the adipocyte derived cytokine, adiponectin, to regulate the recruitment of human T cells to inflamed endothelium. Previously, adiponectin deficient mice were shown to have a two-fold increase in leukocyte adhesion to endothelial cells and importantly, leukocyte recruitment was normalized by the addition of recombinant adiponectin. In our in vitro studies we used static transwell assays, as well as flow based adhesion assays, to track the migration of T cells (which were in crude isolates of peripheral blood lymphocytes [PBL]) across TNF-α and IFN-γ stimulated endothelial cells. T cell migration was dose dependently blocked by adiponectin (Figure 1).

The effect of adiponectin on T cell transmigration was mediated by signalling through the adiponectin receptors (AR1 and AR2). AMP-activated protein kinase (AMPK) is a crucial intermediate in the down stream signalling from AR1 and AR2 and when PBL were pre-treated for 30 minutes with the AMPK inhibitor, compound C, the effects of adiponectin on the inhibition of T cell migration were ablated, i.e. T cell migration returned to the levels

observed in the absence of adiponectin (Figure 2). Compound C did not have any effects on migration in the absence of adiponectin.

Importantly, we found that the adiponectin mediated inhibition of T cell migration was significantly compromised in patients with T1D i.e., the ability of adiponectin to modulate T cell recruitment in our *in vitro* migration assays was lost when PBL isolated from T1D were used (Figure 3). We have now shown that both AR1 and AR2 are significantly down regulated on lymphocytes in T1D (Figure 4), and the levels of adiponectin mediated inhibition of T cell migration in vitro correlate exquisitely with expression of these receptors in T1D, to the extent that patient and healthy control cohorts cluster independently when receptor density is plotted against sensitivity to adiponectin in the endothelial cell transmigration assay (Figure 5).

We do not believe that adiponectin represents a suitable target for regulating T cell recruitment in T1D. Its concentration in the circulation is not altered in T1D, indicating that aspects of adiponectin biology other than its bioavailability are important arbiters of function. Moreover, adiponectin is a pleiotropic agent with important roles in metabolic homeostasis, raising the possibility of serious off target side effects.

Rather, we believe that targeting pathways down stream of adiponectin, which regulate T cell migration, would provide a therapeutic modality of greater precision. Thus, we have now gone on to show unequivocally that adiponectin achieves its effects on T cell migration by the induction of a novel mediator, which we believe is a peptide inhibitor of trans-endothelial migration that is released from B lymphocytes. Importantly, B lymphocytes express adiponectin receptors, so can respond in an appropriate manner to stimulation by this agent (Figure 6).

Moreover, the inhibition of T cell migration by adiponectin is lost if B cells are removed from mixed lymphocyte preparations (PBL), and inhibition of T cell migration is regained if isolated B cells are added to purified preparations of T cells (Figure 7a). Interestingly, natural killer lymphocytes (NK cells), which also express high levels of adiponectin receptors (Figure 6) are not capable of regulating the migration of T cells (Figure 7b), indicating that the regulation of T cell migration is mediated exclusively by B lymphocytes and not other cellular components of the PBL population.

B cells mediate their effects in this system by secretion of the peptide. Thus, supernatants conditioned by adiponectin stimulated B cells, could effectively inhibit T cell migration (Figure 8). Moreover, the effects of conditioned supernatants were lost when Brefeldine A, which is

an inhibitor of B cell secretory pathways, was used to inhibit the release of the peptide from B cells in to the conditioned medium, see (Figure 8).

We have now definitively identified the secreted peptide released from B cells in response to adiponectin stimulation. Using mass spectrometric analysis adiponectin conditioned B cell supernatant, as well as the relevant control supernatants were purified and analysed by LC-MS/MS. Comparative analysis of a protein sequence database revealed a single candidate peptide unique to the adiponectin conditioned B cell supernatant, described in Table 1, below.

m/z	Elution time (min)	Score	Modification	Association protein	Sequence
774.88	13.2	63.1	NA	14-3-3 zeta/delta	SVTEQGAELSNEER

Table 1: Candidate peptide for the peptide revealed by comparative analysis of B cell supernatants

Due to the statistically stringent nature of the fragmentation analysis, the software was able to provide a definitive sequence with a high probability of accuracy and to identify the 14.3.3 zeta/delta (14.3.3.ζδ) protein as the precursor protein. Indeed the peptide represents amino acids 28-41 of the 14.3.3 ζδ protein, which in turn is a 245 amino acid product of the *YWHAZ* gen. Stringent database searches demonstrate that the peptide sequence is unique to this protein and is not shared, even by the other six members of the 14.3.3 family of proteins (Figure 9). The peptide is not a member of any known family of immuno-regulatory molecules and due to its chemistry, has attractive therapeutic potential.

We have been able to successfully synthesise the peptide. Comparative analysis of the B cell derived peptide and the synthetic version show identical mass: charge ratios in mass spectrometry analysis, showing that the native peptide has not been subject to post-translational modification prior to excision from the 14.3.3. zeta/delta protein and secretion from B cells (Figure 10).

The peptide has efficacy both in vitro and in vivo. Using the synthetic peptide we constructed a dose response curve in our in vitro assay of T cell migration (Figure 11). The peptide has an EC50 of ≈ 20pM in this assay. We have also utilised the peptide in an *in vivo* model of acute, zymosan induced peritonitis (Figure 12). In this model we first showed that the knockout of B lymphocytes (the cellular source of the peptide) resulted in an increase in the

recruitment of T lymphocytes into the peritoneal cavity. We then conducted the experiment after injection of the peptide into the blood and peritoneum cavity of the B cell knockout mice. The peptide was able to significantly reduce the recruitment of T cells to the peritoneum after challenge with zymosan (Figure 12).

Without being bound by theory, we understand that the following represents the paradigm by which PEPITEM regulates T cell trafficking across endothelial cells during inflammation: Adiponectin, operating through the receptors Adipo-R1 and Adipo-R2 (AR1/2), stimulates the release the immune-regulatory peptide, PEPITEM, from B cells, which are recruited to the endothelial cell surface during inflammation. PEPITEM stimulates endothelial cells through its cognate receptor, promoting the formation and release of sphingosine-1-phosphate (S1P). S1P in turn stimulates T cells recruited to the endothelial cell surface during Inflammation through the S1P-receptor(s) S1PR1/4, a signal that inhibits the ability of T cells to traffic across the endothelial cell barrier and enter inflamed tissue.

The following Examples present experimental proofs for the function of this pathway in both *in vitro* and *in vivo* studies, demonstrate changes in pathway function associated with chronic auto-immune disease in humans, and describe the identity the PEPITEM peptide.

Example 1

Adiponectin inhibits the transendothelial cell migration of peripheral blood lymphocytes (PBL). Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PBL were isolated and treated with adiponectin at 0.0001 to 15μg/ml for one hour.

The results are shown in Figure 1, where part (a) shows that PBL transmigration was significantly and dose dependently reduced by adiponectin in a static adhesion assay; part (b) shows that adiponectin had an EC50 of 0.94μg/ml as determined by linear regression; and part (c) shows that Adiponectin was equally effective at inhibiting PBL migration in a flow based adhesion assay. Data is representative of at least three independent experiments and were analysed using t-test, one-way ANOVA and Dunnett's multiple comparisons post-test. *p≤0.01, **p≤0.001, ***p≤0.0001.

Inhibition of AMPK with compound C restores the migration of PBL.

Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours. Compound C was added to PBL at 10μg/ml for 30 minutes prior to addition of adiponectin at 15μg/ml for 1 hour. Adiponectin treatment induced a decrease of transmigration, which was restored to normal, control levels in the presence of compound C. The results are shown in Figure 2, where data is representative of three experiments and were analysed using one-way ANOVA and Dunnet's multiple comparisons post-test. **p≤0.001, ***p≤0.0001.

PBL from T1D patients are released from the inhibitory effect of adiponectin on transendothelial cell migration.

Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in absence of adiponectin. The results are shown in Figure 3. Part a) shows that Adiponectin-mediated inhibition of PBL transmigration is lost in T1D; and part b) shows that the percentage of inhibition was calculated by dividing the percentage of transmigration with adiponectin treatment by the percentage of transmigration of untreated PBL. n= 13 for HC groups and n=12 for T1D group. Data was analysed using t-test and one-way ANOVA and Bonferonni's multiple comparisons post-test. ***p≤0.0001.

The expression of adiponectin receptors on PBL is reduced in patients with T1D.

The frequency of PBL expressing adiponectin receptors AR1 or AR2 were determined for each healthy or diseased subject and are shown in Figures 4a) and 4b), respectively. Data is represented as mean ± SEM and was analysed using t-test or Mann Whitney t-test when data did not pass the Kolmogorov-Smirnov normality test.

The expression of adiponectin receptors in T1D or healthy control subjects correlates with the inhibition of lymphocyte migration by adiponectin.

Figure 5a) shows the correlation between the expression of AR1 and inhibition of lymphocyte migration, whilst Figure 5b) shows the correlation between the expression of AR2 and inhibition of lymphocyte migration. Correlations were determined using linear regression analysis.

The expression of adiponectin receptors on different leukocyte subsets.

Figure 6a) and b) show the expression of AR1 (Figure 6a) and AR2 (Figure 6b) on different cell types. Data is mean ± SEM and are representative of seven healthy controls. Data was analysed using one-way ANOVA and Bonferonni's multiple comparisons post-hoc test. ***p≤0.0001.

B cells mediate the adiponectin-induced inhibition of T cell migration.

Figure 7a) shows that the migration of PBL is lost when they are depleted of B cells (Bs) and regained when B cells are added back to isolated T cells. Figure 7b) shows that the migration of natural killer cells is not affected by adiponectin and addition of NKs to T cells does not regulate the migration of the T cells. Data is mean ± SEM and are representative of at least three independent experiments. Data was analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. **p≤0.001, ***p≤0.0001.

B cells modulate PBL transmigration through secretion of a peptide.

B cells were isolated and incubated in presence or absence of adiponectin at 15µg/ml. Supernatant was taken after one hour and added to Bs-ve PBL which significantly restored the adiponectin inhibition of PBL transmigration. For some experiments, B cells were treated with Brefeldin A, an inhibitor of B cell secretion. These supernatants were not able to regulate the migration of T cells. This is shown in Figure 8, where the data is shown as mean ± SEM and is representative of three independent experiments analysed using one-way ANOVA and Bonferroni's multiple comparison post test. ***p<0.001, ns=non significant.

The sequence of the peptide was determined and it is shown in Figure 9 together with the different isoforms of the 14.3.3 proteins. See also Table 1 above.

Comparison of MS/MS parent ion m/z 774.88 from B cell supernatants and a synthetic version of the peptide.

The ion m/z 774.88 is a fragmentation product of the analysis protocol and is generally only of use for identification using MS/MS, but can be an important parameter for comparison.

A comparison of the Mass Spec profiles of parent ion m/z 774.88 from B cell supernatants and a synthetic version of the peptide analysis is shown in Figure 10, revealing identical mass: charge ratios. This confirmed sequence identity and showed that the peptide is not subject to post-translational modification prior to secretion.

The peptide inhibits T cell migration across endothelial cells in vitro.

PBL were treated with Adiponectin (15µg/ml as positive control) or the peptide at concentrations between 0.001 and 10ng/ml, a scramble peptide was used as a negative control (used 10ng/ml). Other bioactive peptides were also used to demonstrate specificity of the peptide (i.e. tetanus toxoid peptide (TTp) at 10ng/ml and pro-insulin (PI) at 10ng/ml). The results are shown in Figure 11. Figure 11 a) shows that PBL transmigration was dose-dependently reduced in presence of the peptide but not in the presence of the scrambled peptide, TTp or PI controls. Figure 11b) shows that the EC50 of the peptide (18.6pM) was calculated using non linear regression analysis. Data is representative of three independent experiments and was analysed using one-way ANOVA and Bonferroni's multiple comparison post test. *p≤0.01, **p≤0.001, ***p≤0.0001.

Absolute number of T cells in the inflamed peritoneum of wild type or B cell knockout mice in the presence or absence of the peptide.

Leukocytes were collected from the peritoneum after 48 hours injection of zymosan (or PBS as control) with or without the peptide or a scrambled peptide. T cells were identified by expression of CD3. The peptide or a scrambled peptide was injected at a final concentration of 300µg / mouse. The results are shown in Figure 12, where data for each group is the mean and was analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. *p≤0.01.

Example 2

This Example shows the results of further work undertaken and thus compliments Example 1.

The effect of adiponectin (AQ) on the transendothelial cell migration of peripheral blood lymphocytes (PBL).

Refer to Figure 13. Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PBL were isolated and treated with adiponectin at 0.0001 to 15μg/ml for one hour. Part (a) shows that PBL transmigration was significantly and dose dependently reduced by adiponectin in a static adhesion assay and that adiponectin had an EC50 of ~40nM as determined by linear regression; and part (b) shows that Adiponectin was equally effective at inhibiting PBL migration in a flow based adhesion assay; and part (c) shows that Adiponectin is effective on endothelial cells isolated from different tissues such as HUVEC (Umbilical cord), HSEC (liver sinusoidal endothelial cells) and DMEC (Dermal microvascular endothelium) but not HSAVEC (saphenous vein). In part (d), compound C, an AMP-kinase inhibitor, was added to PBL at 10μg/ml for 30 minutes prior to addition of adiponectin at 15μg/ml for 1 hour. AMPK is a signalling adapter that is required for adiponectin-receptor signalling. Adiponectin treatment induced a decrease of transmigration, which was restored to normal control levels in the presence of compound C. These data indicate that adiponectin has a strong capacity to regulate the transmigration of lymphocytes through action on its receptors expressed on PBL. Data is a pool of at least three independent experiments and were analysed using t-test, one-way ANOVA and Dunnett's multiple comparisons post-test.**$p \leq 0.01$, ***$p \leq 0.001$.

T cells do not posses Adiponectin receptors.

The simplest interpretation of the previous experiment is that T cells are under the direct control of Aq. However, T cells lack the appropriate receptors. However, other leukocytes do have Adipo-R1/2 and both monocytes and B cells show high levels of expression.

Expression of both adiponectin receptors, AdipoR1 and AdipoR2, was measured on PBMC by flow cytometry using rabbit anti-human adiponectin receptor 1 and 2 antibodies (Phoenix peptides). Adiponectin receptor expression is shown on the horizontal axis against pan markers of PBMC sub-populations (vertical axis). AdipoR1 and AdipoR2 are highly

expressed on monocytes (CD14+) and on B cells (CD19+) but at very low levels on T cells (CD3+). This indicates that adiponectin cannot directly control T cell migration.

B cells are required for the adiponectin mediated inhibition of T cell trafficking.

Refer to Figure 14. Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PBL transmigration was measured after removal of B cells using bead positive selection and after reconstitution with B cells that were isolated using bead negative selection in presence or absence of adiponectin (15 µg/ml). Supernatants from adiponectin-treated B cells or B cell treated with Brefeldin A to block protein secretion were added to PBL.

Removing B cells form the peripheral blood lymphocyte preparation completely inhibited this response. This could be reconstituted using supernatants from Adiponectin stimulated B cells that could also effectively inhibit lymphocyte migration, but this effect was lost when supernatants were prepared in the presence of Brefeldin-A, an inhibitor of B cell secretion. These data demonstrate that a soluble mediator released form B cells is required.

Data is a pool at least three independent experiments and was analysed using one-way ANOVA and Bonferroni's multiple comparison post test. *p≤0.05, ***p≤0.001.

A 14 amino acid peptide released from B cells regulates T cell trafficking

Refer to Figure 15. B cells were isolated using negative selection and incubated with adiponectin for an hour. Supernatants were recovered and purified on a C18 columns to remove large size proteins and acquired by mass spectrometry. The proteomic analysis using mass spectrometry of supernatants from AQ stimulated B cells revealed a 14 amino acid peptide with the sequence SVTEQGAELSNEER. Comparing this to an in silico library of published and predicted sequences, the peptide demonstrated exact sequence homology to a single human protein, and represents amino acids 28-41 of the 14.3.3 zeta/delta (14.3.3.ζδ) protein, which in turn is a 245 amino acid product of the *YWHAZ* gene. The peptide is not a member, nor is it related to, nor does it have sequence similarity to, any of the known families of immune-regulatory peptides. Analysis of synthetic peptide by mass spectrometry showed an identical mass:charge ratio to the native peptide (m/z=774.88), demonstrating that the B-cell derived product was not subject to any form of post

translational modification prior to release . These data indicate that the 14 amino acid peptide identified is the mediator released by B cells under adiponectin stimulation.

PEPITEM inhibits T cells transmigration

Refer to Figure 16. Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PBL were treated with Adiponectin (15µg/ml as positive control) or the peptide at concentrations between 0.001 and 10ng/ml, a scramble peptide was used as a negative control (10ng/ml). Other bioactive peptides were also used to demonstrate specificity of the peptide (i.e. tetanus toxoid peptide (TTp) at 10ng/ml and pro-insulin (PI) at 10ng/ml). Part (a) shows that PBL transmigration was dose-dependently reduced in presence of the peptide but not in the presence of the scrambled peptide, TTp or PI controls. Part (b) shows that the EC50 of the peptide (18.6pM) was calculated using non linear regression analysis. The data indicates that PEPITEM is able to inhibit PBL transmigration similarly to adiponectin. Data is a pool of at least three independent experiments and was analysed using one-way ANOVA and Bonferroni's multiple comparison post test. *p≤0.05, **p≤0.01, ***p≤0.001.

PEPITEM inhibits T cell migration AND promotes the recruitment of anti-inflammatory regulatory T cells

Refer to Figure 17. Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PBL and the different subsets and PEPITEM were added to different endothelial cells and transmigration was measured. The different subsets were isolated using negative selection for Treg, CD4+ and CD8+ memory and naïve T cells. Positive selection was used to isolate the different monocyte subsets.

Part (a) shows that PEPITEM inhibits T cell migration across EC with the same pattern as adiponectin on different endothelial cell type. Part (b) shows that PEPITEM is effective at inhibiting the transmigration of memory CD4+ and CD8+ T cells, but it has no effect neutrophils, or monocytes (including CD16- and CD16+ subsets. Naïve lymphocytes were not assessed in this analysis as they do not adhere to the endothelial cell monolayer. Part (c) shows the efficiency of the migration of regulatory T cells (Treg), which have anti-inflammatory functions, was increased by PEPITEM. These data indicate that PEPITEM is able to specifically modulate transmigration of memory T cells and Treg.

Data is a pool of at least three independent experiments and was analysed using t-test and one-way ANOVA and Bonferroni's multiple comparison post test. *p≤0.05, **p≤0.01, ***p≤0.001.

PEPITEM does not directly regulate T cell migration

Refer to Figure 18. Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PEPITEM was added with the PBL on the endothelial cells or endothelial cells were pre-treated with PEPITEM and PBL added after washes or PBL were pre-treated with PEPITEM, washed and added to the endothelial cells.

When PBL were treated with PEPITEM and the agent was washed away prior to assay on endothelium, the efficiency of lymphocyte migration was not affected. However, pre-treating the endothelial cells with PEPITEM resulted in inhibition of lymphocyte trafficking. These data indicate that PEPITEM operates by stimulating endothelial cells to release an agent that inhibits T cell trafficking.

Data is a pool of three independent experiments and was analysed using paired t-test *p≤0.05, **p≤0.01.

The induction of sphingosine-1-phosphate (S1P) synthesis by endothelial cells inhibits T cell migration.

Refer to Figure 19. PBL or B cell depleted PBL transmigration across IFN-γ/TNF-α treated HUVEC was measured after blockade of S1P signalling using S1PR antagonist (W146, 10 μM) in presence or absence of (part **a**) adiponectin (15 μg/ml) or (part **b**) PEPITEM. B cell depleted PBL were pre-treated with S1P at different concentrations (0 – 100 μM) and transmigration across IFN-γ/TNF-α treated HUVEC was measured (part c). Levels of SPHK1 and SPHK2 mRNA expression determined by real-time PCR of RNA from B cells and HUVEC (part d, n=2). PBL transmigration was measured across IFN-γ/TNF-α treated HUVEC pre-treated with SPHK1 specific inhibitor (5μM) in presence of PEPITEM (10 ng/ml) (part e).

The data shows that antagonism of the S1P receptor on T cells results in loss of adiponectin and PEPITEM inhibition on T cell transmigration (part a, b). Part (c) shows that addition of

S1P to B cell depleted T cells restores the inhibition of transmigration; and part (d) shows high expression of S1P kinase 1 and 2 in HUVEC (SPHK1 and 2); and part (e) shows that inhibition of SPHK1 releases lymphocytes from the inhibitory effect of PEPITEM. These data indicates that PEPITEM stimulates endothelial cells to release S1P, which in turn inhibits lymphocyte transmigration.

Data is a pool of at least three independent experiments and was analysed using t-test and one-way ANOVA and Bonferroni's multiple comparison post test. *p≤0.05, **p≤0.01, ***p≤0.001.

S1P regulates the affinity of the lymphocyte integrin LFA-1.

Refer to Figure 20. 96 well plates were coated with 50ug/ml of recombinant ICAM overnight at 4°C. The plate was blocked using PBS 4% BSA for an hour at room temperature and PBL treated with IP-10 (10ng/ml) and/or S1P (10uM) were added for 30 minutes. Excess of unbound PBL was washed and PBL were labelled for the intermediate affinity site of the lymphocyte integrin LFA-1 (CD11a/CD18; αLβ2) using the KIM127 antibody (10ug/ml) and for the high affinity site using antibody 24 (10ug/ml) at 4 °C. The expression of both affinity site was measured on memory T cells using mean fluorescence intensity (MFI). The data shows that the expression of both intermediate and high affinity sites increased upon IP-10 stimulation is down-regulated in presence of S1P. The data indicates that S1P regulate lymphocyte transmigration by modulating the affinity of the integrin LFA-1 that is essential to lymphocyte transmigration. Data is a pool of two independent experiments.

Absolute number of T cells in the inflamed peritoneum of wild type or B cell knockout mice in the presence or absence of the peptide.

Refer to Figure 21. In part (a), wild-type or B cell knock-out (Jh-/-) BALB/c mice were injected with 100ug zymosan. Leukocytes were collected from the peritoneum after 48 hours injection of zymosan (or PBS as control) with or without the peptide or a scrambled peptide. T cells were identified by expression of CD3. The peptide or a scrambled peptide was injected at a final concentration of 300μg / mouse. The results are shown in part (a), where data for each group is the mean and was analysed using one-way ANOVA and Bonferroni's

multiple comparisons post-test. *p≤0.01. In part (b), wild-type or B cell knock-out C56BL/6 mice were injected with Salmonella typhirium. After 5 days, liver were collected and sections stained for T cells. The data in part (b) shows the number of T cells per infection loci in liver sections.

The data shows that absence of B cells in mouse results in higher recruitment of T cells in the peritoneum upon zymosan-induced inflammation and Salmonella infection. This is reduced in the zymosan treated B cell knock-out mice by PEPITEM but not by the scrambled control. These data indicates that B cells are essential to regulate recruitment of T cells during inflammation *in vivo* by release of PEPITEM at sites of inflammation.

The adiponectin/PEPITEM pathway is altered in patients with type 1 diabetes

Refer to Figure 22. The frequency of PBL expressing adiponectin receptors AR1 or AR2 were determined for each healthy or diseased subject by flow cytometry and are shown in part (a) and (b), respectively. Data is represented as mean ± SEM and was analysed using t-test or Mann Whitney t-test when data did not pass the Kolmogorov-Smirnov normality test. Endothelial cells were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in the absence of adiponectin. PBL were isolated from healthy controls and patients with type 1 diabetes and treated with adiponectin 15μg/ml for one hour. Part (c) shows a correlation between the expression of AdipoR2 and inhibition of lymphocyte migration, Correlations were determined using linear regression analysis. Part (d) shows the transmigration of PBL from newly diagnosed patient with type 1 diabetes, pre-treated with adiponectin or PEPITEM (n=5). Data was analysed using t-test **p<0.01.

The results show in part (a and b), lower expression of both adiponectin receptors (AdipoR1/2) on PBL from patients with type 1 diabetes; and part (b) shows that the lower expression of AdipoR2, the lower is the capacity of adiponectin to inhibit lymphocyte transmigration; and in part (d), PEPITEM was still able to inhibit lymphocyte transmigration.

The data indicates that lymphocytes from patients with type 1 diabetes are released from the inhibitory effects of adiponectin because they express lower adiponectin receptors and this can be restored by exogenous addition of PEPITEM.

Claims:

1. A method for treatment and/or prophylaxis of a condition associated with T cell mediated chronic inflammatory disease by administration, to a patient, of a peptide comprising N'-SVTEQGAELSNEER-C' (SEQ ID NO: 1) or an analogue thereof that inhibits T cell migration.

2. A method according to claim 1, wherein the condition is selected from the group consisting of T cell auto-reactivity, T cell mediated chronic inflammatory disease and autoimmune disease.

3. A method according to claim 1 or 2, wherein the condition is diabetes mellitus (type 1).

4. A method according to claim 1 or 2, wherein the condition is selected from the group consisting of: juvenile onset diabetes; rheumatoid arthritis; Crohn's disease; atherosclerosis; psoriasis; inflammatory and fibrotic liver disease(s) including steatohepatitis and cirrhosis; and uveitis.

5. A method according to claim 1 or 2, wherein the condition is selected from the group consisting of nephropathy; diabetic kidney disease; peripheral neuropathy; diabetic retinopathy; and cardio-cerebral disease.

6. A method according to any preceding claim, wherein the migration of the T cells is trans-endothelial across the endothelium of the pancreatic microvasculature separating the islet cells from the blood supply.

7. A method according to any preceding claim, wherein the T cells are auto-reactive T cells.

8. A method according to claim 7, wherein the peptide serves to inhibit the recruitment of auto-reactive T cells to the islets of the pancreas.

9. A method according to any preceding claim, wherein a polynucleotide sequence encoding the peptide or analogue thereof is administered.

10. A method according to claim 9, wherein the polynucleotide is selected from the group consisting of: SEQ ID NO: 2; SEQ ID NO: 3; the DNA form or DNA/RNA hybrid forms thereof; and any complementary sequence thereof.

11. A method according to claim 10, wherein the polynucleotide is operably linked to a suitable promoter, for instance a pancreas-specific promoter.

12. A method according to any of claims 9 to 11, wherein a plasmid comprising the polynucleotide is administered.

13. A method according to claim 12, wherein a viral vector comprising the plasmid is administered.

14. A method according to any preceding claim, wherein a pharmaceutically-acceptable composition is administered, the composition comprising the peptide or analogue thereof as defined in any of claims 1-8, the polynucleotide as defined in claims 9-10, the plasmid as defined in claim 12 or a viral vector as defined in claim 13.

15. A peptide or analogue thereof as defined in any of claims 1-8, the polynucleotide as defined in claims 9-10, the plasmid as defined in claim 12 or a viral vector as defined in claim 13, for use in the methods of treatment and/or prophylaxis of a condition as defined in any of claims 1-8.

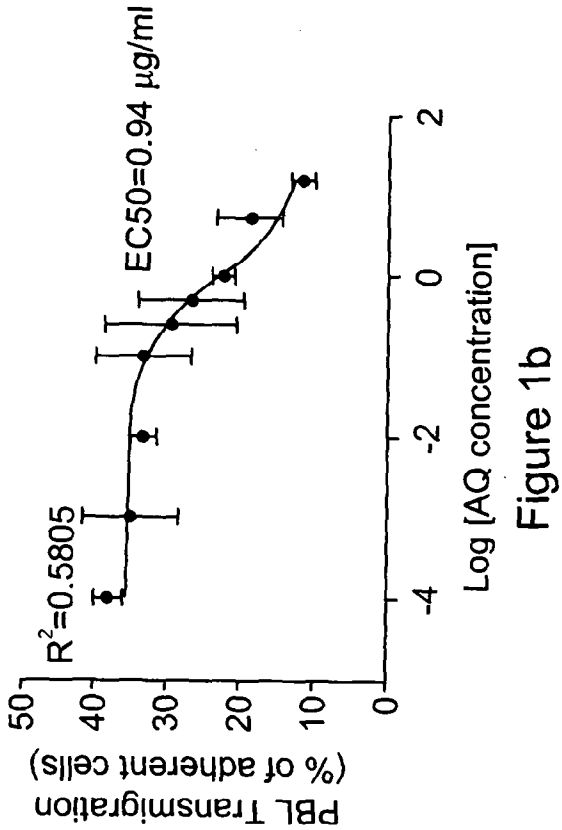


Figure 1b

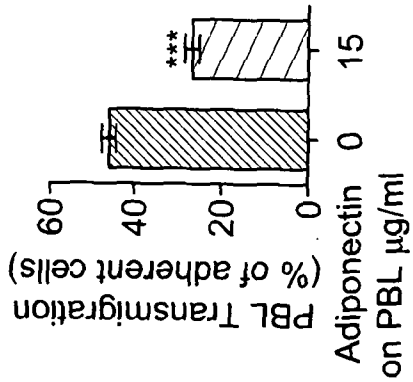


Figure 1c

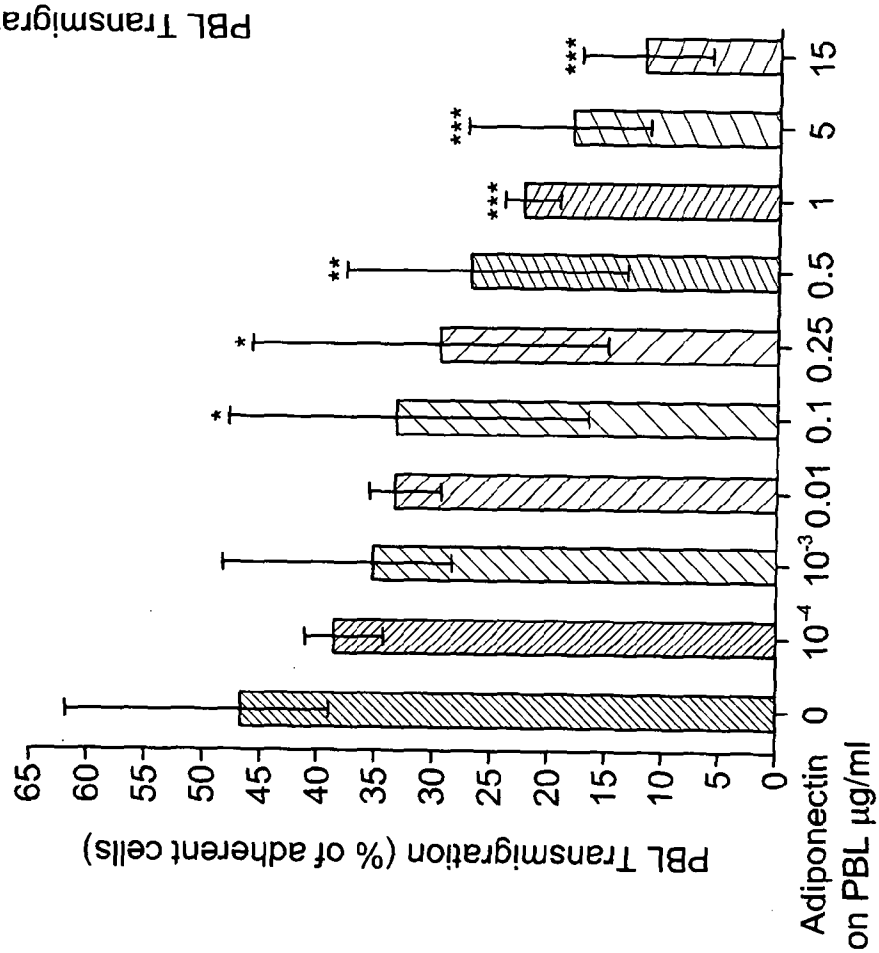


Figure 1a

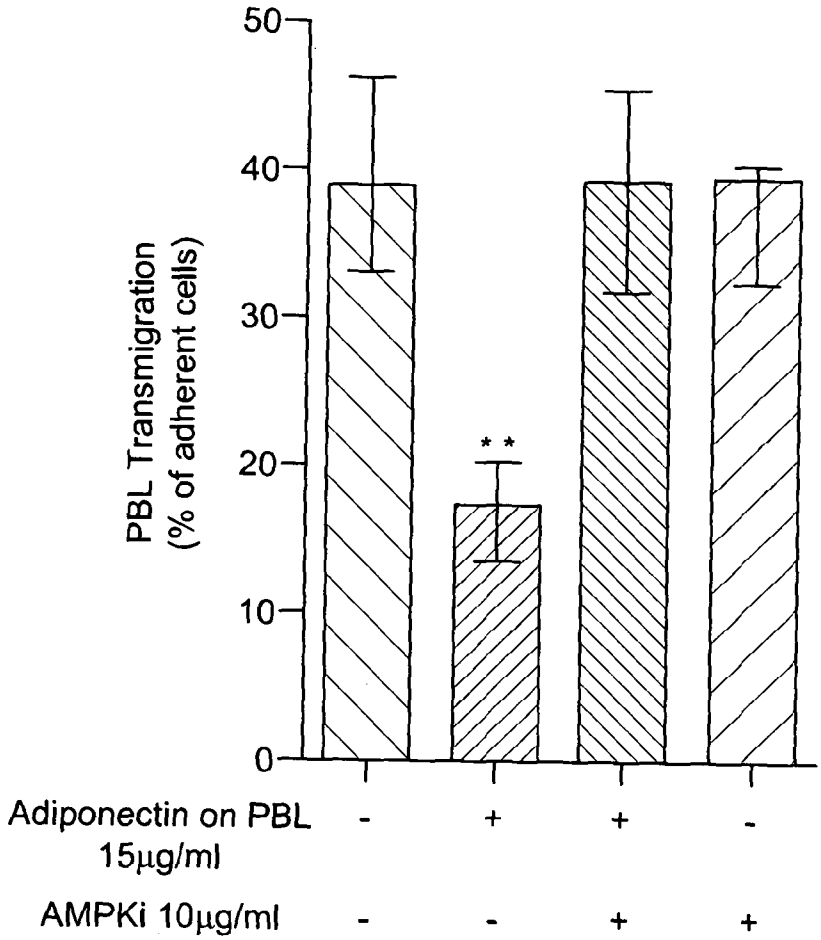

50
40
30
20
10
0
PBL Transmigration
(% of adherent cells)
* *
Adiponectin on PBL
15µg/ml
- + + -
AMPKi 10µg/ml
- - + +

Figure 2

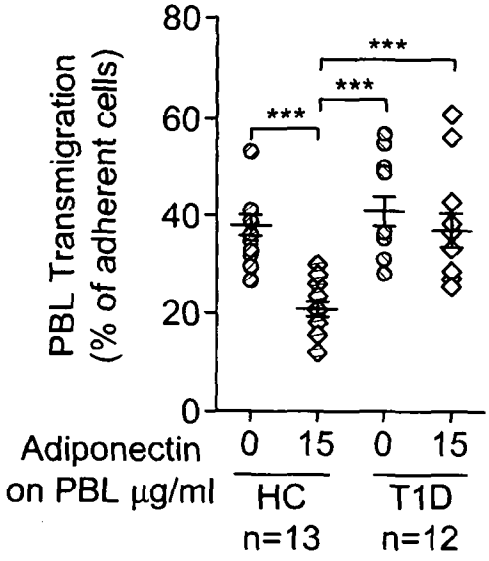


Figure 3a

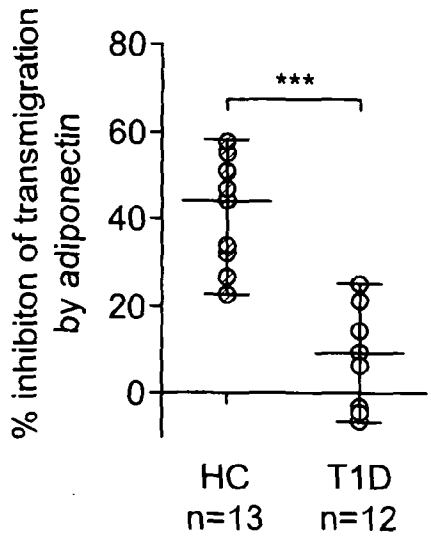


Figure 3b

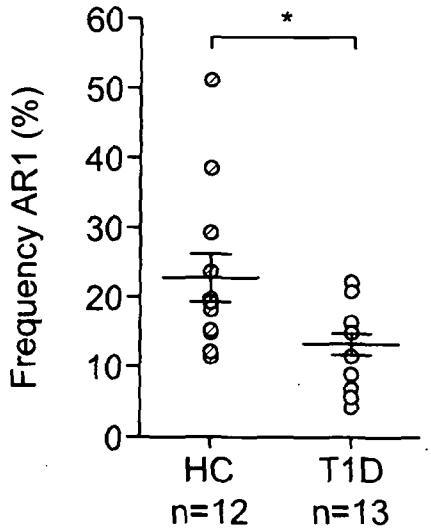


Figure 4a

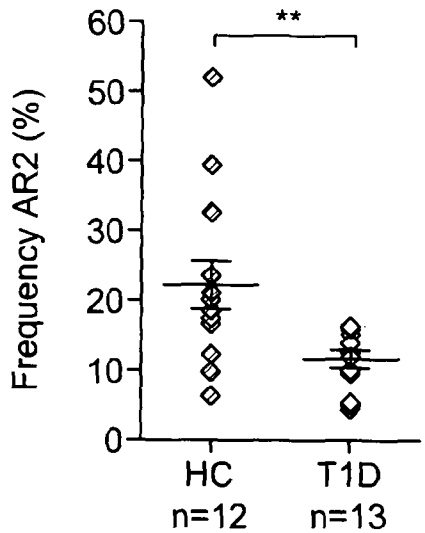


Figure 4b

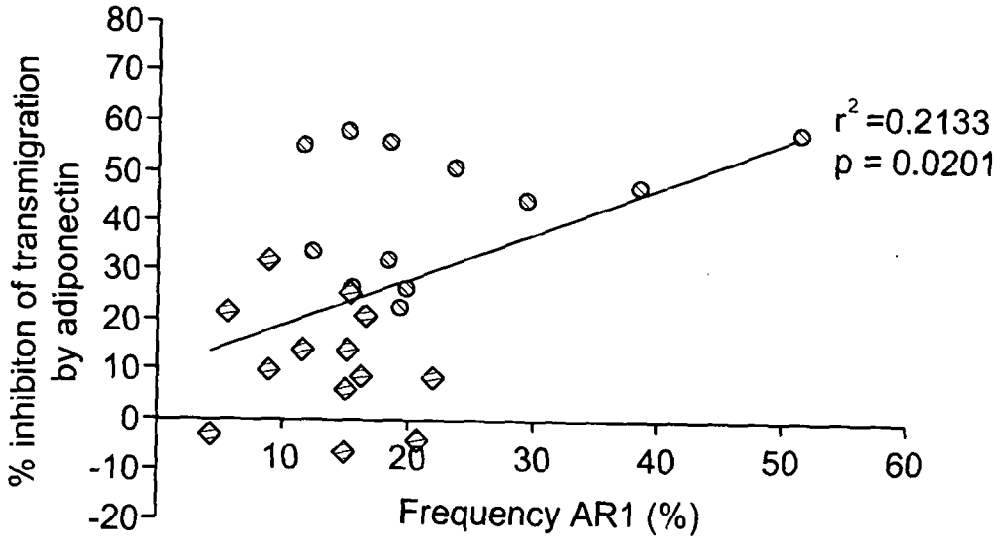


Figure 5a

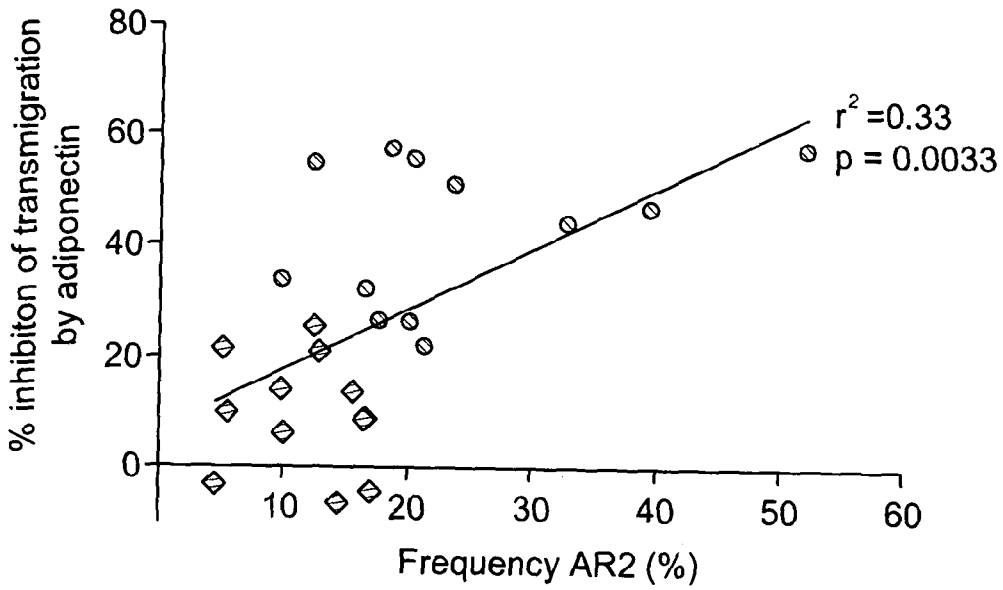


Figure 5b

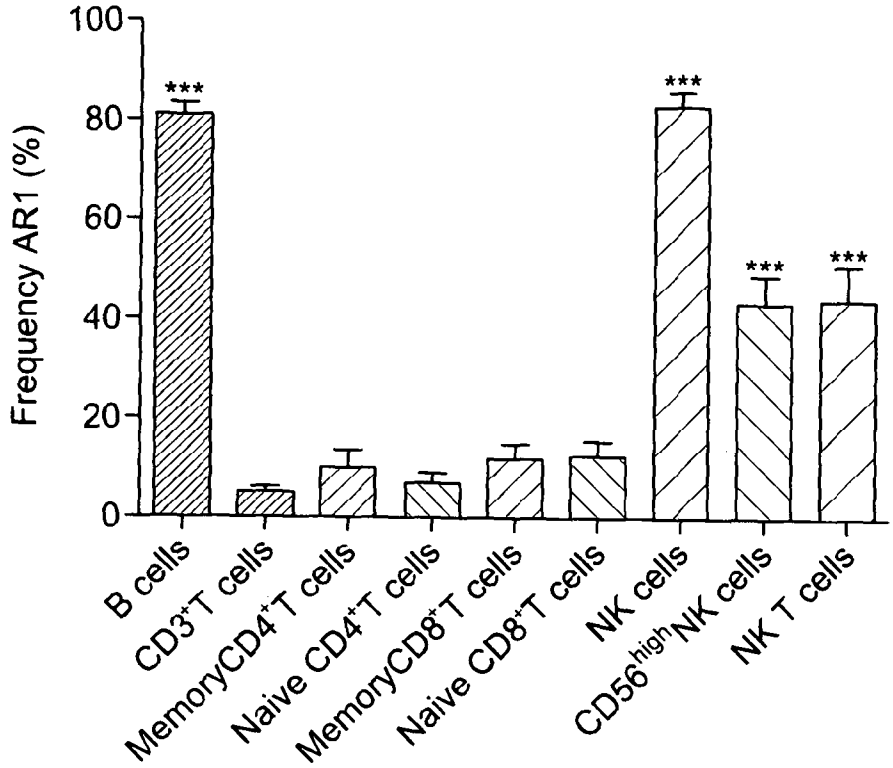


Figure 6a

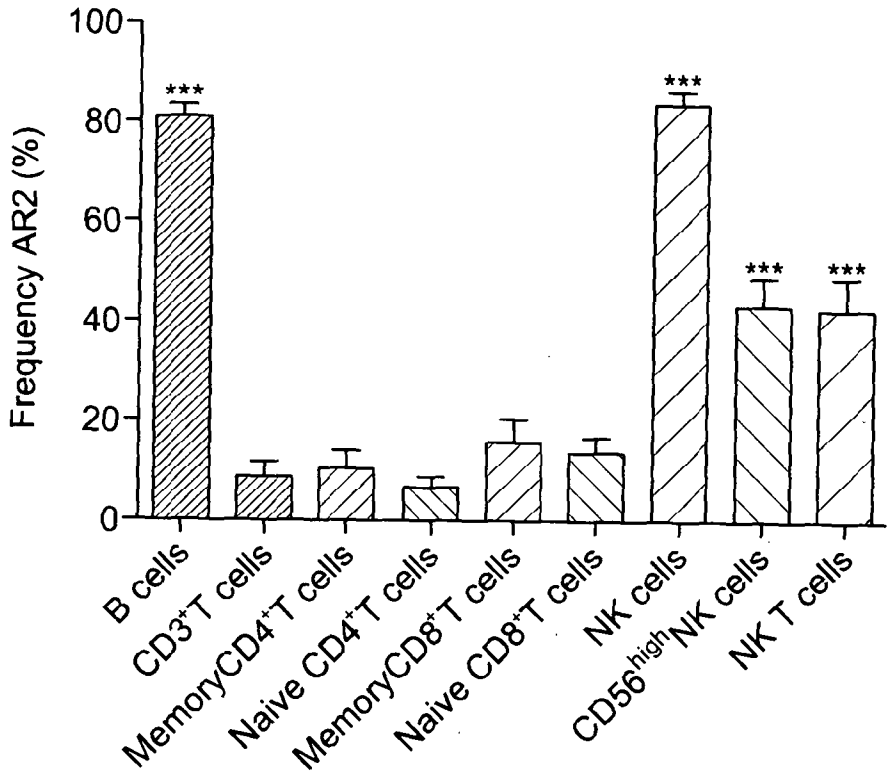


Figure 6b

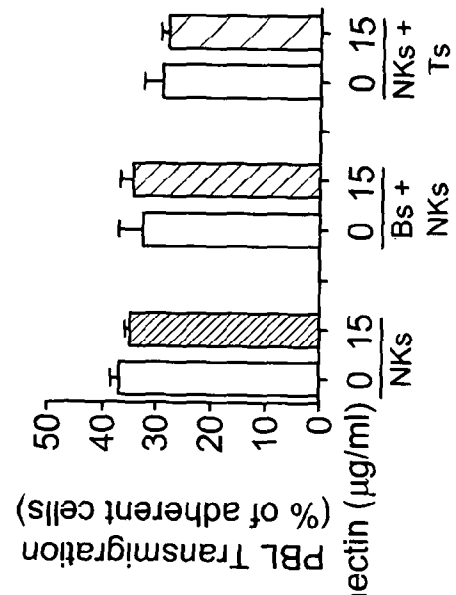


Figure 7a

Figure 7b

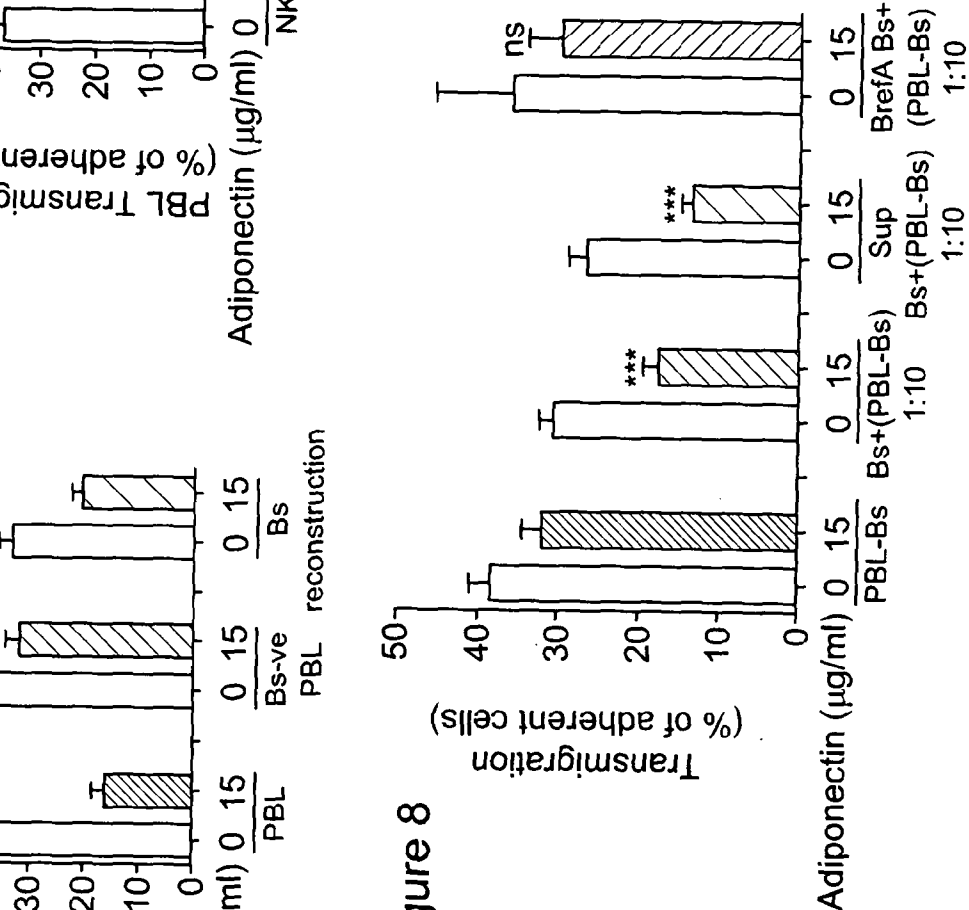


Figure 8

Sequences of 14.3.3. isoforms and presence of the peptide therin.

	10	20	30	40	50	60
Beta/Alpha	MTMDKSELVQ	KAKLAEQAER	YDDMAAAMKA	VTEQGHELSN	EERNLLSVAY	KNVVGARRSS
Epsilon	MDDREDLVYQ	AKLAEQAERY	DEMVESMKKV	AGMDVELTVE	ERNLLSVAYK	NVIGARRASW
Eta	MGDREQLLQR	ARLAEQAERY	DDMASAMKAV	TELNEPLSNE	DRNLLSVAYK	NVIGARRASW
Gamma	MVDREQLVQK	ARLAEQAERY	DDMAAAMKNV	TELNEPLSNE	ERNLLSVAYK	NVIGARRASW
Sigma	MERASLIQKA	KLAEQAERYE	DMAAFMKGAV	EKGEELSCEE	RNLLSVAYKN	VVGGQRAAWR
Theta	MEKTELIQKA	KLAEQAERYD	DMATCMKAVT	EQGAELSNEE	RNLLSVAYKN	VVGGRRSAWR
Zeta/Delta	MDKNELVQKA	KLAEQAERYD	DMAACMKSVT	EQGAELSNEE	RNLLSVAYKN	VVGARRSSWR

	70	80	90	100	110	120
Beta/Alpha	WRVISSIEQK	TERNEKKQQM	GKEYREKIEA	ELQDICNDVL	ELLDKYLIPN	ATQPESKVFY
Epsilon	RIISSIEQKE	ENKGGEDKLK	MIREYRQMVE	TELKLICCDI	LDVLDKHLIP	AANTGESKVF
Eta	RVISSIEQKT	MADGNEKKLE	KVKAYREKIE	KELETVCNDV	L SLLDKFLIK	NCNDFQYESK
Gamma	RVISSIEQKT	SADGNEKKIE	MVRAYREKIE	KELEAVCQDV	LSLLDNYLIK	NCSETQYESK
Sigma	VLSSIEQKSN	EEGSEEKGPE	VREYREKVET	ELQGVCDTVL	GLLDSHLIKE	AGDAESRVFY
Theta	VISSIEQKTD	TSDKKLQLIK	DYREKVESEL	RSICTTVLEL	LDKYLIANAT	NPESKVFYLK
Zeta/Delta	VVSSIEQKTE	GAEKKQQMAR	EYREKIETEL	RDIC NDVLSL	LEKFLIPNAS	QAESKVFYLK

	130	140	150	160	170	180
Beta/Alpha	LKMKGDYFRY	LSEVASGDNK	QTTVSNSQQA	YQEAFEISKK	EMQPTHPIRL	GLALNFSVFY
Epsilon	YYKMKGDYHR	YLAEFATGND	RKEAAENSLV	AYKAASDIAM	TELPPTHPIR	LGLALNFSVF
Eta	VFYLKMKGDY	YRYLAEVASG	EKK NSVVEAS	EAAYKEAFEI	SKEQMQPTHP	IRLGLALNFS
Gamma	VFYLKMKGDY	YRYLAEVATG	EKRATVVESS	EKAYSEAHEI	SKEHMQPTHP	IRLGLALNYS
Sigma	LKMKGDYYRY	LAEVATGDDK	KRIIDSARSA	YQEAMDISKK	EMPPTNPIRL	GLALNFSVFH
Theta	MKGDYFRYLA	EVACGDDRKQ	TIDNSQGAYQ	EAFDISKKEM	QPTHPIRLGL	ALNFSVFYYE
Zeta/Delta	MKGDYYRYLA	EVAAGD DKKG	IVDQSQQAYQ	EAFEISKKEM	QPTHPIRLGL	ALNFSVFYYE

Figure 9

	190	200	210	220	230	240
Beta/Alpha	YEILNSPEKA	CSLAKTAFDE	AIAELDTLNE	ESYKDSTLIM	QLLRDNLTLW	TSENQGDEGD
Epsilon	YYEILNSPDR	ACRLAKAAFD	DAIAELDTLS	EESYKDSTLI	MQLLRDNLTL	WTSDMQGDGE
Eta	VFYYEIQNAP	EQACLLAKQA	FDDAIAELDT	LNEDSYKDST	LIMQLLRDNL	TLWTSDQQDE
Gamma	VFYYEIQNAP	EQACHLAKTA	FDDAIAELDT	LNEDSYKDST	LIMQLLRDNL	TLWTSDQQDD
Sigma	YEIANSPEEA	ISLAKTTFDE	AMADLHTLSE	DSYKDSTLIM	QLLRDNLTLW	TADNAGEEGG
Theta	ILNNPELACT	LAKTAFDEAI	AELDTLNEDS	YKDSTLIMQL	LRDNLTLWTS	DSAGEECD AA
Zeta/Delta	ILNSPEKACS	LAKTAFDEAI	AELDTLSEES	YKDSTLIMQL	LRDNLTLWTS	DTQGDEAEAG

Beta/Alpha	AGEGEN
Epsilon	EQNKEALQDV EDENQ
Eta	EAGEGN
Gamma	DGGEGNN
Sigma	EAPQEPQS
Theta	EGAEN
Zeta/Delta	EGGEN

Figure 9 (Continued)

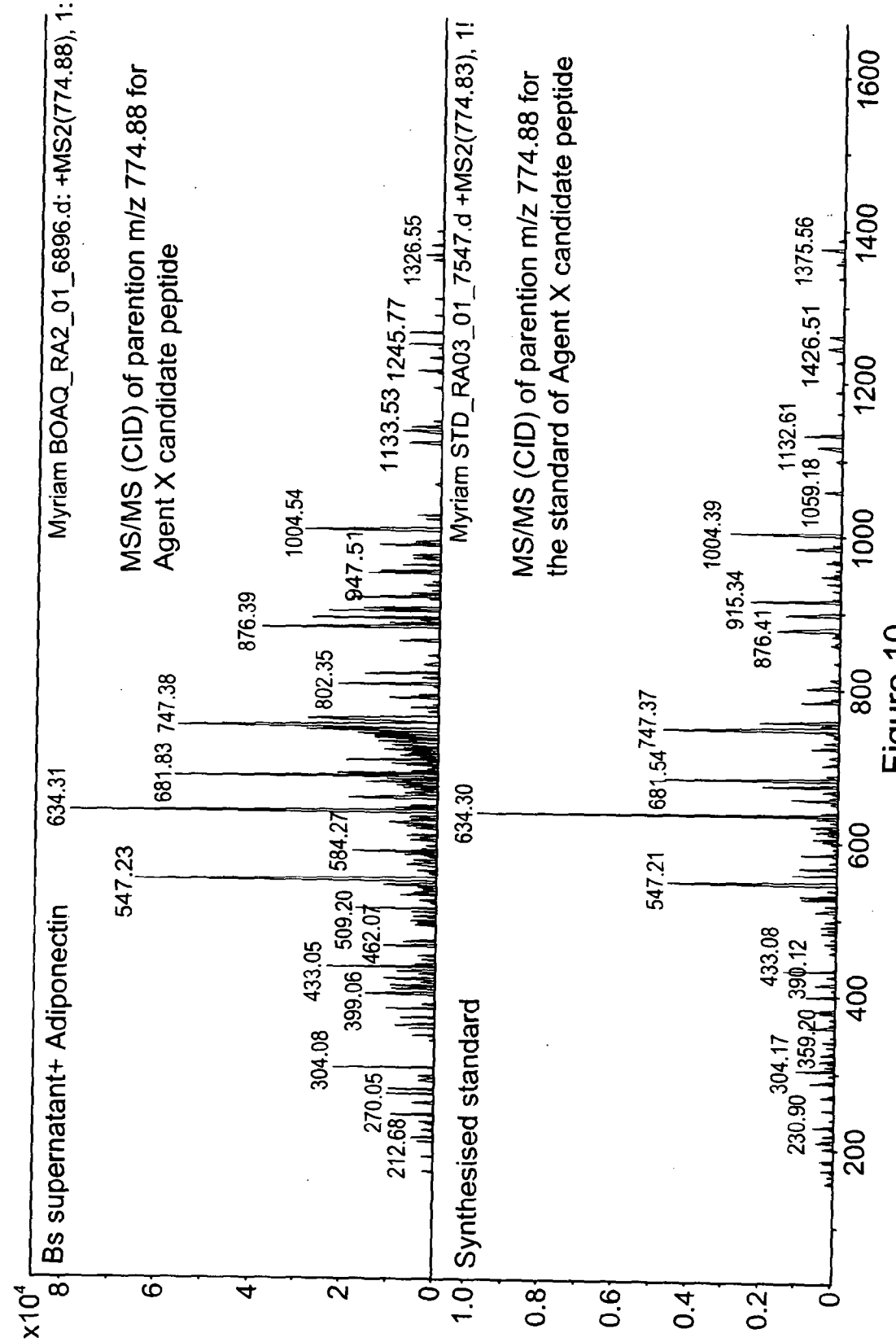

Bs supernatant+ Adiponectin
Myriam BOAQ_RA2_01_6896.d: +MS2(774.88), 1:
MS/MS (CID) of parention m/z 774.88 for Agent X candidate peptide
Synthesised standard
Myriam STD_RA03_01_7547.d +MS2(774.83), 1!
MS/MS (CID) of parention m/z 774.88 for the standard of Agent X candidate peptide
x10^4
634.31
681.83
747.38
547.23
876.39
802.35
1004.54
947.51
584.27
509.20
462.07
433.05
399.06
304.08
270.05
212.68
1133.53
1245.77
1326.55
634.30
681.54
747.37
547.21
915.34
1004.39
876.41
1132.61
1059.18
1426.51
1375.56
433.08
390.12
359.20
304.17
230.90

Figure 10

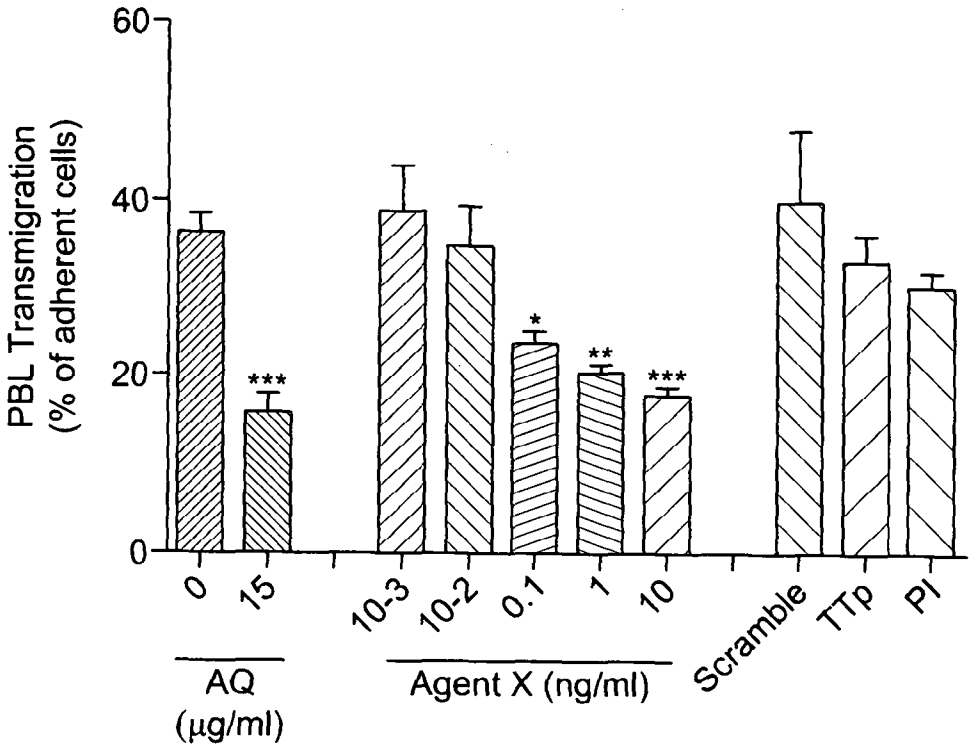


Figure 11a

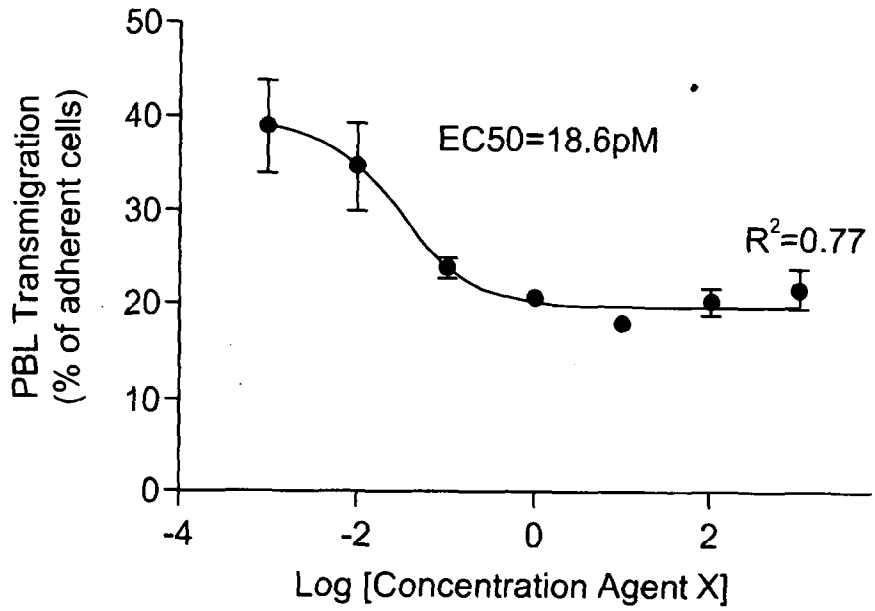


Figure 11b

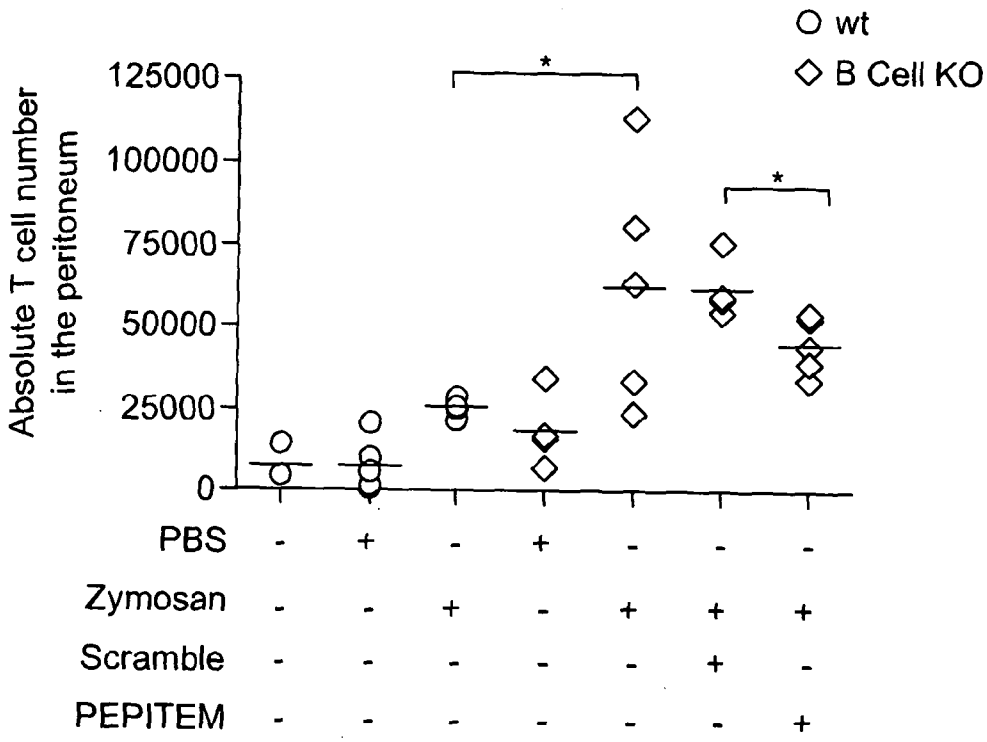

wt
B Cell KO
Absolute T cell number in the peritoneum
125000
100000
75000
50000
25000
0
*
*
PBS - + - + - - -
Zymosan - - + - + + +
Scramble - - - - - + -
PEPITEM - - - - - - +

Figure 12

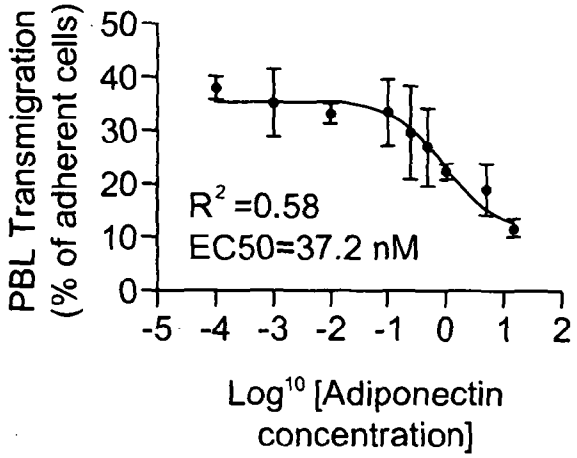


Figure 13a

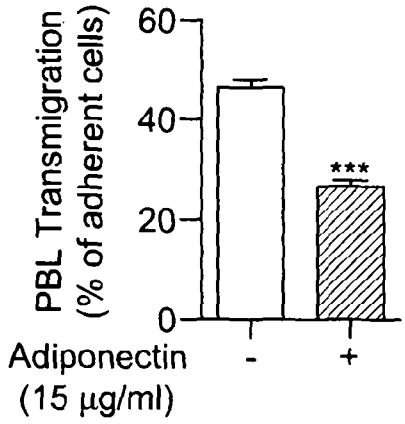


Figure 13b

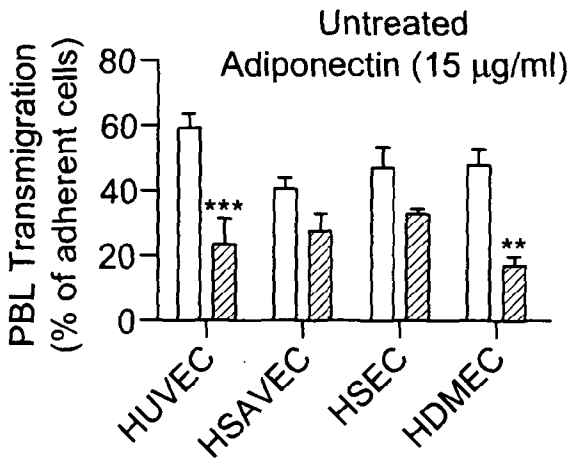


Figure 13c

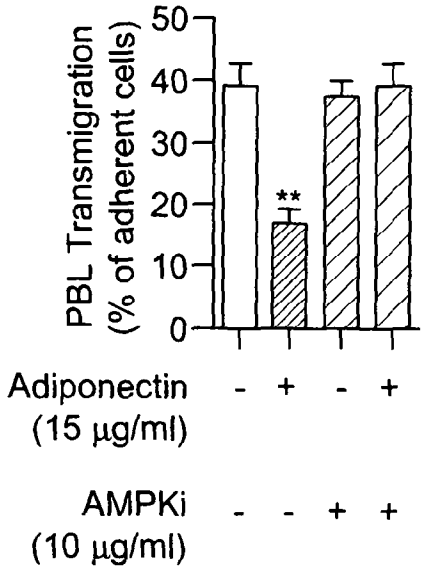


Figure 13d

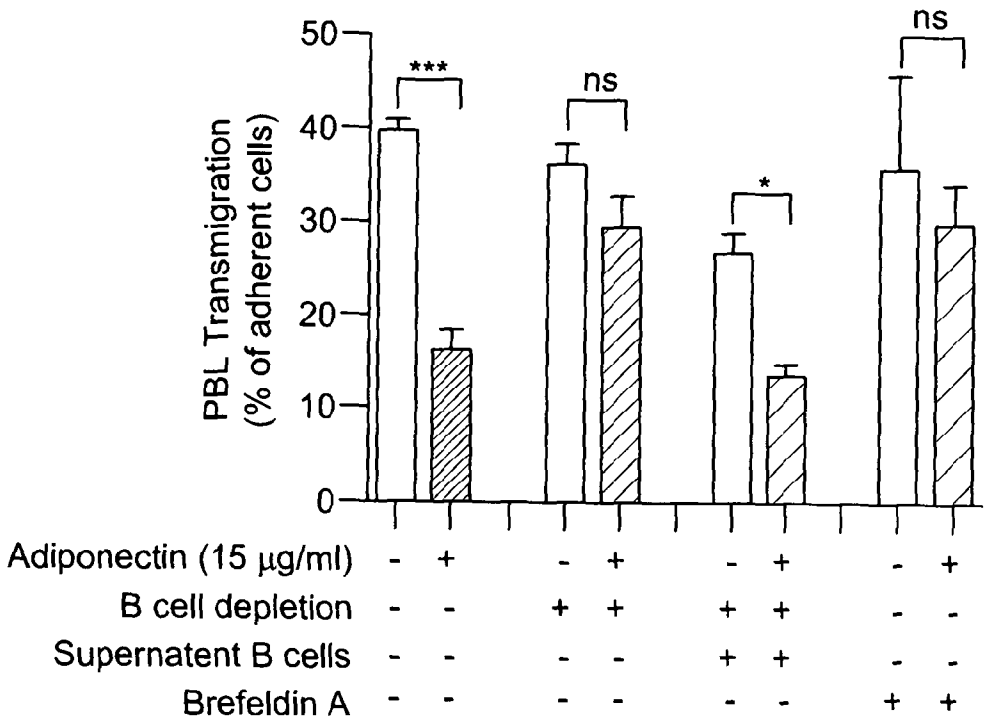
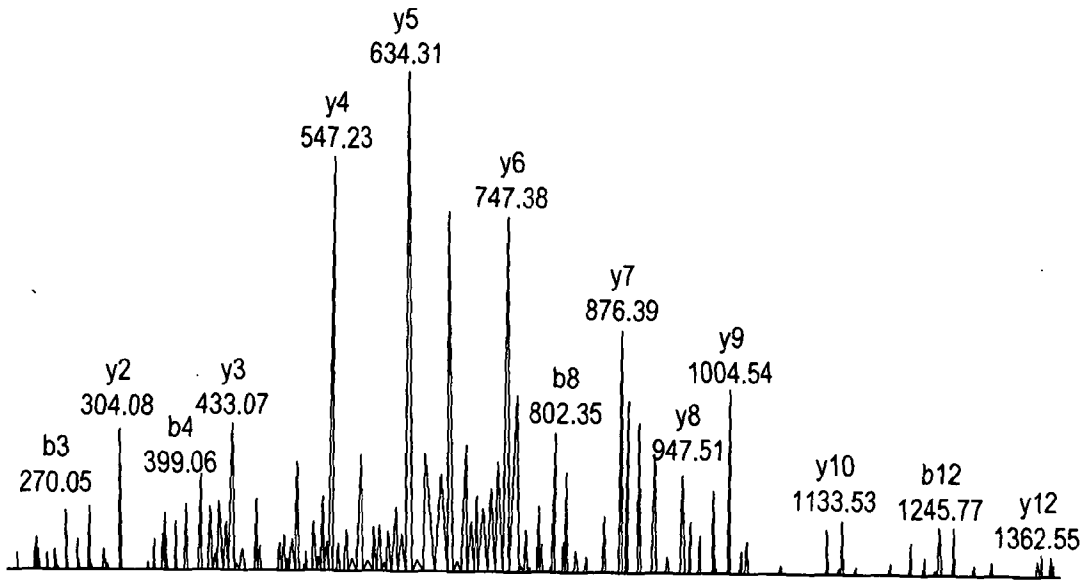


Figure 14



m/z	Elution time (min)	Score	Modification	Type	Association protein	Sequence
774.88	13.2	63.1	NA	CID	14-3-3 zeta/delta	SVTEQGAELSNEER

Figure 15

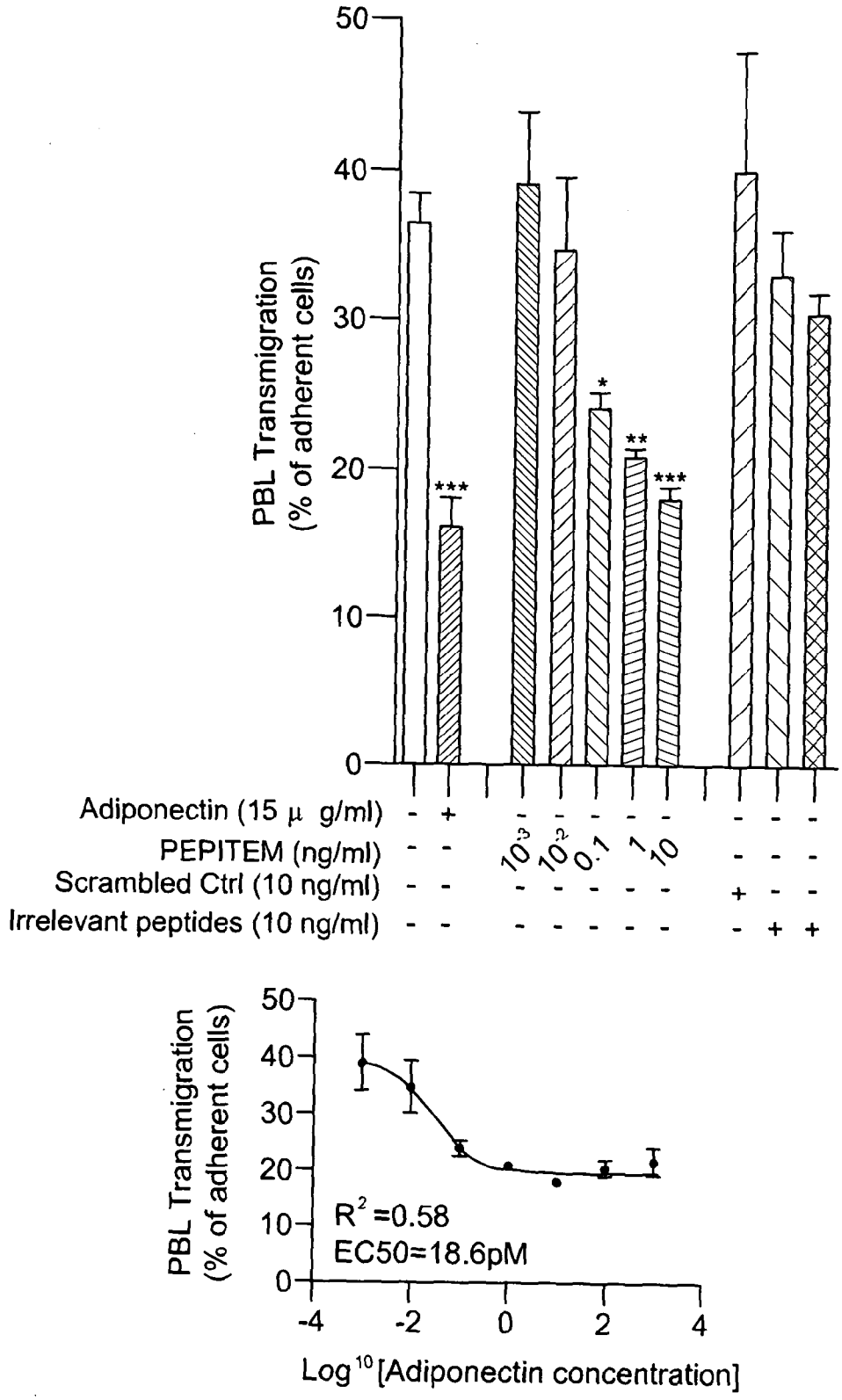

PBL Transmigration
(% of adherent cells)
50
40
30
20
10
0
***
*
**
***
Adiponectin (15 μ g/ml) - + - - - - - - - -
PEPITEM (ng/ml) - - 10⁻³ 10⁻² 0.1 1 10 - - -
Scrambled Ctrl (10 ng/ml) - - - - - - - + - -
Irrelevant peptides (10 ng/ml) - - - - - - - - + +
PBL Transmigration
(% of adherent cells)
50
40
30
20
10
0
R² =0.58
EC50=18.6pM
-4
-2
0
2
4
Log[10][Adiponectin concentration]

Figure 16

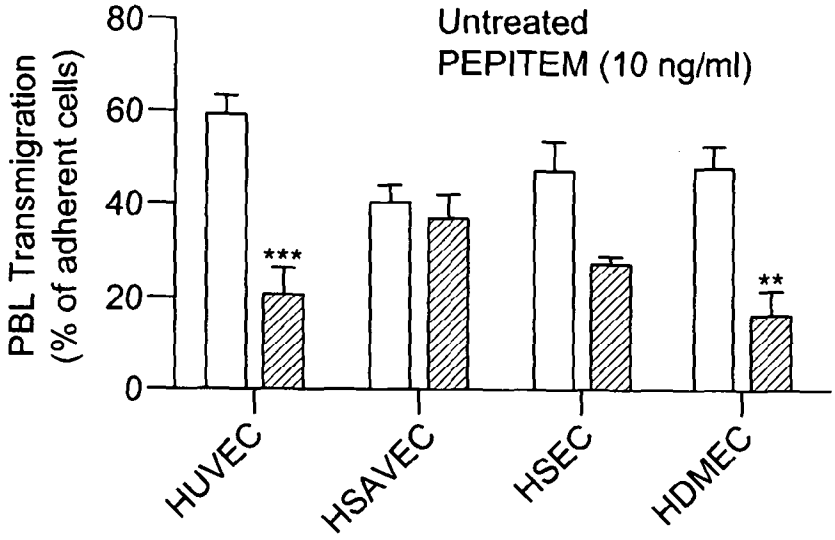


Figure 17a

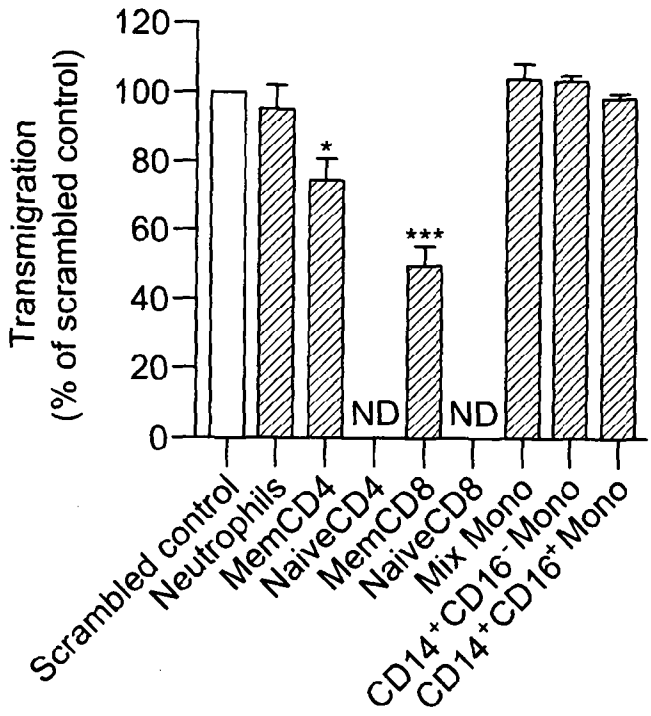


Figure 17b

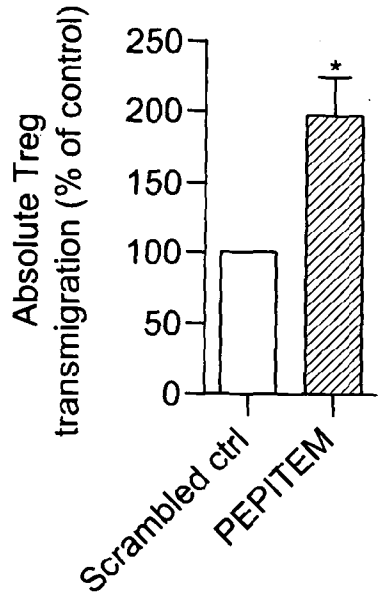


Figure 17c

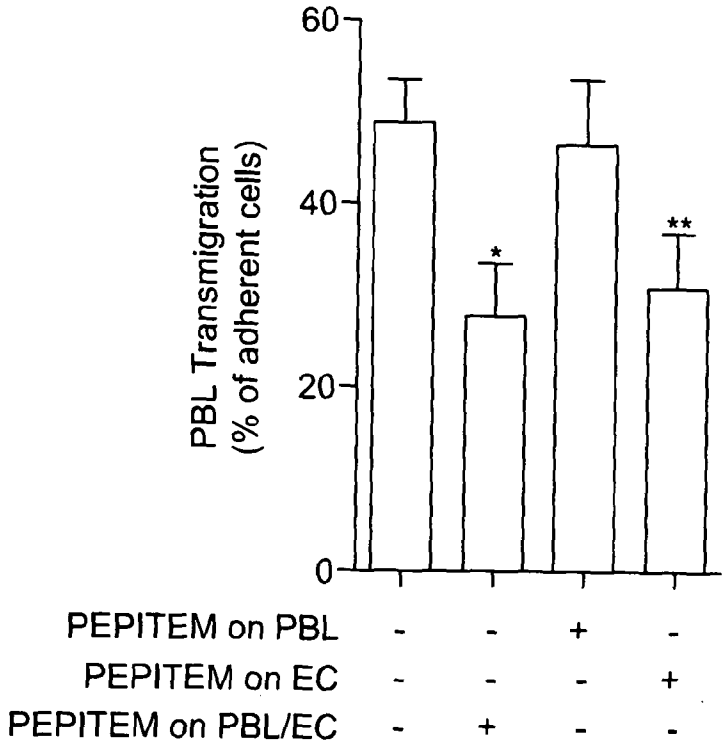


Figure 18

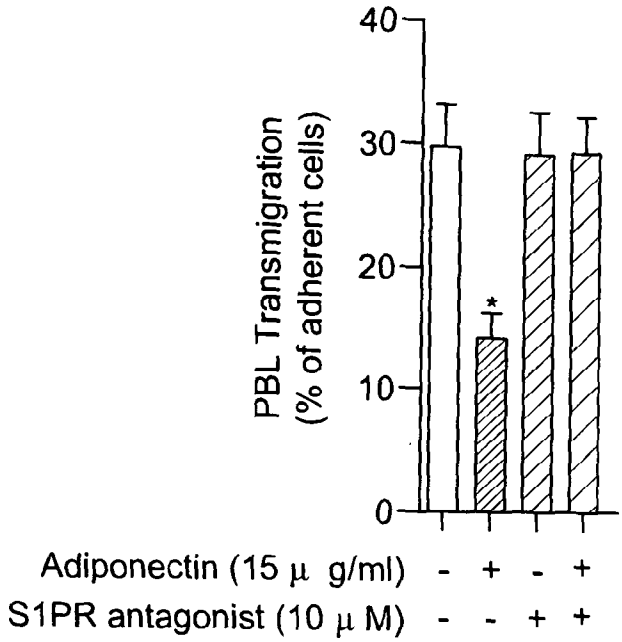


Figure 19a

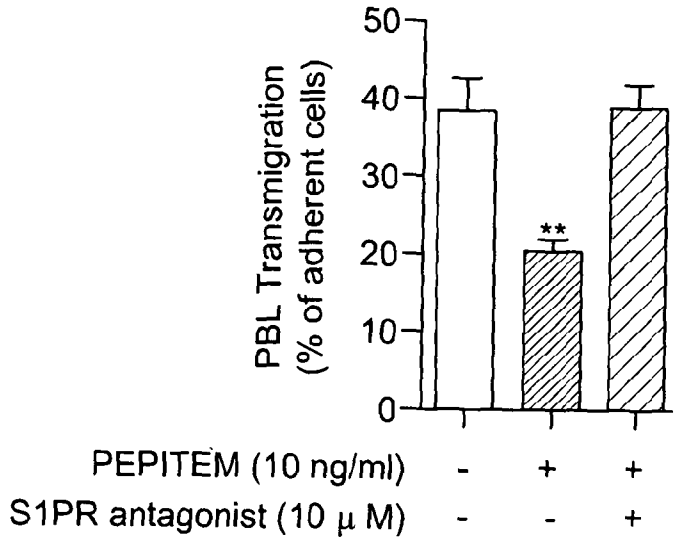
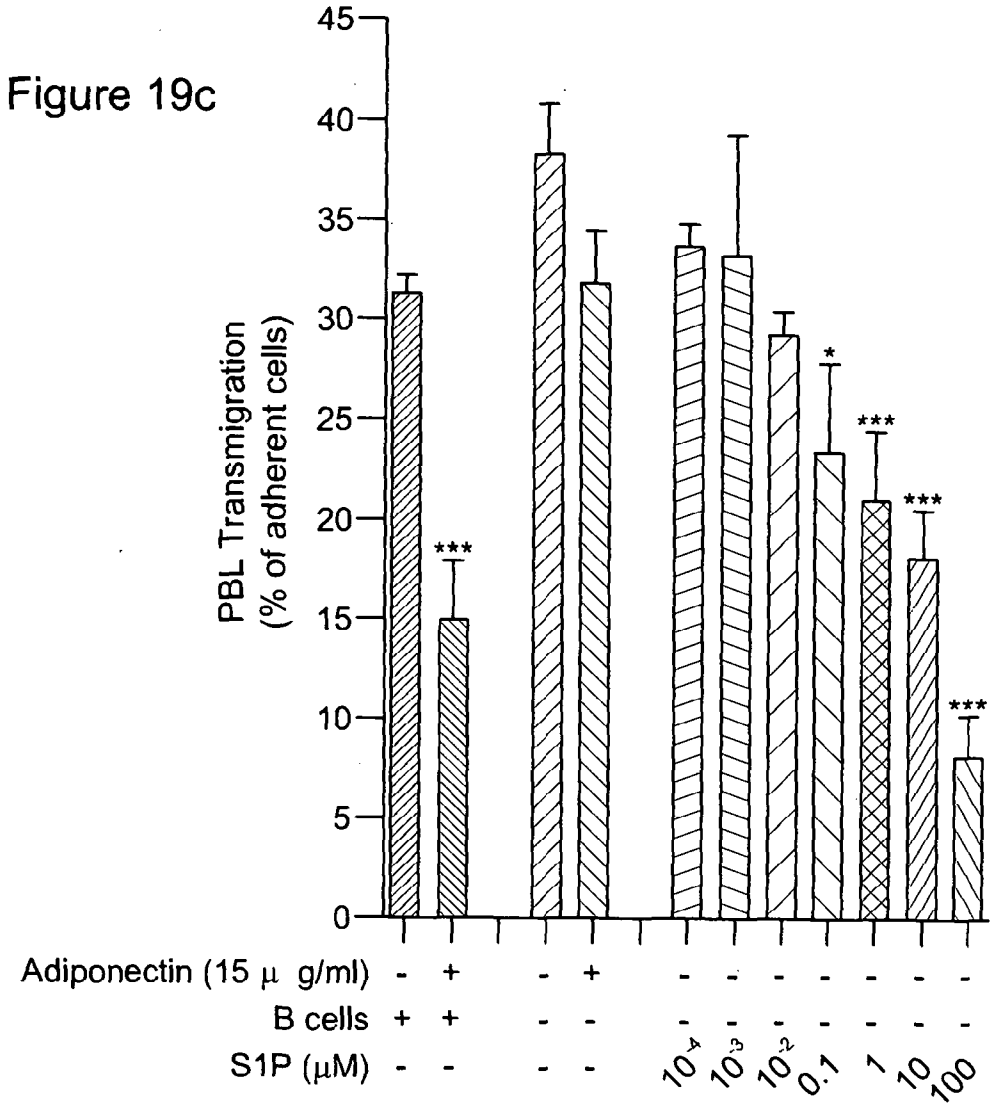


Figure 19b

Figure 19c



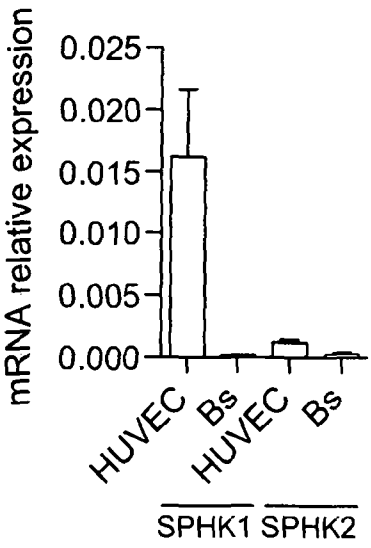


Figure 19d

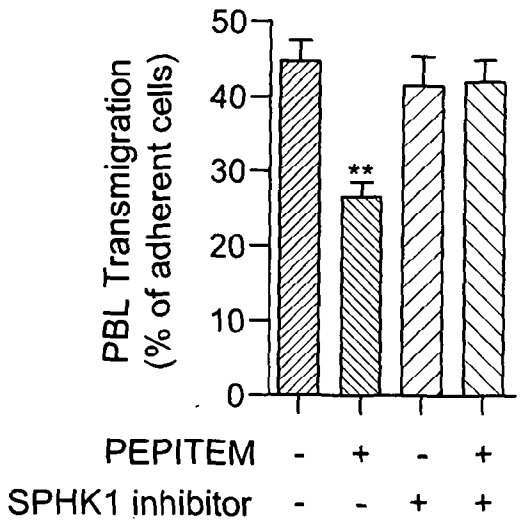


Figure 19e

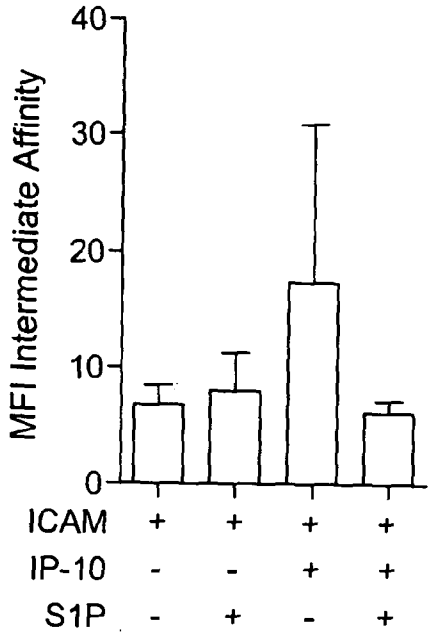


Figure 20a

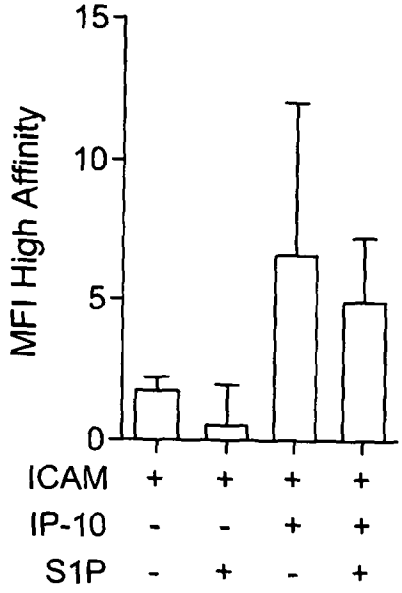


Figure 20b

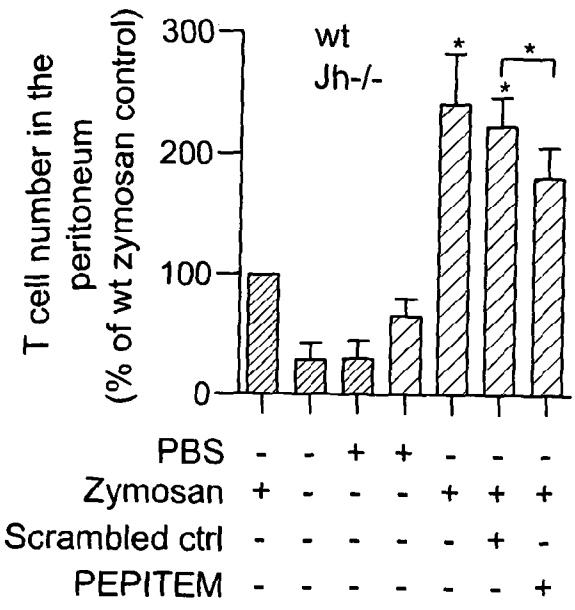


Figure 21a

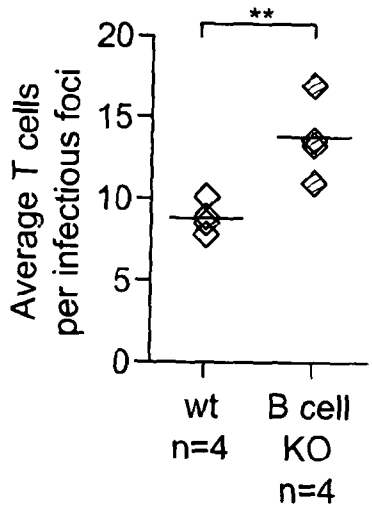


Figure 21b

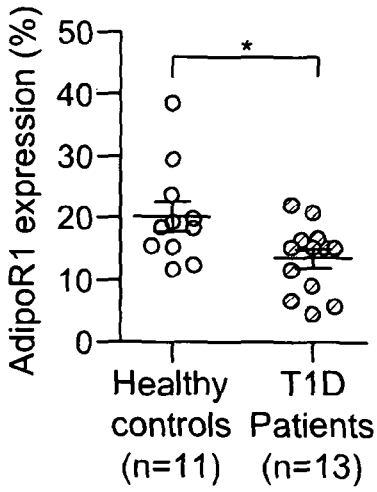


Figure 22a

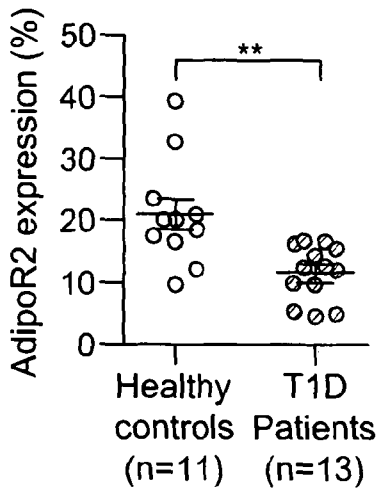


Figure 22b

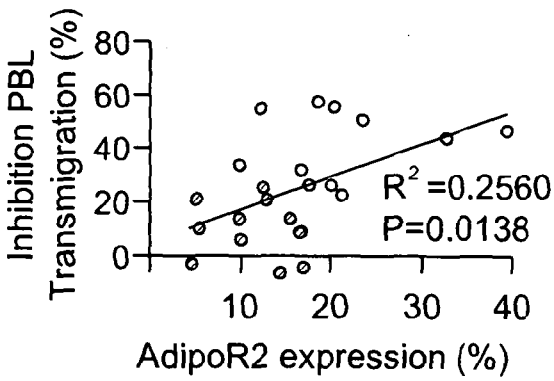


Figure 22c

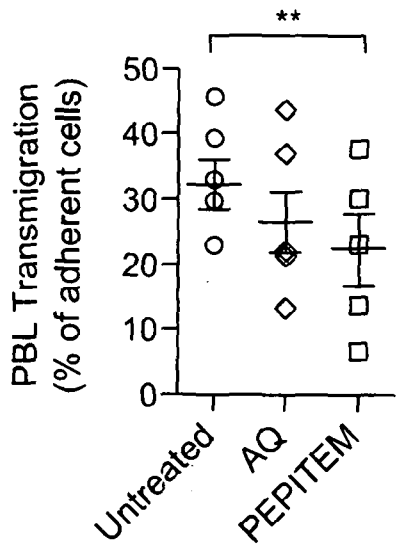


Figure 22d

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/050068

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LANKISCH T O ET AL: "Bile proteomic profiles differentiate cholangiocarcinoma from primary sclerosing cholangitis and choledocholithiasis", HEPATOLOGY, WILEY, USA, vol. 53, no. 3, 1 March 2011 (2011-03-01), pages 875-884, XP002673392, ISSN: 0270-9139, DOI: 10.1002/HEP.24103 [retrieved on 2011-01-03]	1-15
A	US 2011/130415 A1 (SINGH RAJINDER [US] ET AL) 2 June 2011 (2011-06-02)	1-15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2013	02/05/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vandenbogaerde, Ann

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2013/050068

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011130415 A1	02-06-2011	CA 2780759 A1	09-06-2011
		EP 2507227 A1	10-10-2012
		US 2011130415 A1	02-06-2011
		WO 2011068898 A1	09-06-2011