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(54) Title: ANTI-INFLAMMATORY AGENTS

(57) Abstract: Anti-inflammatory agents are described which act as inhibitors of part of the C-terminal domain of the adhesion molecule ICAM-1 and thus restrict transendothelial migration of lymphocytes. The agents may have an amino acid sequence which is the same as or which mimics the relevant portion of this domain. Alternatively, the agents may interfère with the interaction of this domain with one or more of its binding partners.

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ANTI-INFLAMMATORY AGENTS

This invention relates to anti-inflammatory agents and compositions, in particular for the treatment of neuroinflammation.

Interaction of T cells with the vascular endothelium is a critical step during T cell extravasation from blood into tissue in immunosurveillance and inflammation. Cell adhesion molecules (CAMs) expressed on the T cell and endothelial cell surfaces play essential roles in this intercellular interaction. Lymphocyte interaction with the endothelium *in vivo* is initiated by selectin mediated rolling followed by activation of integrins, firm attachment of the lymphocyte and subsequent migration across the endothelium. The intercellular adhesion molecules (ICAM)-1 and ICAM-2, members of the immunoglobulin – superfamily, were both identified by their ability to bind the same ß2-integrin ligand LFA-1, which is expressed on all leukocytes. Binding of T cells via LFA-1 to antigen presenting cells or target cells is necessary for T cell activation and T cell effector functions and for firm attachment and migration of leukocytes across the endothelium.

Both ICAM-1 and ICAM-2 are expressed at low levels on endothelial cells and ICAM-1 expression is strongly inducible by inflammatory cytokines. Strikingly, ICAM-1 or ICAM-2 deficient mice are both viable and show relatively mild defects in their immune responses. ICAM-1 deficient mice suffer from a moderate leukocytosis while activation and migration of leukocytes to places of inflammation are reduced resulting in impaired immune and inflammatory responses.

However, molecules such as ICAM-1 do not appear to be simply points of attachment but are involved in transducing leukocyte adhesion-mediated signalling responses to endothelial cells.

It has previously been demonstrated that the expression of the adhesion molecule ICAM-1 on endothelial cells is pivotal in supporting lymphocyte migration across vascular endothelium in both peripheral tissue and the CNS.

Endothelial cells of the blood-central nervous system (CNS) barriers display characteristics that differentiate them from endothelium of other organs. In particular these cells express well-developed intercellular tight junctions and are responsible for exceptionally low paracellular permeability. The endothelia, which facilitates the passage of migratory cells across the vessel wall through the expression of cell adhesion molecules such as ICAM-1. There is emerging evidence that ICAM-1 may mediate leukocyte migration under inflammatory conditions whereas ICAM-1 may play the major role in mediating basal infiltration of leukocytes in immunosurveillance. It is also interesting to note that ICAM-1 and ICAM-2 appear to control different signaling responses in endothelial cells (Adamson et al., 1999: Thompson et al., 2002)

It is known that following ICAM-1 cross-linking or co-culture with T-lymphocytes, brain endothelial cell ICAM-1 is capable of evoking signal transduction events, suggesting that endothelial cells actively respond to leukocyte adhesion (Etienne et al., 1998; Adamson et al., 1999; Durieu-Trautmann, 1994). However, it has previously been unclear how ICAM-1 elicits these signals. Although the short cytoplasmic domain of ICAM-1 is known to bind a variety of molecules such as α-actinin, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase and ezrin, ICAM-1 has no catalytic activity associated with this region. It lacks intrinsic kinase activity or known protein-protein interaction domains that could recruit downstream signalling components. Previous attempts to reduce transendothelial migration using β Interferon have produced less than promising results.

Aspects of the present invention seek to overcome or reduce the above mentioned problems.

The cytoplasmic domain of ICAM-1 consists of 27 amino acids in rat and mouse and 28 amino acids in human. The amino acid sequence is known from GENBANK Accession number NM-000201 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

The human sequence is as follows:

RQRKIKKYRLQQAQKGTPMKPNTQATPP (SEQ. ID. NO:1)

The mouse sequence is as follows:

RQRKIRIYKLQKAQEEAIKLKGQAPPP (SEQ. ID. NO:2)

The portion of the cytoplasmic domain which we have now found to be important for the transendothelial migration of lymphocytes is underlined in the above sequences. In humans, this corresponds to the sequence from residue 505 to residue 518, i.e.

QRKIKKYRLQQAQ (SEQ. ID. NO:3)

or subsequences contained therein.

In mice, this corresponds to the sequence

QRKIRIYKLQKAQ (SEQ. ID. NO:4)

or subsequences contained therein.

We have now found that by targeting a part of the ICAM-1 C-terminal domain, having the sequence given above, with certain blocking agents having amino acid sequences which are either the same as or which mimic the relevant portion of this domain or which

interfere with its interaction with binding partners, we can inhibit the function of this molecule and thereby restrict transendothelial migration and thus alleviate neuroinflammation.

We have found a novel class of anti-inflammatory agents which can antagonise ICAM-1 mediated intracellular signal transduction and subsequent transendothelial migration. In particular, we have now identified sequences within the ICAM-1 intracellular (C-terminal) domain which can be mimicked with short peptides resulting in the reduction or inhibition of transendothelial migration of T-lymphocytes. The peptides effectively act as an inhibitor which blocks the endothelial support of T-lymphocyte transmigration mediated through endothelial ICAM-1 molecule.

Examples of representative peptide sequences effective against at least one of human, rat and mouse ICAM-1 are those having or containing the sequence:
QRKIKKYRLQQAQ (SEQ. ID NO:3)
QRKIRIYKLQKAQ (SEQ. ID NO:4)
QRKIKK(YP)RLQQAQ (SEQ. ID NO:5)
QRKIRI(YP)KLQKAQ (SEQ. ID NO:6)

The present invention comprises an anti-inflammatory agent which reduces or inhibits transendothelial migration of T -lymphocytes. The anti-inflammatory agent preferably mimics at least a part of the cytoplasmic domain of ICAM-1. The anti-inflammatory agent may comprise a peptide consisting of, or containing the amino acid sequences described above (SEQ. ID NOS: 3 to 6), or sub-sequences contained therein or other amino acid sequence within the ICAM-1 cytoplasmic domain or sequences from binding partners that recognise the ICAM-1 cytoplasmic domain. The peptide is preferably cell permeant and may be linked to a peptide, such as a penetratin peptide, consisting of or containing the amino acid sequence:

RQIKIWFQNRRMKWKK (SEQ. ID NO:7)

As used herein the term "amino acid" and any reference to a specific amino acid is meant to include naturally occurring proteogenic amino acids as well as non-naturally occurring amino acids such as amino acid analogues. One of skill in the art would know that this definition includes, unless otherwise specifically indicated naturally occurring proteogenic (L) amino acids, chemically modified amino acids (including amino acid analogues such as penicillamine, 3-mercapto-D-valine), naturally occurring non-proteogenic amino acids such as norleucine and chemically synthesised compounds that have properties known in the art to be characteristic of an amino acid (e.g D-amino acids). As used herein the term "proteogenic" indicates that the amino acid can be incorporated into a protein in cell through well-described metabolic pathways. As used herein the term "peptide" is used in its broadest sense to refer to compounds containing amino acid equivalents or other non-amino acid groups, while still retaining the desired functional activity of a peptide. Peptide equivalents can differ from the representative peptides specified above by the placement of one or more amino acids with related organic acids (such as PABA) amino acids or the like, or the substitution or modification of side chains or functional groups. In particular, certain amino acids in the peptide may be phosphorylated.

It is to be understood that limited modifications can be made to a peptide without destroying their biological function. Thus modification of the peptides of the present invention that do not completely destroy their activity discovered in accordance with the present invention are within the definition of the compound claims as such. Modifications can include for example additions, deletions or substitutions of amino acid residues with compounds that can mimic amino acid structure or functions, as well as the addition of chemical moieties such as amino or acetyl groups. The modifications can be deliberate or accidental, and can be modifications of composition of the structure. Variants can be determined by simple experiments in the light of the present disclosure as a whole.

The inhibitor may comprise a peptidomimetic which functions in a manner equivalent to the peptide inhibitors specified above. Peptidomimetic agents are compounds with a chemical structure which mimic the structure of peptide sequences.

Advantageously, the invention further comprises a pharmaceutical composition comprising an inhibitor as defined above, in particular for human or vetinary use. The invention further comprises a method of inhibiting or reducing inflammation, for example neuroinflammation by administration of a defined peptide or peptidomimetic to a patient in need thereof. One skilled in the art would know that a pharmaceutical composition comprising a peptide or peptidomimetic of the present invention can be administered to a subject having inflammation, by a variety of routes for example by an oral or intravenous route.

In specific cell permeant peptide sequences directed against the rat ICAM-1 molecule prevented transendothelial migration of T-lymphocytes across rat endothelial cells expressing only rat ICAM-1 (Figure 1). Whereas cell permeant peptide comprising part of the sequence of the human ICAM-1 C-terminal (intracellular domain) prevented the enhanced migration of T-lymphocytes associated with ectopic expression of human ICAM-1 in rat CNS endothelial cells (Figure 2). Similar results were obtained in mouse brain endothelial cells, which were derived from mice in which gene disruption rendered such endothelial cells devoid of both ICAM-1 and ICAM-2. Peptide sequences directed against the C-terminal domain of mouse ICAM-1 (which was re-introduced following transfection with mouse ICAM-1) also showed reduced ability of mouse ICAM-1 positive endothelial cells to support transendothelial migration of T-lymphocytes. In all cases an cell permeant peptide comprising the sequence of rat rod opsin (irrelevant) was unable to prevent transendothelial migration.

Example 1

Methods

Endothelial cells

The immortalised Lewis rat brain microvascular endothelial cell (EC) line GP8/3.9 (22) was maintained in Ham's F-10 medium supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100μg/ml streptomycin. GP8/3.9 cells transfected with RSVpuro human ICAM-1 constructs were maintained in GP8/3.9 medium containing puromycin (20μg/ml).

cDNA constructs

Wild type human ICAM-1 and mutant ICAM-1 molecules were generated using RT-PCR from CHO cells overexpressing human ICAM-1 (a gift from Dr. J. Pearson, Kings College London, UK). RNA was extracted from ICAM-1 expressing CHO cells using an RNeasy® kit (Qiagen, West Sussex UK) according to the manufacturer's instructions. Reverse transcriptase was used to generate cDNA, from oligo$(dT)_{15-18}$ primed RNA, which was used as a template for PCR. PCR reactions were performed using the forward primer 5'-GGAAGCTTCTAGAATGGCTCCCAGCAGCCCCC-3' (SEQ. ID. NO:8) and one of three reverse primers 5'-GGGTCGACTCTAGAGTTATAGAGGTACGTGCTGAGG-3' (SEQ. ID. NO:9), 5'-GGGTCGACTCTAGATCAGTTATAGAGGTACGTGCTGAG-3' (SEQ. ID. NO:10) and 5'-GGGTCGACTCTAGATCAGGGAGGCGTGGCTTGTGTGTTCGGTTTCATGGGGGTCCCTTTTTGGGC CTGTTGTAGTCTGAATTTCTG-3' (SEQ. ID. NO:11) to generate wild type, C-terminal truncation at amino acid 504 and $Tyr_{512}\rightarrow Phe_{512}$ point mutations respectively. *Hind III* and *SalI* sites, which were engineered into the forward and reverse PCR primers respectively were used to clone the PCR fragment into a *Hind III/Xho I* restricted pcDNA3/RSVpuro plasmid. The identity of the PCR generated ICAM-1 inserts was confirmed by DNA sequencing. A GPI anchored human ICAM-1 construct (pCDM8 GPI-ICAM-1) in which both the C-terminal domain and transmembrane domain are deleted by truncating ICAM-1 at codon 480 and fusing this sequence to the GPI-anchor sequence of human LFA3. The GPI-ICAM-1 sequence was excised from pCDM8 using *HindIII* and *Xba1* and directionally cloned into *HindIII/Xba1* digested RSVpuro.

Penetratin-ICAM-1 peptides

Penetratin peptides were N-terminally biotinylated and consisted of 16 residues of the penetratin sequence (RQIKIWFQNRRMKWKK (SEQ. ID. NO:7)). The 13 C-terminal amino acids of human (h) or rat ICAM-1(r) were synthesised distal to the penetratin sequence. Peptides were HPLC purified before use. Sequences used were hICAM-1 (QRKIKKYRLQQAQ (SEQ. ID. NO:3)), YP-hICAM-1 (QRKIKK(YP)RLQQAQ (SEQ. ID. NO:5)), rICAM-1 (QRKIRIYKLQKAQ (SEQ. ID. NO:4)) YP-rICAM-1 (QRKIRI(YP)KLQKAQ (SEQ. ID. NO:6)) and an irrelevant sequence from the soluble part of rat rod opsin (CKPMSNFRFGENH (SEQ. ID. NO:12)). Penetratin peptides were localised in cells using streptavidin-FITC (1:50, Jackson, USA) following fixation in 3.7% paraformaldehyde and permeabilisation with 0.2% triton-X100.

Generation of stable GP8/3.9 rat brain EC lines overexpressing human ICAM-1

Pre-confluent GP8/3.9 EC (approx. $0.5x10^6$ cells) were transfected with 3-6 μg of each of the ICAM-1 constructs or with the pCDNA3/RSVpuro vector (no insert) using Fugene transfection reagent according to the manufacturer's instructions. After 24-48h, puromycin (20 μg/ml) was added to cultures to select for ICAM-1 expressing cells. In subsequent studies transfected cells were maintained in medium containing 20μg/ml puromycin and removed prior to co-culture with T-lymphocytes.

Flow cytometric analysis of ICAM-1 transfectants

Puromycin resistant GP8/3.9 brain EC clones were generated and assessed by flow cytometric analysis using a human specific anti-ICAM-1 antibody (clone BBA4) to demonstrate the presence of human ICAM-1 expression. After detachment with collagenase (1 mg/ml) for 20 min cells were washed and incubated with the BBA4 mAb (10μg/ml) for 1h on ice. After washing, cell pellets were resuspended in 100μl of FITC-conjugated goat-anti-mouse IgG (1/100 dilution) and incubated for a further 30 min prior to standard paraformaldehyde fixation and flow cytometric analysis. Data was quantified and rendered using Cellquest® software. A secondary isotype matched IgG control sample for each cell line was also acquired.

Adhesion of peripheral lymph node lymphocytes to EC and T-lymphocyte transendothelial migration

Adhesion assays and transendothelial migration assays were carried out as previously described using cells harvested from Lewis rat peripheral lymph nodes and MBP-antigen specific T-lymphocyte lines (Greenwood et al., 1995; Pryce et al., 1997; Greenwood and Calder 1993). The results are expressed as the means ± SEM and significant differences between groups determined by Student's t-test.

Experimental findings

Cell permeant peptides comprising the C-terminal domain of ICAM-1 attenuate transendothelial migration of lymphocytes but not adhesion

It was determined whether ICAM-1 mediated support of transendothelial lymphocyte migration could be inhibited with cell permeant peptide mimicking the intracellular domain of ICAM-1. Peptides comprising the membrane proximal part of the rat or human ICAM-1 sequence within the intracellular domain were used to antagonise the binding potential of ICAM-1 binding/signalling partners. Such ICAM-1 C-terminal sequences were fused to the penetratin sequence to facilitate uptake of peptides into cells (Hall et al, 1996; Peck and Isacke 1998). A penetratin peptide fused to the 13 amino acids of the intracellular domain of rat ICAM-1 immediately adjacent to the plasma membrane (rICAM-1: QRKIRIYKLQKAQ (SEQ. ID. NO:4)) or an identical peptide in which the conserved tyrosine residue corresponding to codon 512 of the rat ICAM-1 sequence was phosphorylated (YP-rICAM-1: QRKIRI(YP)KLQKAQ (SEQ. ID. NO:6)) were both effective in attenuating transendothelial migration of T-lymphocytes through untransfected rat brain EC following a pre-incubation with 100μg/ml peptides for 2 h followed by removal and addition of lymphocytes. The rICAM-1 C-terminal peptide reduced transendothelial migration to 45.9% ± 4.3% of control value ($p<0.0001$, $n=29$). Moreover, the phosphopeptide (YP-rICAM-1) was also effective in significantly inhibiting transendothelial migration of T-lymphocytes to 50% ± 4.6% of the control ($p<0.0001$, $n=29$) (Figure 1A). Significantly, both the phosphopeptide and the non-

phosphorylated peptide were equally effective. However, treatment of untransfected brain EC with these peptides did not reduce lymphocyte adhesion, which was contrary to our findings with EC expressing human ICAM-1 lacking the intracellular domain. Thus, treatment with either the rICAM-1 peptide or the YP-rICAM-1 peptide resulted in adhesion values of 96.1% ± 1.8% (n=32) and 100.3% ± 2.2% (n=32) of control values respectively (Figure 1B). When identical experiments were conducted in untransfected rat brain EC using penetratin ICAM-1 peptides comprising an identical region of the human ICAM-1 molecule, both phosphorylated and non-phosphorylated human peptides were also effective in inhibiting transendothelial lymphocyte migration. Incubation of EC with hICAM-1 (QRKIKKYRLQQAQ (SEQ. ID. NO:3)) or YP-hICAM-1 (QRKIKK(YP)RLQQAQ (SEQ. ID. NO:5)) peptides caused a reduction of transendothelial migration to 79.9% ± 3.8% ($p<0.01$, n=27) and 53.9% ± 4.2% ($p<0.001$, n=28) of control values respectively (Figure 1C). In a similar fashion to the penetratin rat ICAM-1 peptides, human ICAM-1 peptides were also without effect on lymphocyte adhesion being 109.0% ± 1.1% (n=32) and 99.5% ± 2.4% (n=32) for hICAM-1 and YP-hICAM-1 respectively (Figure 1D). An irrelevant control peptide sequence corresponding to the soluble part of rat rod opsin (CKPMSNFRFGENH (SEQ. ID. NO:12)) had no significant effect on either lymphocyte adhesion or transendothelial migration. N-terminal biotinylation of penetratin peptides was employed to evaluate the entry of penetratin-peptides into rat brain EC. Following fixation and visualisation of biotin peptides with streptavidin-TRITC it was confirmed that in all cases there was efficient uptake of peptide. Thus, treatment with intracellular peptides comprising a sequence from the intracellular domain of either rat or human ICAM-1 appears to be effective in inhibiting transendothelial lymphocyte migration through rat brain EC without affecting lymphocyte adhesion.

Human ICAM-1 intracellular domain peptides abolish the enhanced transendothelial migration of lymphocytes mediated through ectopic expression of hICAM-1 in rat brain EC without affecting lymphocyte adhesion

When penetratin peptides containing the human ICAM-1 sequence were used to treat rat brain EC expressing WT-hICAM-1, both non-phosphorylated and tyrosyl-phosphopeptides were able to abolish the increase in transendothelial lymphocyte migration associated with the ectopic expression of WT-hICAM-1. Lymphocyte migration was reduced to 56.3% ± 5.7% ($p<0.001$ verses cells expressing WT-hICAM-1, n=22) and 57.5% ± 8.2% of control value ($p<0.001$ verses cells expressing WT-hICAM-1, n=32) following treatment with hICAM-1 C-terminal peptide and YP-hICAM-1 peptide respectively (Figure 2A). It is noteworthy that both peptides were effective in significantly reducing migration to values significantly below that observed with control rat brain EC ($p<0.001$ verses control brain EC), which is consistent with the effects of these peptides on EC expressing only endogenous rat ICAM-1 (Figure 1C). Rat brain EC expressing WT-hICAM-1 treated with penetratin peptides containing the human ICAM-1 sequence were unable to significantly reduce lymphocyte adhesion which again is consistent with those experiments conducted on rat brain EC expressing only endogenous ICAM-1. Thus, adhesion of lymphocytes to brain EC expressing WT-hICAM-1 was 299.0% ± 12.4% and following treatment of EC with hICAM-1 and YP-hICAM-1 peptides was 278.0% ± 11.5% (n=32, NS) and 251.3% ± 14.9% (n=32, NS) respectively (Figure 2B).

In summary, cell permeant ICAM-1 C-terminal peptides mimicking the ICAM-1 intracellular domain were shown to be effective in inhibiting transendothelial migration of lymphocytes. Furthermore, inclusion of a phosphorylated tyrosine residue at a position in the peptide corresponding to codon 512 of ICAM-1 was no more potent an inhibitor of transendothelial migration than the non-phosphorylated peptide, suggesting phosphorylation of the conserved tyrosine residue is not required for ICAM-1 mediated signal transduction enabling transendothelial migration of T-cells. It is interesting to note that although the amino acid sequence of human and rat ICAM-1 are different, they are similar in the juxta-membrane region to which the peptides were targeted. Thus peptides comprising the human ICAM-1 were effective in inhibiting transendothelial migration of lymphocytes across monolayers of rat brain EC expressing only endogenous ICAM-1 and were almost as effective as rat specific sequences. This suggests that common effector

molecules bind both the rat and human ICAM-1 C-terminal sequence. Such studies further implicate the intracellular domain of ICAM-1 in T cell mediated signalling functions and the subsequent EC support of lymphocyte migration. Similar studies using mouse ICAM-1 peptides also lead to attenuation of transendothelial migration of lymphocytes without affecting lymphocyte adhesion to mouse brain EC.

Example 2

Endothelioma Cell Lines

Endothelioma cell lines were established by infection of primary brain endothelial cells with a recombinant retrovirus coding for the Polyoma middle T oncogene (Kiefer et al, 1994) exactly as described before The wild type line bEnd.5 served as a control cell line. The ICAM-1 deficient brain endothelioma cell line bEndI1.1 and its daughter cell line bEnd I1.1-ICAM-1 re-expressing wild type ICAM-1 after retroviral transduction into bEndI1.1 were described in detail before (Reiss et al., 1998). The ICAM-$1^{-/-}$ICAM-$2^{-/-}$ brain endothelioma cell line bEndI1/2.1 was established by retroviral transduction of primary brain endothelial cells derived form ICAM-$1^{-/-}$ICAM-$2^{-/-}$ mice with the Polyoma middle T oncogene. ICAM-$1^{-/-}$ICAM-$2^{-/-}$ mice were obtained by cross-breeding the ICAM-1- and the ICAM-2-deficient mice

T cells

The proteolipid protein (peptide aa 139 – 153) -specific T cell lines SJL.PLP3 to SJL.PLP9 were established from draining lymph nodes of SJL/N mice previously immunized with PLP in CFA (Bomholtgard, Denmark) and have been described in great detail before (Engelhardt et al., 1998). They were used for experiments starting after the third round of antigen-specific stimulation, when proliferation only occured in response to the specific-antigen as measured by the incorporation of ^{3}H-thymidine. These PLP-specific T cell lines are T_{H1} memory/effector T cells and were used in the assays at days 3 to 5 after antigen-specific restimulation.

Generation of ICAM-1 mutants

A pBluescript plasmid clone coding for the full length open reading frame of murine ICAM-1 was used as a template to generate the ICAM-1 mutants by PCR-mutagenesis and PCR fragment amplification, which were subcloned into the retroviral vector pBABEpuro. Oligonucleotides used were as follows: RL01: cct gat gtc gac tca gcg gtt ata aac ata aga ggc (SEQ. ID. NO:13), RL02: cgt gat ggc agc tag ctt tgt ttt t (SEQ. ID. NO:14), RL03: gcc aga gaa aga tac gta tat tca agc tgc aga agg ctc agg (SEQ. ID. NO:15), RL04: cgc aat taa ccc tca cta aag g (SEQ. ID. NO:16), RL06: ggc agg agt cga ctc cag cag gct cag gg (SEQ. ID. NO:17), RL11: ggt aca tac gtg tgc cat gc (SEQ. ID. NO:18), RL13: ccg tat gtc gac tca gcg gtt aaa aac aaa gct agc (SEQ. ID. NO:19). PCR mutagenesis of internal DNA sequences was based on a two-step PCR procedure using the proof reading VENT DNA-polymerase (Biolabs, Schwalbach, Germany) and combinations of mutagenesis primers with two peripheral primers. In the first round the mutagenesis primer and the opposite peripheral primer were used. After purification the generated PCR fragment was used as a megaprimer together with both peripheral primers for the second round of PCR.

The cytoplasmic deletion mutant pICAM-1(M1) was generated by introducing a stop codon into the reading frame of the cDNA for murine ICAM-1 by PCR using the oligonucleotides RL01 and RL04. The resulting PCR fragment was cloned *Eco* RI/*Sal* I into pBluescript. In order to reduce the fragment size generated by PCR a *Spe* I/*Sal* I insert from pBluescript was cloned into pBABEpuro (*Spe* I/*Sal* I) to obtain a shuttle construct (pRL202). Finally, a *Hind* III fragment from pBABEpuro-ICAM-1 was substituted for the *Hind* III fragment of pRL202. The ICAM-1 mutant M2 where the tyrosines 507 and 509 were replaced by phenylalanines was derived by PCR mutagenesis using the oligonucleotides RL02, RL04 and RL06. The PCR fragment was cloned into *Hind* III/*Sal* I cut pBluescript. To complete the ICAM-1 cDNA and to reduce the PCR fragment size a *Eco* RI/*Nco* I insert from pICAM-1(M1) was substituted for the corresponding insert of the construct created resulting in pBluescriptICAM-1(M2). Full length mutated ICAM-1 was shifted into pBABEpuro *Eco* RI/*Sal* I to give the final pICAM-1(M2) cDNA construct. PCR mutagenesis to obtain pICAM-1(M3) was performed using the oligonucleotides RL03, RL04 and RL06. The PCR fragment was

cloned into pBluescript *Hind* III/*Sal* I. To complete the ICAM-1 cDNA and to minimize the PCR fragment size a *Eco* RI/*Nco* I insert from pICAM-1 (M1) was substituted for the corresponding insert of pBluescript resulting in pICAM-1(M3). Full length mutated ICAM-1 was shifted I into pBABEpuro *Eco* RI/*Sal* to give the final pICAM-1(M3) construct. In order to obtain pICAM-1(M4) the *Eco* RI/*Sna* BI insert from pBluescript ICAM-1(M2) was ligated with the *Sna* BI/*Sal* I insert from pBluescript ICAM-1 (M3) and cloned into pBluescript (*Eco* RI/ *Sal* I). Full length mutated ICAM-1 was shifted into pBABEpuro *Eco* RI/*Sal* I to give the final pICAM-1(M4) retroviral construct. To obtain pICAM-1(M5), a PCR fragment was generated with use of the oligonucleotides RL11 and RL13 and as template pBluescript-ICAM-1(M2). After restriction digest of this PCR fragment with *Nco* I/*Sal* I it was substituted for the corresponding insert of pBluescript ICAM-1(M3). Full length mutated ICAM-1 was shifted into pBABEpuro *Eco* RI/*Sal* I to give the final pICAM-1(M5) retroviral construct. All fragments generated by PCR were sequenced to verify the DNA sequence.

Retroviral transduction of endothelioma cell lines

The resulting plasmids were transfected into the retrovirus packaging cell line GP+ E86 (Markowitz et al, 1988) by $CaPO_4$ precipitation. Stably transfected GP+E-86 clones were selected in the presence of puromycin (2 μg/ml) or in case of the ICAM-2 construct in the presence of hygromycin (50 μg/ml). bEndI1.1 or bEndI1/2.1 were grown up to 2/3 confluency in 100 mm petri-dishes prior to infection. The cells were incubated twice for 16 hours with supernatants from the different GP+E-86 transfectants supplemented with 8 μg/ml Polybrene (Hexadimethine Bromide; Sigma). Selection was started 24 hours after the second infection. Clones were grown to passage 5 and expression of ICAM-1 or ICAM-1 mutants or ICAM-2 in retransfected endothelioma cells was analyzed by FACS analysis and immunofluorescence.

Penetratin-ICAM-1 peptides

Penetratin peptides were synthesised at the Kennedy Institute of Rheumatology, UK. Peptides were N-terminally biotinylated and consisted of 16 residues of the penetratin sequence (RQIKIWFQNRRMKWKK (SEQ. ID. NO:7)). 13 C-terminal amino acids of

murine ICAM-1 were synthesised distal to the penetratin sequence. Peptides were HPLC purified before use. Sequences used were biotinylated protein (BP)-ICAM-1wt (QRKIRIYKLQKAQ (SEQ. ID. NO:4)), BP-ICAM-1PY (QRKIRI(YP)KLQKAQ (SEQ. ID. NO:6)), and an irrelevant sequence from the soluble part of rat rodopsin BP-irrelevent-(CKPMSNFRFGENH (SEQ. ID. NO:12)). Penetratin peptides were localised in cells using streptavidin-Cy3 (1:100, Jackson, USA) following either fixation of the cells in 1% formaldehyde in PBS or permeabilisation with cold (-20°C) methanol and rehydration with PBS.

Flow cytometry

FACS analysis was performed exactly as described before. Endothelioma cells were harvested by incubating with 5 mM EDTA in HBSS at 37°C, washed and cell populations ($5x10^5$ cells/sample) were incubated with primary rat mAb for 30 minutes at 4°C, washed twice with FACS buffer (PBS supplemented with 1% bovine serum albumin (BSA) and 0.1% NaN_3 followed by incubation with a Phycoerythrin-conjugated goat anti-rat IgG $F(ab')_2$ mAb (Biosource, Camarillo, CA, USA) for 30 minutes at 4°C, washed twice with FACS buffer and fixed in 1% formaldehyde in PBS. Cells were either analyzed immediately or stored at 4°C for later analysis. Flow cytometric analysis was performed on a FACScan using CellQuest software (Becton Dickinson, Heidelberg, Germany). For analysis, light scatter gates were drawn around live endothelial cell populations.

Adhesion and Transmigration Assays

Adhesion and transmigration assays were performed exactly as described in detail before Briefly, adhesion assays have been carried out using 16-well glass chamber-slides (Nunc, Wiesbaden, Germany) and analyzed by video associated light microscopy (NIH Image software, NIH, USA). Bound cells per pre-defined field were determined by counting 5 fields per well. Assays were performed in triplicates for each value. Transmigration assays were performed using 6.5mm Transwells (Costar, Bodenheim, Germany) with 5 μm pore size. Migrated T lymphocytes were collected for cell counting (CASY, Schärfe-System, Reutlingen, Germany). Confluency of the endothelial monolayer was confirmed

after each assay on formalin fixed and 2.6% Giemsa stained inserts. Assays were performed as triplicates for each value.

Statistical analysis

Within each assay, parameters were tested in triplicates. The numbers of repeats of the individual assays is indicated in the results section. The mean ± standard deviation within one assay was evaluated according to the Student's t-test using the Macintosh-software Instat, where $p < 0.05$ = significant; $p < 0.005$ = very significant and $p < 0.0005$ = extremely significant.

Experimental findings

bEndI1/2.1 do not support transendothelial migration (TEM) of PLP-specific T cells

transendothelial migration (TEM) of PLP-specific T cells across unstimulated ICAM-$1^{-/-}$ ICAM-$2^{-/-}$.

bEndI1/2.1 and wild-type bEnd5 were always investigated simultaneoulsy within the same experiments. During a 4 hour time period about 40 % of T cells spontaneously migrated across a monolayer of bEnd5, whereas migration of T cells across bEndI1/2.1 over the same time period was negligible (Fig. 3A). Although absolute numbers of migrated PLP-specific T cells varied between assays depending on the activation stage of the T cells (day after restimulation) and the specific T cell line used, relative numbers of T cells transmigrating across bEnd5 versus bEndI1/2.1 within each assay were always highly reproducible. Figure 3A shows one representative experiment of 7 replicate experiments reproducing the same relative results. Here, 43.1±4.8% of SJL.PLP3 spontaneously migrated across a monolayer of bEnd5. At the same time only 1.6±0.9% of SJL.PLP3 migrated across bEndI1/2.1 within 4 hours. Migration of SJL.PLP3 across TNF-α stimulated bEnd5 increased to 70±4.4% though migration across bEnd I1/2.1 only increased to 5.1±0.2%, respectively. Thus, migration of T cells across bEndI1/2.1 compared to unstimulated or stimulated wild-type endothelium, was dramatically reduced by 97.3±2.8% and 92.8±0.3%, respectively. Taken together, in the absence of endothelial

ICAM-1 and ICAM-2 TEM of PLP-specific T lymphocytes *in vitro* was almost completely abolished.

ICAM-1 and ICAM-2 reconstitute TEM of PLP-specific T cells across bEndI1/2.1

We next investigated whether re-expression of ICAM-1 and /or ICAM-2 on the cell surface of bEndI1/2.1 would re-establish TEM of T cells. For this purpose ICAM-1, ICAM-2 or both were retrovirally transduced into bEndI1/2.1 and derivative cell lines of bEndI1/2.1 expressing ICAM-1 (bEndI1/2.1-ICAM-1), ICAM-2 (bEndI1/2.1-ICAM-2) or both ICAM-1+ICAM-2 (bEndI1/2.1-ICAM-1/2) were established. Surface expression of both, ICAM-1 and ICAM-2, was verified by FACS-analysis and immunofluorescence staining using 3 different anti-ICAM-1 monoclonal antibodies and one anti-ICAM-2 monoclonal antibody. Additionally, the presence of ICAM-1 and ICAM-2 protein was confirmed by Western blot analysis. Ectopic expression of ICAM-1 and/or ICAM-2 neither affected expression of other endothelial cell surface molecules such as endoglin, the MECA-32 antigen, VE-cadherin nor the inducibility of VCAM-1 as investigated by FACS analysis .

When directly comparing the TEM of PLP-specific T cells across unstimulated or TNF-α stimulated bEndI1/2.1 (1.7±0.2% and 18.5±3.1%) respectively with their migration across bEndI1/2.1-ICAM-1, we found that surface expression of ICAM-1 alone was sufficient to re-establish TEM of T cells to 57.9±2.2% and 56.8±4.4% respectively (Fig. 3B). In contrast, cell surface expression of ICAM-2 in bEndI1/2.1-ICAM-2 restored spontaneous migration of PLP-specific T cells across unstimulated and stimulated bEndI1/2-ICAM-2 to a lower degree namely to 15.9±1.7% and 32.0±7.9%, respectively. TEM of SJL.PLP3 across EndI1/2.1-ICAM-1/2 expressing both, ICAM-1 and ICAM-2, established TEM rates of SJL.PLP3 to 41.0±2.8% and 49.9±3.5%. Thus, surface expression of ICAM-1 but not of ICAM-2 was sufficient to achieve migration rates of PLP-specific T cells comparable to those obtained with wild-type endothelium (Fig. 3A and Reiss et al., 1998; 1999). Furthermore, ICAM-1 and to a lower degree ICAM-2 can reconstitute TEM of T cells in the absence of the respective other ICAM-molecule whereby ICAM-1 in the absence of ICAM-2 but not ICAM-2 in the absence of ICAM-1 reconstituted TEM to wild type migration levels.

Cell permeable peptides derived from the C-terminal part of ICAM-1 attenuate TEM of PLP-specific T cells *in vitro*

In order to obtain more direct proof for the involvement of the cytoplasmic tail of endothelial ICAM-1 in the TEM of T cells we finally asked whether cell-permeable penetratin peptides harbouring 13 amino acids of the cytoplasmic domain of murine ICAM-1 or an identical peptide, in which the tyrosine residue corresponding to position 518 of the murine ICAM-1 sequence was phosphorylated, would interfere with TEM of PLP-specific T cells. Thus, effects of membrane permeable peptides of cytoplasmic ICAM-1 on TEM of T cells could only be addressed in bEndI1-ICAM-1. Uptake of the control peptide resulted in a slight, however, not significant reduction of TEM of T cells across bEndI1-ICAM-1 when compared to untreated endothelial cells. In contrast, uptake of either peptide harbouring the cytoplasmic tail of ICAM-1 resulted in a significant reduction of the TEM of PLP-specific T cells across bEndI-ICAM-1 when compared to migration across bEndI1-ICAM-1 pretreated with control peptide, irrespective of the presence of a phosphotyrosine (Fig. 4). Taken together, cell permeable peptides derived from the cytoplasmic part of ICAM-1 inhibited TEM of T cells in a dominant negative fashion indicating the involvement of the cytoplasmic part of endothelial ICAM-1 in TEM of T cells directly at the site of T cell/endothelial interaction.

Identification of ICAM-1 C-terminal domain binding partners

By performing a Yeast-two-Hybrid Screen with the cytoplasmic tail of murine ICAM-1 as bait and a pray library from murine brain endothelioma cell lines we obtained 59 clones, 10 of which are already characterized. Four of these clones code for the calmodulin-related S100A10/p11, which has been shown to form heterotetramers with Annexin II. Annexin II is a member of a multigene family of cytosolic proteins, which bind to cell membranes in a Ca2+ dependent manner. The annexin II/p11 complex is thought to have an important cellular function in linking cellular membranes with the cytoskeleton. GST-pulldown experiments confirm a weak interaction of S100A10/p11 with the cytoplasmic tail of ICAM-1. Accordingly, we believe that S100A10/p11 is a missing link mediating the contact between ICAM-1 and the cytoskeleton which is

required for transendothelial migration (TEM) of T cells. Furthermore, we have demonstrated that cross-linking of endothelial ICAM-1 specifically induced a transient phosphorylation of VE-cadherin. Phosphorylation of VE-cadherin correlates to an increased vascular permeability. In addition, VE-cadherin is known to be involved in TEM of leukocytes *in vitro* and *in vivo*. Thus inhibiting the cytoplasmic link of endothelial ICAM-1 to the cytoskeleton is a successful strategy to inhibit both TEM of leukocytes and increase vascular permeability during inflammation.

In summary, in order to delineate the functional involvement of endothelial ICAM-1 versus ICAM-2 in transendothelial migration (TEM) of T lymphocytes we have established endothelial cell lines deficient for both ICAM-1 and ICAM-2 from ICAM-$1^{-/-}$ ICAM-$2^{-/-}$ mice. Here we show that endothelial cells lacking ICAM-1 and ICAM-2 failed to support TEM of T cells. Re-expression of ICAM-1 restored TEM of T cells to wild-type levels whereas re-expression of ICAM-2 only partially rescued TEM of T cells. Re-expression of various ICAM-1 mutants in an ICAM-$1^{-/-}$ICAM-$2^{-/-}$ endothelial cell line (bEndI1/2.1) or in an ICAM-$1^{-/-}$ endothelioma cell line (bEndI1.1) demonstrated that the extracellular domain of ICAM-1 is sufficient to support T cell adhesion, while TEM was strictly dependent on the presence of the cytoplasmic tail of ICAM-1. Tyrosine-phosphorylation of the transmembrane or cytoplasmic tyrosines of endothelial ICAM-1 was not required for TEM of T cells. Cell permeable peptides derived from the cytoplasmic tail of ICAM-1 interfered with TEM in a dominant negative fashion indicating that endothelial ICAM-1 delivers signals via its cytoplasmic tail into endothelial cells, which are essential for TEM of T cells. In addition, peptides having an amino acid sequence which is the same as or which mimics the amino acid sequence of a binding partner of the ICAM-1 C terminal domain, also act in a dominant negative fashion to interfere with TEM of T cells. Examples of this are peptides having substantial sequence similarity with the binding partner S100A10/p11.

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Legends to Figures

Figure 1.

Lymphocyte adhesion to and migration through rat brain EC monolayers in the presence of human and rat penetratin-ICAM-1 peptides.

(A and C) Migration of antigen specific (MBP) T cells through or (B and D) adhesion of [^{3}H]-labelled activated rat peripheral lymph node lymphocytes to control rat brain EC monolayers or EC pretreated with rat (A and B) or human (C and D) penetratin peptides. Penetratin peptide containing C-terminal 16 amino acids of rat ICAM-1 (rICAM-1), penetratin peptide containing C-terminal 16 amino acids of rat ICAM-1 in which the tyrosine residue is phosphorylated (YP-rICAM-1), penetratin peptide containing C-terminal 16 amino acids of human ICAM-1 (hICAM-1), penetratin peptide containing C-terminal 16 amino acids of human ICAM-1 in which the tyrosine residue is phosphorylated (YP-hICAM-1) and penetratin peptide containing 16 amino acids or rat opsin (irrelevant). Observations are a minimum of three independent experiments (n=6/experiment). Data is expressed as Mean ± SEM percent of control of a minimum of three independent experiments (n= >6 per experiment). Significant differences compared to control were determined by Student's t-test *$p<0.01$, **$p<0.001$, ***$p<0.0001$

Figure 2.

Lymphocyte adhesion to and migration through rat brain EC monolayers expressing human ICAM-1: Effect of penetratin-human ICAM-1 peptides.

(A) Migration of antigen specific (MBP) T cells through or (B) adhesion of [^{3}H]-labelled activated rat peripheral lymph node lymphocytes to control EC monolayers, EC transfected with RSVpuro empty vector (rsvpuro) or EC expressing human wild type ICAM-1 (WT-hICAM-1). EC expressing WT-hICAM-1 were also pretreated with either penetratin peptide containing C-terminal 16 amino acids of human ICAM-1 (hICAM-1) or penetratin peptide containing C-terminal 16 amino acids of ICAM-1 in which the tyrosine residue is phosphorylated (YP-hICAM-1). Observations are a minimum of three independent experiments (n=6/experiment). Data is expressed as Mean ± SEM percent of control of a minimum of three independent experiments (n= >6 per experiment).

Significant differences were determined by Student's t-test. **p<0.001 compared to wild type human ICAM-1. † † p<0.001 compared to RSVpuro control.

Figure 3:

Endothelial ICAM-1 and ICAM-2 are required for TEM of T cells.

A: One representative experiment comparing TEM of the T cell line SJLB.PLP3 across unstimulated and stimulated bEnd5 and ICAM-$1^{-/-}$ICAM-$2^{-/-}$ bEndI1/2.1 is shown. Bars represent mean +/- SD (*n* = 3). TEM of T cells across bEndI1/2.1 is reduced to an extremely significant level ($p < 0.0005$) when compared to bEnd5. This assay was reproduced 7 times.

B: One representative experiment comparing transmigration of SJLB.PLP3 across bEndI1/2.1 deficient for ICAM-1 and ICAM-2, bEndI1/2.1-ICAM-1 (bEndI1/2.1 transduced with ICAM-1), bEndI1/2.1-ICAM-2 (bEndI1/2.1 transduced with ICAM-2), and bEndI1/2.1-ICAM-1/ICAM-2 (bEndI1/2.1 transduced with ICAM-1+ ICAM-2) is shown. Re-expression of ICAM-1, ICAM-2 or both ICAM-1 and ICAM-2 in bEndI1/2.1 reconstitutes TEM of PLP-specific T cells to an extremely significant level when compared to migration across bEndI1/2.1 ($p < 0.0005$). This assay was reproduced 4 times. Bars represent mean +/- SD (*n* = 3). Significant differences compared to control were determined using Student's t-test.

Figure 4:

TEM of T-cells across bEndI1.1-ICAM-1 in presence of murine penetratin-ICAM-1 peptides. Both penetratin-ICAM-1 peptides containing 13 amino acids of the cytoplasmic tail of murine ICAM-1 without (BP-ICAM-1-wt) or with the tyrosine residue phosphorylated (BP-ICAM-1-PY) significantly reduced TEM of T cells across bEnd I1-ICAM-1 when compared to an irrelevant control penetratin peptide. No significant difference could be observed comparing BP-ICAM-1-wt versus BP-ICAM-1-PY. Bars represent mean +/- SD (n = 3). Significant differences compared to control were determined using Student's t-test.

CLAIMS

1. An anti-inflammatory agent active as an inhibitor of part of the C-terminal domain of the adhesion molecule ICAM-1 which mediates transendothelial migration of lymphocytes.

2. An anti-inflammatory agent according to claim 1, wherein the agent is active as an inhibitor of that part of the C-terminal domain having the following sequence or subsequences therein:
QRKIKKYRLQQAQ (SEQ. ID NO:3)
QRKIRIYKLQKAQ (SEQ. ID NO:4)

3. An anti-inflammatory agent according to claim 1 or 2, selected from peptides having the following sequences or subsequences therein:
1. QRKIKKYRLQQAQ (SEQ. ID NO:3)
2. QRKIRIYKLQKAQ (SEQ. ID NO:4)
3. QRKIKK(YP)RLQQAQ (SEQ. ID NO:5)
4. QRKIRI(YP)KLQKAQ (SEQ. ID NO:6)

4. An anti-inflammatory agent according to any preceding claim linked to a peptide sequence which renders the molecule cell permeant.

5. An anti-inflammatory agent according to claim 4, wherein the cell permeant linker consists of or contains the amino acid sequence of
RQIKIWFQNRRMKWKK (SEQ. ID NO:7)

6. An anti-inflammatory agent comprising a mimetic of a peptide specified in claim 3.

7. The use of an agent according to any preceding claim in the treatment of inflammation in humans or animals.

8. The use of an agent according to claim 7, wherein the agent is administered orally or intravenously.

9. The use of an agent according to claim 7 or 8, wherein the agent is administered in a daily dose such that the steady state serum concentration is in excess of 10μg/ml.

10. A method of treatment of inflammation comprising the administration of an agent according to any one of claims 1 to 6 to a human or animal in need thereof.

11. A method of treatment of inflammation according to claim 10, wherein the agent is administered orally or intravenously.

12. A method of treatment of inflammation according to claim 10 or 11, wherein the agent is administered in a daily dose of such that the steady state serum concentration is in excess of 10μg/ml.

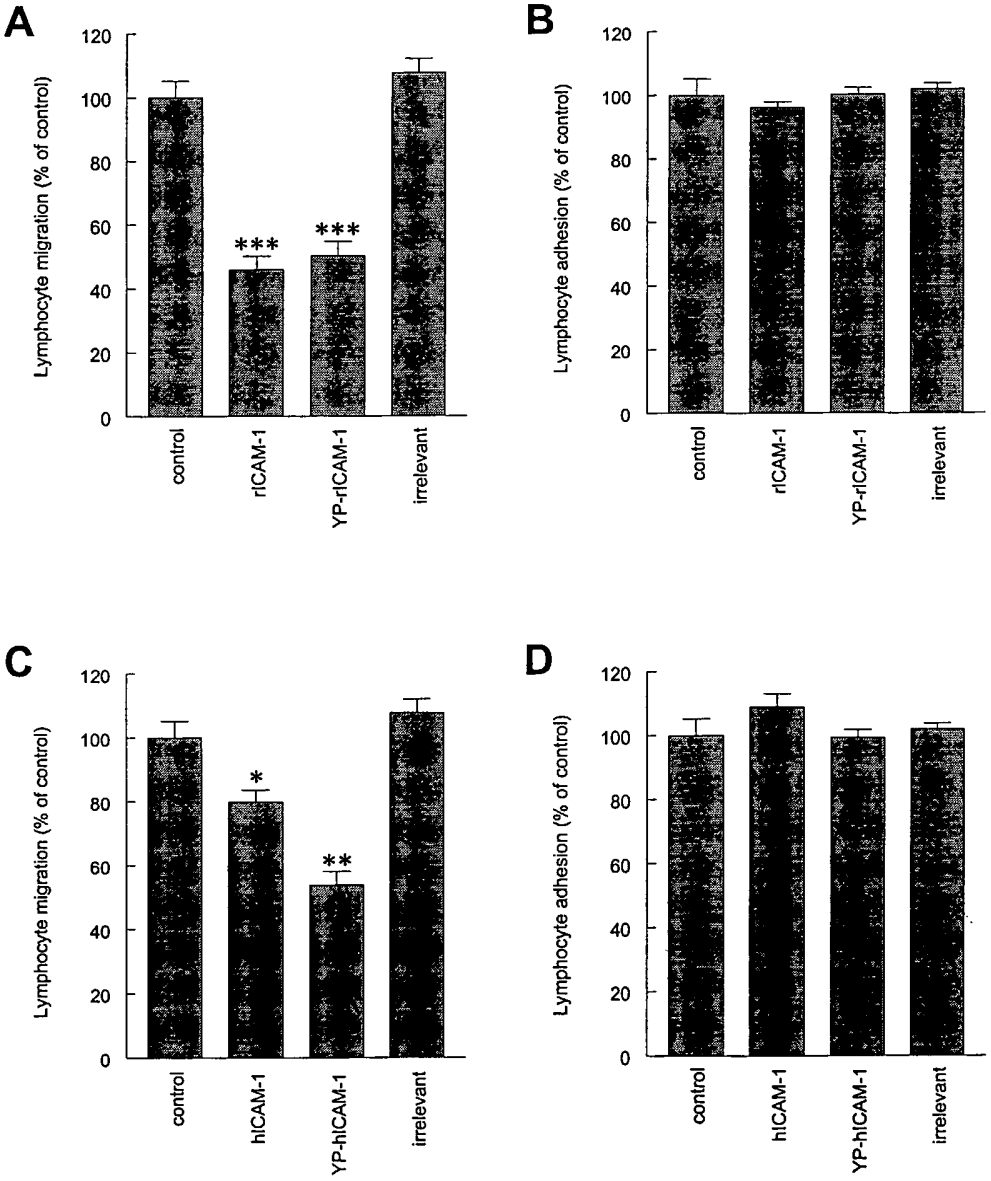

A
Lymphocyte migration (% of control)
120
100
80
60
40
20
0
***
***
control
rICAM-1
YP-rICAM-1
irrelevant
B
Lymphocyte adhesion (% of control)
120
100
80
60
40
20
0
control
rICAM-1
YP-rICAM-1
irrelevant
C
Lymphocyte migration (% of control)
120
100
80
60
40
20
0
*
**
control
hICAM-1
YP-hICAM-1
irrelevant
D
Lymphocyte adhesion (% of control)
120
100
80
60
40
20
0
control
hICAM-1
YP-hICAM-1
irrelevant

Figure 1

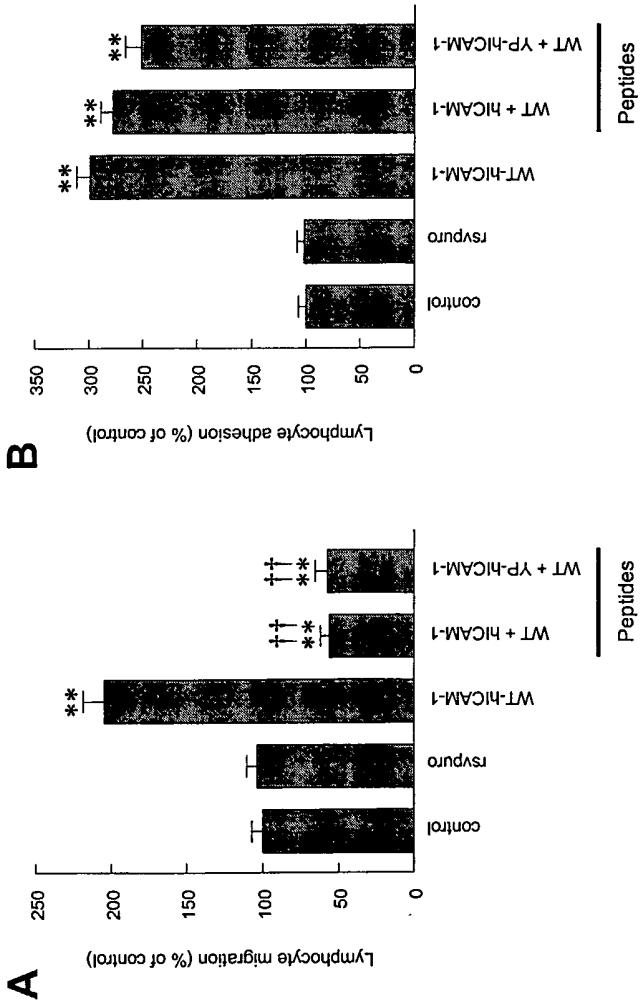

A
Lymphocyte migration (% of control)
250
200
150
100
50
0
control
rsvpuro
WT-hICAM-1
WT + hICAM-1
WT + YP-hICAM-1
Peptides
B
Lymphocyte adhesion (% of control)
350
300
250
200
150
100
50
0
control
rsvpuro
WT-hICAM-1
WT + hICAM-1
WT + YP-hICAM-1
Peptides

Figure 2

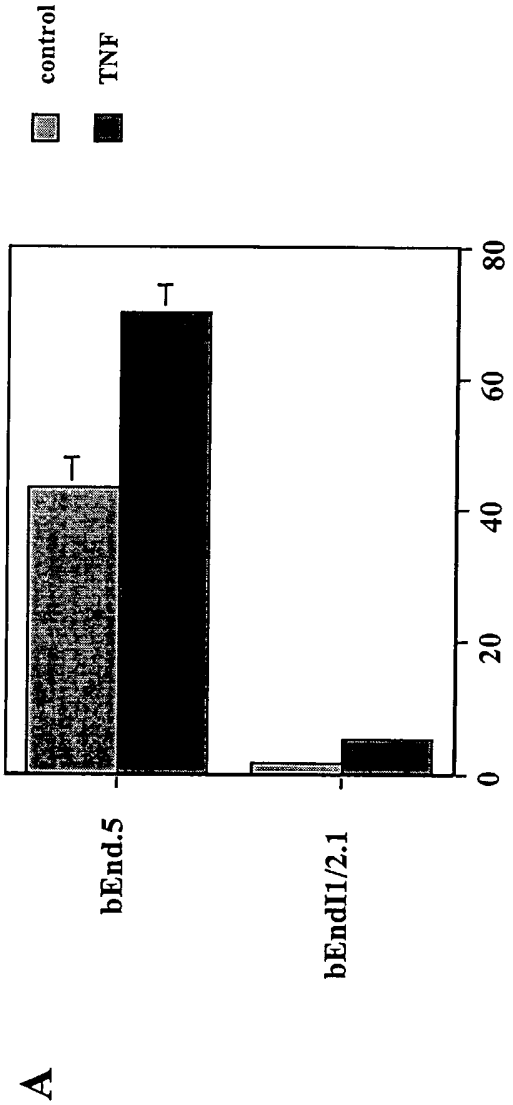


Figure 3A

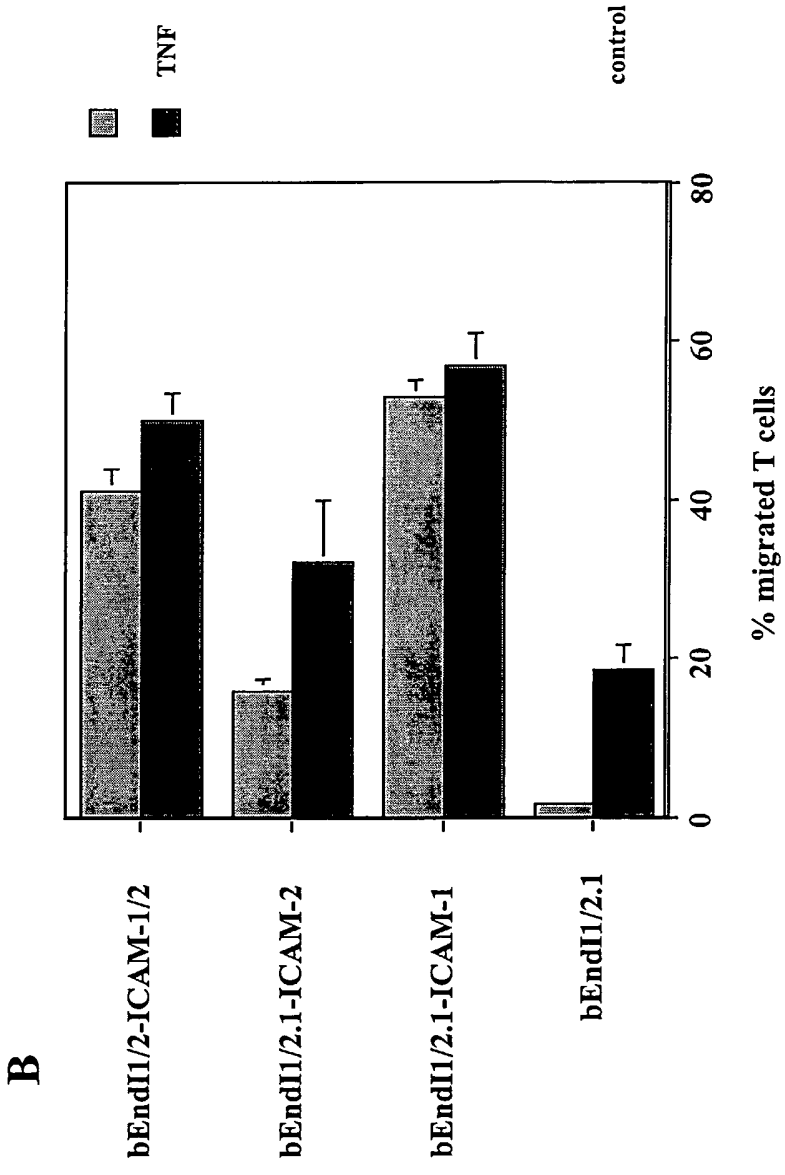

B
TNF
control
bEndI1/2-ICAM-1/2
bEndI1/2.1-ICAM-2
bEndI1/2.1-ICAM-1
bEndI1/2.1
% migrated T cells
0
20
40
60
80

Figure 3B

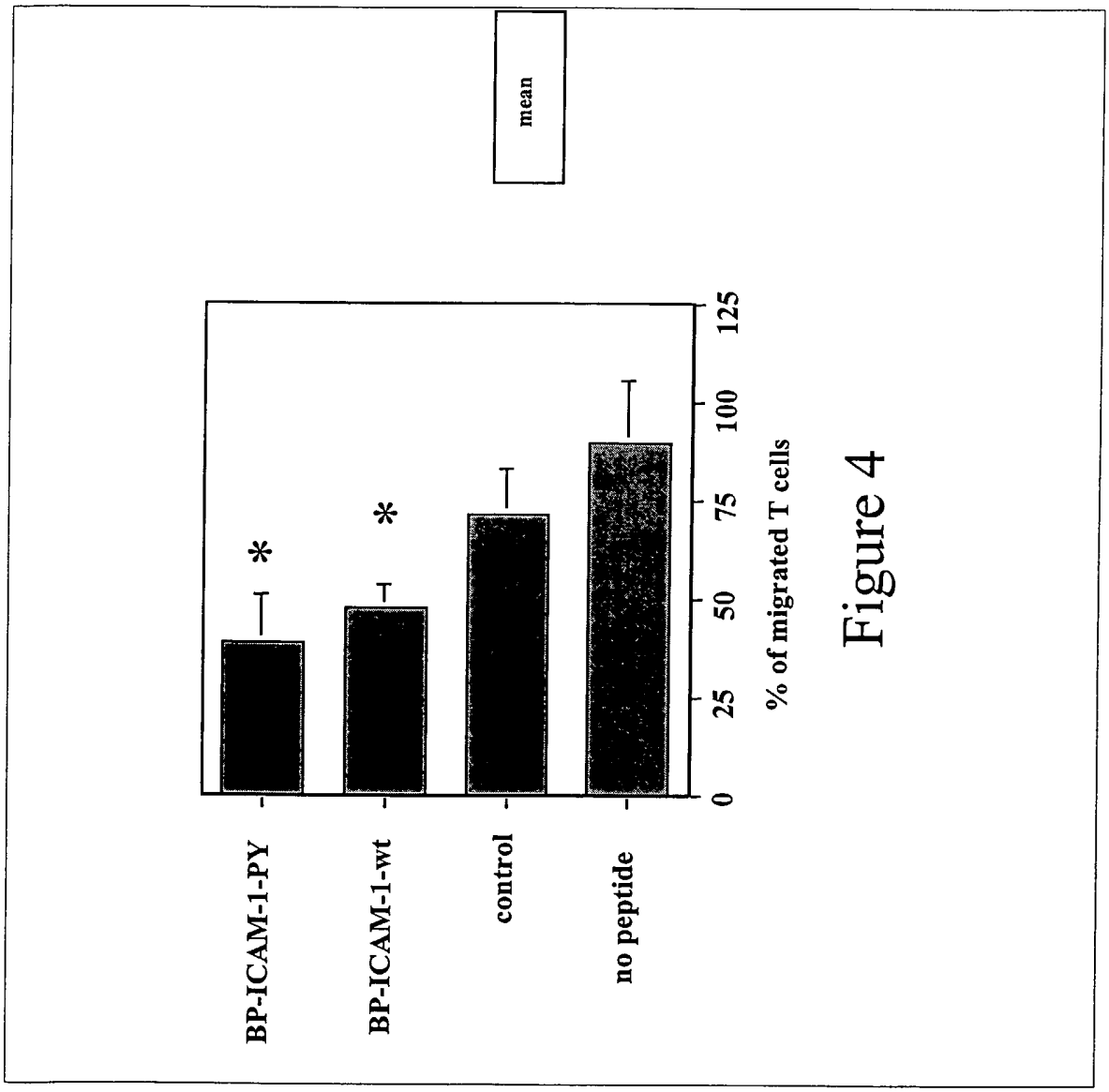

mean
BP-ICAM-1-PY
BP-ICAM-1-wt
control
no peptide
*
*
0
25
50
75
100
125
% of migrated T cells

Figure 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT /GB2004/002163

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K7/04 C07K7/06 C07K7/08 A61K38/10 A61P25/00

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)
IPC 7 C07K 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 practical, search terms used)
EPO-Internal, PAJ, WPI Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO 01/94570 A (LOEWENHEIM HUBERT ; ANTZ CHRISTOF (DE); HAHN HARTMUT (DE); OTOGENE AG) 13 December 2001 (2001-12-13) *cf. abstract, page 5, line 5, bridging with page 6, line 23, page 7, line 12 to page 8, line 21, page 12, 2nd para.*	1-12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2004	06/10/2004

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stoltner, A

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/002163

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEROSSI D (REPRINT) ET AL: "Trojan peptides: the penetratin system for intracellular delivery" TRENDS IN CELL BIOLOGY, ELSEVIER SCIENCE LTD, XX, vol. 8, no. 2, February 1998 (1998-02), pages 84-87, XP002122131 ISSN: 0962-8924 *cf. page 84, abstract and figure 1*	1-12
Y	FISCHER ET AL: "Structure-activity relationship of truncated and substituted analogues of the intracellular delivery vector penetratin" JOURNAL OF PEPTIDE RESEARCH, MUNKSGAARD INTERNATIONAL PUBLISHERS, COPENHAGEN, DK, vol. 55, no. 2, 2000, pages 163-172, XP002140331 ISSN: 1397-002X *cf. abstract, page 165, table 1, page 171, right col., "conclusion"*	1-12

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International Application No
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