Therapeutic and imaging potential of peptide agents in cardiocascular disease

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Targeting CD40 with a Selective Phage Display Derived Peptide

Haixiang Yu*, Andrea M. Woltman[†], Peter Boross[§], Karen Sliedregt-Bol#, Sjef Verbeek[§], Herman Overkleeft #, Gijs A. van der Marel#, Cees van Kooten[†], Theo J.C. van Berkel*, Erik A.L. Biessen*‡

*Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research and #Department of Bio-organic Chemistry, Leiden University, P. O. Box 9502, 2300 RA Leiden, the Netherlands. [†] Department of Nephrology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, the Netherlands. §Human and Clinical Genetics, Leiden University Medical Center, Wassenaarseweg 72, 2333AL Leiden, the Netherlands

Running title: phage display derived peptide selective for CD40

Abstract

The CD40/CD154 dyad is deemed to play a central role in several immunogenic and inflammatory processes, including atherosclerosis. As CD40 activation induces the expression of various pro-atherogenic cytokines, chemokines, growth factors, matrix metalloproteinases and as interruption of CD40 signalling was found to retard atherogenesis and stabilize vulnerable plaques, we sought to investigate whether CD40 could be an attractive target for therapeutic and targeted imaging in atherosclerosis. In this study, we have designed a novel peptide ligand selective for CD40 by phage display. An enriched phage pool was seen to bind not only human CD40 but also inflammatory joints in a murine model of rheumatoid arthritis. Peptides corresponding with the phage insert displayed nanomolar affinity for CD40 and after docking as a tetramer its avidity and biologic potency was further enhanced, which is in agreement with the presumed trimeric interaction between CD40 and CD40L. In conclusion, we have isolated a novel peptide ligand which selectively binds to human CD40 and may have potential in targeted imaging approaches or drug delivery agent in CD40 dependent inflammatory disorders such as atherosclerosis.

Introduction

The CD40/CD154 (CD40 ligand, CD40L) dyad is deemed to play a central role in several immunogenic and inflammatory processes, including atherosclerosis[1]. The expression and function of CD40/CD154 are not restricted to B and activated T cells for lymphocytic communication, but on a variety of other cell types, such as monocytes, platelets, dendritic cells, fibroblasts, mast cells, endothelial cells, smooth muscle cells and macrophages. Ligation of CD40 triggers the expression of cytokines, adhesion molecules, growth factor, matrix metalloproteinases, tissue factors and apoptotic mediators. Accordingly, CD40 signaling has been associated with the pathophysiology of immunodeficiency, collagen-induced arthritis, neurodegenerative disorders, graft-versus-host disease, cancer, and atherosclerosis[2-7]. Disruption of CD40/CD154 was seen to lead to inhibition of immune responses and blockade of CD40/CD154 by monoclonal antibodies was shown to be beneficial in the treatment of autoimmune diseases[3], transplantation[8,9] and atherosclerosis[7,10,11]. However, antibody therapy to interrupt CD40/CD154 signaling often was accompanied with thromboembolic symptoms, which has raised major concerns and precluded further clinical studies on CD40 therapy in humans[12]. Therefore, alternative strategies to intervene in CD40 signaling are eagerly awaited.

Phage display is a powerful high throughput strategy for the unbiased design of novel ligands with improved affinity. In this study, we describe the use of phage display to identify selective peptide ligands that are specific for CD40 and demonstrate that the peptide when exposed on phage is able to target inflamed joints in an acute rheumatoid arthritis mouse model in a CD40 dependent manner. We further demonstrate functionality of a synthetic peptide of the encoded phage insert, NP31, at which the nanomolar affinity could be further enhanced after tetramerization on streptavidin scaffold, which is in agreement with the presumed trimeric interaction between CD40 and CD40L. Furthermore we show that the NP31 peptide behaves as a partial antagonist blocking CD40 elicited cytokine production at low micromolar levels.

Material and methods

Phage libraries

The cysteine-constrained peptide phage library pComb8 $CX_{15}C$, at which X is any amino acid and C is a fixed cysteine residue, was generated at the Department of Biochemistry, University of Amsterdam, The Netherlands. The pIF15 phage library containing randomized linear 15-mer amino acids peptide sequence was kindly provided by Dr. Monaci, IRBM, Rome, Italy.

Antibody and plasmid

Goat anti-mouse IgG (Fc specific) antibody was from Sigma (St. Louis, MO, USA). Human CD40-murine IgG recombinant protein was from Ancell (Bayport, MN, USA). Human CD4-murine IgG was kindly provided by Dr. Appelmelk (Vrije Universiteit, Amsterdam, the Netherlands). Biotin, streptavidin, horseradish

peroxidase conjugated streptavidin (StrepHRP) was from Amersham Bioscience (Buckinghamshire, England). Streptavidin-R-phycoerythrin (StrepPE) was from Sigma (St. Louis, MO, USA). Recombinant human soluble CD40L and murine soluble CD40L were from Santa Cruz (CA, USA).

Cell culture

Mouse fibroblast L cells stably transfected with human CD40 and negative control L-cells (ATCC: CCL 1.3, murine fibroblasts) were cultured as described[13]. Human endothelial cells Ea.Hy.926 and murine H5V endothelial cells were cultured in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.

Selection of CD40 binding phage

10 μg/mL of goat anti-mouse IgG in coating buffer (50 mmol/L NaHCO3, pH 9.6) was incubated overnight at 4°C in a high binding 96 well plate (Costar, Corning, UK) at 100 μL/well. Subsequently, wells were washed 3x with 200 μL assay buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L CaCl2, pH 7.4) and incubated for 1h at 37°C with 200 μL blocking buffer (3% BSA in assay buffer). Wells were then incubated with 100 μL 3 μg/mL of human CD40-muIgG in 100 μL of binding buffer (0.1% BSA, 0.5% Tween 20 in assay buffer, 2h 37°C). After incubated for 2h at room temperature (RT) with the phage libraries at 10^9 colony forming units (CFU) in 100 μL of binding buffer, wells were washed 10x with 200 μL binding buffer and bound phage were eluted by incubation for 5 min at RT with 100 μL of elution buffer (0.1 mol/L glycine/HCl, pH 2.2) and neutralized by addition of 50 μL of neutralization buffer (1 mol/L Tris/HCl, pH 8.5). Phage were titrated, amplified and purified as described[14]. Amplified phage was used in further selection rounds. For DNA sequencing of enriched phage pools, plasmid DNA was isolated from single colonies using the Wizard plus SV Miniprep DNA Purification System (Promega, Madison, USA). DNA sequencing of the plasmids was conducted at the DNA-sequencing facility of the Leiden University Medical Center using a standard M13 primer. Unless otherwise stated, the phage pool amplified from a single phage clone isolated after 5 rounds of selection was used for all further experiments.

Peptide synthesis

NP31 (CMSYEGSWRKWVMWGGCG), scrambled NP31 control peptide (NP31c, CEMGWMWGWRGSKYSVCG), biotinylated NP31 (CMSYEGSWRKWVMWGGCGGGK-biotin, truncated and alanine mutated NP31 peptides were synthesized using Fmoc solid-phase peptide synthesis on a Multisyntech Syro Multiple Peptide Synthesizer. Crude peptides were purified on a preparative C_{18} RP-HPLC column (Alltech) using a BIOCAD VISION automated purification system. Purified peptides were characterized by LC-MS, MALDI-TOF MS and found to be at least 95% pure.

Competition experiment of CD40 selective phage binding

In analogy to the phage selection procedure, 10 μg/mL of goat anti-mouse IgG in coating buffer was incubated overnight at 4°C in a high-binding 96-well plate, 100 μL/well. Wells were washed 3x with assay buffer and incubated for 1 h at 37 °C with blocking buffer. After incubation for 2h at 37°C with 3 μg/mL of human CD40-muIgG (100 μL/well), wells were washed 3x with binding buffer, after which the enriched phage pool at 10^9 cfu in 100 μL binding buffer was added and incubated for 2h at RT in the presence of NP31 or NP31c peptides. Wells were washed 10x with binding buffer and bound phage were eluted by incubation for 5 min at RT with 100 μL elution buffer and neutralized with 50 μL neutralization buffer. Residual phage binding was calculated from the output/input ratio.

Competition ELISA of selective peptide binding to CD40

StrepHRP was incubated for 2h at RT in phosphate-buffered saline (pH 7.0) with NP31-biotin a 1:4 molar ratio, thus forming a tetrameric NP31-strepHRP complex. For competition studies, high binding microtiter wells were coated with human CD40-muIgG as described under "Selection of CD40 binding phage". Wells were incubated with 250 nmol/L NP31-strepHRP in assay buffer for 1h at 4ºC in the presence or absence of tittered amount of peptides. After washing 6x with 200 μL assay buffer, the wells were incubated for 15 min at RT with 100 μL TMB/H_2O_2 (Pierce, Rochford, USA). The reaction was stopped by adding 50 μL 2 mol/L H_2SO_4 and the absorbance was read at 450nm.

NP31 peptide selectively binds to cells overexpressing CD40

L-cells stably transfected with human CD40 and CD40 null control L-cells were collected by centrifugation. 3×10^5 cells were suspended in 100 μL phosphate buffered saline containing 1% normal mouse serum and incubated in v-shape 96 well plate for 1h at 4ºC. NP31-strepPE and strepPE-biotin complex was freshly prepared by incubation of strepPE with NP31-biotin or biotin in a 1:4 molar ratio for 2h at RT. After 2x washing with 100 μL PBS, cells were incubated with 50 μL (250 nmol/L) StrepPE-biotin or NP31-strepPE for 1h at 4ºC. Cells were then washed 3x by 100 μL PBS, resuspended in 150 μL PBS, and then subjected to FACS analysis.

IL-6 cytokines production in endothelial cells

Cells cultured in 24 well plates were treated with and without sCD40L (10 ng/mL) in the presence of NP31 peptide at indicated concentrations. After 24h, IL-6 that had been released into the culture medium was measured by ELISA. (BD Biosciences Pharmingen, USA)

[^{35}S]-labeled CD40 selective binding phage effectively home to inflamed joints in a K/BxN arthritis mouse model

K/BxN mouse model (maintained on a C57Bl/6 background) was set up in the Dept of Human and Clinical Genetics, Leiden University Medical center, the Netherlands. In brief, repeated injection of 200 μL sera from arthritic K/BxN mice into healthy C57Bl/6 recipients induces acute joint damage and cartilage erosion in the bone within 15 days[15]. Each swollen paws scored 1 point, resulting in a maximum arthritis score of 4 per mouse. Mice with a score of 3 or higher were

considered arthritic and used in further experiments. CD40 specific binding phage pool NP31 and non-specific control phage eluted from the second round of selection were radiolabeled as previously described[16] and injected at 10^9 CFU (~200,000 DPM) in 100 µL Tris-buffered saline via the tail vein into anesthetized C57BL/6 mice or K/BxN mice inflamed with swollen joints (20 weeks old; male; n=3). After 2h, mice were subsequently perfused for 2 min with DMEM and PBS at 10 mL/min via a cannula inserted in the left ventricle. Organs were removed and weighed. Organ homogenates were obtained by overnight incubation of ~0.1g tissue with 500 µL Solvable at 56ºC (Packard bioscience, Groningen, the Netherlands) and analyzed for associated [^{35}S] radioactivity in a beta counter after adding 15 mL Hi-ionic Fluor (Perkin-Elmer, Boston, USA) .

Statistical Analysis and animal handling ethics

Values are expressed as mean ± SEM. A two-tailed unpaired Student's t-test was used to compare individual groups. A level of $P<0.05$ was considered significant. Animal experiments were performed at the Gorlaeus laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

RESULTS

Selection and selectivity of phage displayed peptide against human CD40

Peptide phage libraries pIF15 and pComb8, encoding random linear and cysteine-constrained 15-mer peptide sequences respectively, were screened for novel human CD40 binding peptide ligands. To favor the selection of peptides binding to the actual binding pocket of CD40, biopanning was performed on wells coated with anti-mouse IgG antibody to which recombinant human CD40 murine IgG was immobilized comprising the 193 amino acid extracellular domain of CD40. The selection protocol involved gentle wash steps in the first two rounds followed by more stringent washes combined with 10-fold reduced coating densities of recombinant CD40 protein in later rounds led to an approximately 200-fold enrichment of CD40 binding phage for the pComb8 constrained library in the 5th round (Fig.1A).

Table 1 Alignment of CD40 binding peptide sequence selected by phage display

Library	Phage clone	Name
PIF15	DGNVVTCWACREKRW	NP2
pComb8	CMSYEGSWRKWVMWGGC	NP31
	CDLFVMAVGTNLDWWGC	NP26
	CVERCLASTSSGVKALC	NP58
BLAST search	MSY----RYHWVMWG	ABC transporter permease protein

DNA sequence analysis of the CD40 binding phage clones isolated after 5 rounds of selection revealed a single 15-mer cyclic peptide sequence (CMSYEGSWRKWVMWGGC), termed NP31, in 5 out of 5 phage clones for the cysteine constrained pComb8 library. Interestingly, parallel phage display of a non-constrained pIF15 linear 15-mer peptide library resulted in the identification of a consensus peptide motif WRK. Sequencing of phage clones isolated after 4 rounds of selection from the pComb8 library revealed shared peptide motif (Tab.1). Discontinuous Blast search for homology showed that the ABC transporter permease protein contains a WVMWG motif, suggestive of an interaction between this ABC transporter epitope and CD40.

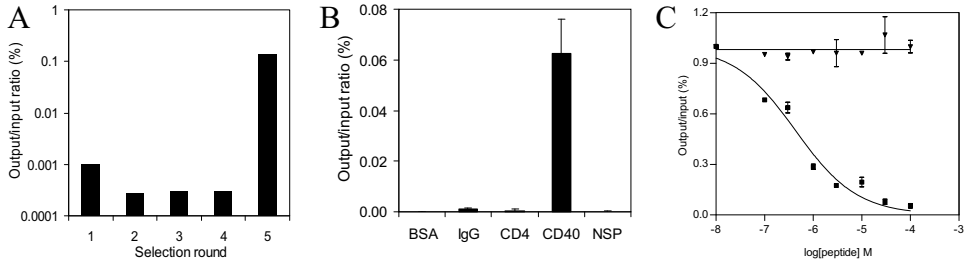

Figure 1: Screening and selectivity of CD40 binding phage. (A) Selection of the pComb8 phage displayed peptide library on immobilized human CD40 recombinant protein led to a sharp enrichment of human CD40 binding phage in the 5th round of selection (~200 fold increase) **(B)** In comparison to a nonspecific phage pool, the enriched phage pool specifically bound to human CD40-muIgG but not to immobilizing agent anti-mouse Ig, blocking agent BSA or human CD4-muIgG fusion protein. Phage binding is expressed as ratio of output to input and represent means ±SD of three individual experiments. **(C)** Binding of the enriched phage pool to human CD40-muIgG was determined in the presence of synthetic peptides encoded by CD40 specific phage clone NP31 (■); a scrambled NP31 sequence was used as control peptide (▼). IC_{50} values, as calculated from the competition curves were 440 nmol/L. Phage binding is expressed as ratio of output to input and represent means ±SD of three individual experiments.

The selectivity pattern of the enriched phage pool was tested in an ELISA based binding assay onto coated recombinant human CD40-muIgG. Of the selected phage clones NP2, NP26, and NP58 were found to interact with human CD40 in a non-specific manner. In contrast, the enriched phage pool NP31 showed selective binding to anti-mouse IgG immobilized human CD40 (Fig. 1B). NP31 phage did not bind to the blocking agent BSA, nor to anti-mouse IgG alone or to immobilized human CD4, establishing that NP31 selectively bound recombinant human CD40.

Competition assay of phage binding to human CD40 by phage encoded synthetic NP31 peptides

We synthesized the cyclic peptide sequence NP31, encoded by the CD40 specific phage clone. The capacity of NP31 peptide to interfere with NP31 phage binding to

human CD40 was tested in a competition assay, at which residual binding of the selected phage to CD40 was determined in the absence or presence of synthetic NP31 or a scrambled control peptide NP31c (Fig.1C). NP31 could dose-dependently and potently inhibit binding of the enriched phage pool to human CD40 at an calculated IC_{50} of 440 nmol/L, whereas the control peptide NP31c was completely ineffective up to 100 μmol/L.

NP31 peptide selectively binds to cells overexpressing human CD40

As CD40 only interacts with CD40L as a trimer or pre-trimer, and is thus expected to display a similar preference for oligomer ligands[17], we argued that the affinity of NP31 for CD40 may benefit from tetrameric presentation on a streptavidin scaffold. A further advantage of streptavidin docking is that it will favorably affect the pharmacokinetics of the peptide in vivo. To this end we synthesized NP31 derivative which was biotinylated at the C-terminal end and incubated it with streptavidin-PE at a molar ratio of 4:1 to prepare tetrameric NP31-strepPE complex. First we verified whether NP31 was also able to bind full length CD40 receptor expressed on cells and we have investigated the ability of strepPE-biotin or NP31-strepPE (Black histogram: strepPE-biotin, Open histogram: NP31-strepPE) to bind murine fibroblast L-cells (Fig. 2A) or L-cells overexpressing human CD40 (L-CD40 cells, Fig.2B) by FACS analysis. A quantitative result of mean fluorescence intensity is given in Fig. 2C. After conjugation to the streptavidin-PE scaffold, NP31 showed a significant higher binding to $CD40^+$ L-cells than to $CD40^-$L-cells ($P<0.001$) at concentrations as low as 250 nmol/L. A strepPE-biotin control did not give any differential binding to CD40+ and CD40- L-cells. In fact the binding of strepPE-biotin to $CD40^+$ cells was very similar to that of NP31-StrepPE to CD40- L-cells and at least 2-fold lower than that of NP31-strepPE to $CD40^+$ L-cells.

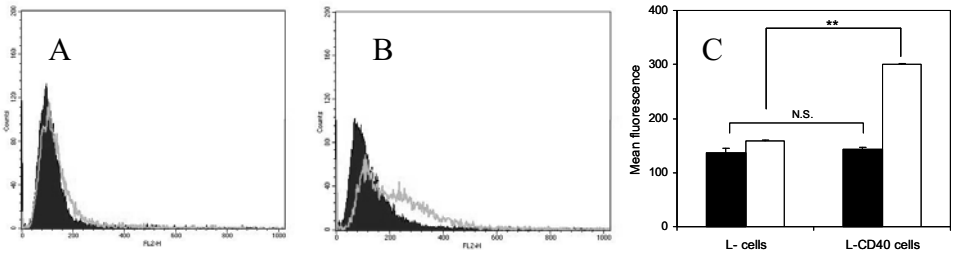

Figure 2: Streptavidin-PE conjugated NP31 complexes selectively and avidly bound to cells overexpressing human CD40. $CD40^-$ L-cells **(A)** or L cells stably transfected with human CD40 **(B)** were incubated for 1h at 4ºC with freshly prepared NP31-strepPE or strepPE-biotin (250 nmol/L). After washing with PBS, cells were resuspended and subjected to FACS analysis. Black histogram: strepPE-biotin, Open histogram: NP31-strepPE. **(C)** Mean fluorescence intensity of PE^+ cells was calculated as a measure of CD40 specific binding of strepPE-biotin (black bars) and NP31-strepPE (open bars). Values represent means ±SD of three individual experiments.

Identification of the minimal essential motif of CD40 binding peptide

Based on the sequence homology of the linear and cyclic peptide inserts of the isolated phage clones, we assumed that recognition of human CD40 only requires part of the NP31 peptide. To identify the crucial amino acid and the minimal binding motif of the peptide, we have set up a CD40 binding ELISA and performed conventional truncation and alanine scanning studies. As ligand in the competition assay, we used a preformed complex of biotinylated NP31 with streptavidin-HRP (NP31-strepHRP). NP31-strepHRP bound specifically and avidly to immobilized human CD40 (Fig. 3A) at 250 nmol/L. In addition, NP31-strepHRP showed only basal interaction with the immobilizing agent anti-mouse IgG protein, with the blocking agent BSA or with normal mouse IgG, which served as a binding reference, indicating that NP31 binding is selective for human CD40 and does not involve the IgG (Fc) part of recombinant human CD40-muIgG.

Competition studies were performed by co-incubation of NP31-strepHRP complex with increasing concentrations of NP31 peptide and NP31-streptavidin. The inhibitory concentration (IC50) for NP31 was found to be 215 nmol/L, which is comparable to the potency of NP31-strepPE binding to CD40+ L-cells and to the value obtained in the phage displacement studies. The tetrameric peptide NP31-streptavidin, prepared by pre-incubation of NP31-biotin with streptavidin at a 4:1 molar ratio for 2h at RT, displayed a 2.5 fold higher affinity as compared with monomeric NP31 (IC50= 84 nmol/L), pointing to a stochastic rather than a multimeric effect.

Subsequent truncation and alanine scan studies were performed by co-incubation of 250 nmol/L of NP31-strepHRP complex with 1 μmol/L of the NP31 analogue. The inhibitory potency of the peptides was expressed as percentage of the specific NP31-strepHRP binding in the absence of peptide after correction for background binding. As a reference we used 1 μmol/L of NP31. Alanine substitution confirmed the crucial requirement of a cyclic configuration for effective CD40 binding. The C-terminal amino acids of NP31 seemed to be essential as well and alanine changes were not tolerated. Also the N-terminal Glu (4) was found to be preferred. Stepwise truncation from the N-terminal end of NP31 showed that up to 4 amino acid truncations did not markedly affect the affinity for CD40. Gly (5) and Ser (6) were replaceable by alanine but could not be left out. Collectively, the truncation and alanine scan studies identify C(x1x2)WRKWVMWGGC as the essential motif for selective CD40 binding, where x can be any amino acid and might in fact only function to provide the proper spatial configuration of the cyclic peptide.

NP31 inhibited sCD40L stimulated IL-6 production in endothelial cells

Recombinant soluble CD40L was shown to induce the expression of the proinflammatory cytokine IL-6 by cultured endothelial cells (EC)[18]. To verify whether NP31 was able to interfere with CD40 mediated effects, we have measured IL-6 production by human endothelial cells (Ea. Hy. 926) and murine endothelial cells (H5V) upon treatment with soluble human or mouse CD40L,

respectively in the presence of NP31. As expected CD40L induction potently stimulated IL-6 secretion by both human and murine endothelial cells and this effect was dose-dependently inhibited by NP31. In addition, NP31 treatment alone had not effect on IL-6 production. At a concentration of 10 µmol/L, NP31 significantly inhibited sCD40L mediated IL-6 production in human and mouse EC (P=0.001, Fig. 4A/B) indicating that NP31 potently binds to murine CD40 as well. However, NP31 only gave a partial, 25% reduction of sCD40L induced cytokine production, which indicates that it might be a partial antagonist of CD40 signaling.

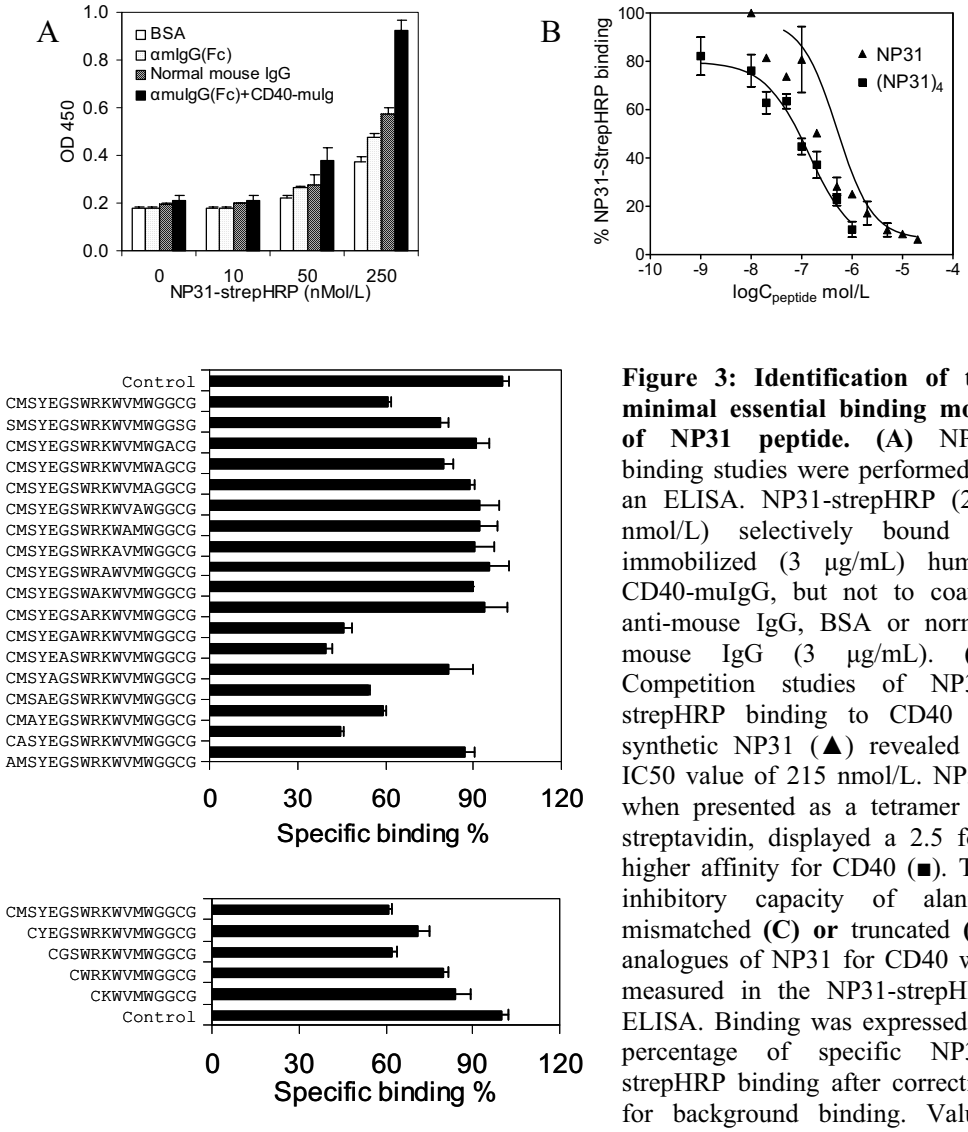

Figure 3: Identification of the minimal essential binding motif of NP31 peptide. (A) NP31 binding studies were performed in an ELISA. NP31-strepHRP (250 nmol/L) selectively bound to immobilized (3 µg/mL) human CD40-muIgG, but not to coated anti-mouse IgG, BSA or normal mouse IgG (3 µg/mL). **(B)** Competition studies of NP31-strepHRP binding to CD40 by synthetic NP31 (▲) revealed an IC50 value of 215 nmol/L. NP31, when presented as a tetramer on streptavidin, displayed a 2.5 fold higher affinity for CD40 (■). The inhibitory capacity of alanine mismatched **(C) or** truncated **(D)** analogues of NP31 for CD40 was measured in the NP31-strepHRP ELISA. Binding was expressed as percentage of specific NP31-strepHRP binding after correction for background binding. Values represent means ±SD of three individual experiments.

CD40 selective binding phage targeted home to the inflamed arthritic joints of K/BxN mouse model in vivo

Next, we have examined the capacity of the CD40 specific phage clone to accumulate in inflamed knee joints in a mouse model of rheumatoid arthritis after intravenous injection. The K/BxN mouse model was previously demonstrated to represent an aggressive form of rheumatoid arthritis and the underlying inflammatory response was shown to be CD40 mediated[19,20]. After circulating for 2h, radiolabeled NP31 phage displayed a significant 10-fold increase in mouse joints (sum of front and hind joints) suffered from arthritis compared with normal mouse joints ((P=0.024; Fig.5B). The control phage tended to accumulate in arthritic joints as well, which might be due to enhanced vascular permeability and/or the abundant presence of macrophages in the inflamed tissue, albeit that this difference did not reach significance. Importantly, accumulation of NP31 and control phage in non-inflamed joints did not differ. Moreover, no differences were observed in the tissue distribution profile of NP31 and control phage, and for both phage liver and spleen appeared to be the most prominent sites of phage uptake (Fig. 5C).

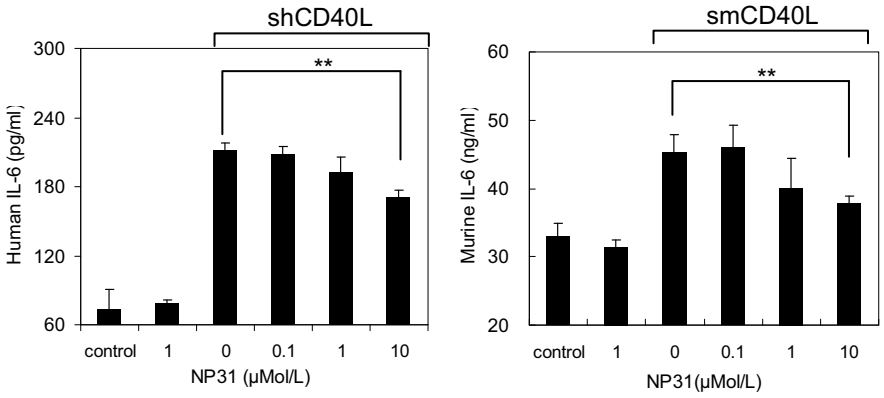

Figure 4: NP31 partially inhibits soluble CD40L elicited IL6 production in endothelial cells. Human endothelial cells (Ea.Hy.926; **A)** and murine endothelial cells (H5V; **B),** cultured in 24 well plates, were treated with shCD40L or smCD40L (10 ng/mL), respectively, in the presence of NP31 at the indicated concentrations. After 24h, media were collected and IL-6 that had been released into the culture medium was measured by ELISA. Values represent means ±SD of three individual experiments (** P<0.01).

Discussion

CD40 signaling plays a key role in various inflammatory diseases such as atherosclerosis and rheumatoid arthritis[21,22]. Therefore, intervention strategies focusing at the inhibition of CD40 activation hold promise for the treatment of autoimmune diseases. In addition, as CD40 was found to be overexpressed in all major inflammatory cell types (e.g. macrophages, B-cells, T-cells), it may be regarded as an attractive target for imaging of sites of acute inflammation and

potentially vulnerable atherosclerotic plaques. Studies in CD40/CD40L deficient mice or animal models in which CD40 signaling was functionally disrupted have established the beneficial effect of blockade CD40 ligation for atherosclerosis and autoimmune diseases[8,9]. Extensive investigations have been focused on CD40/CD40L ligation blocking antibodies. CD40 directed antibody therapy proved effective but suffered from severe side effects such as thromboembolism[12]. Given the central role of CD40 as co-stimulatory molecule in many immune responses, it is surprising that ectopic immune effects were not reported in these studies. Although there is a need for selective partial antagonist of CD40 signaling which does not compromise platelet activity, it is still unclear whether such a targeted intervention in CD40 function will be feasible at all. In this paper, we describe the identification of a 11-mer constrained peptide which selectively binds to human CD40 with nanomolar affinity to confer partial inhibition of CD40L induced IL-6 production and which conveyed phage (but possibly also other vehicles) specifically to sites of inflammation in a mouse model of rheumatoid arthritis.

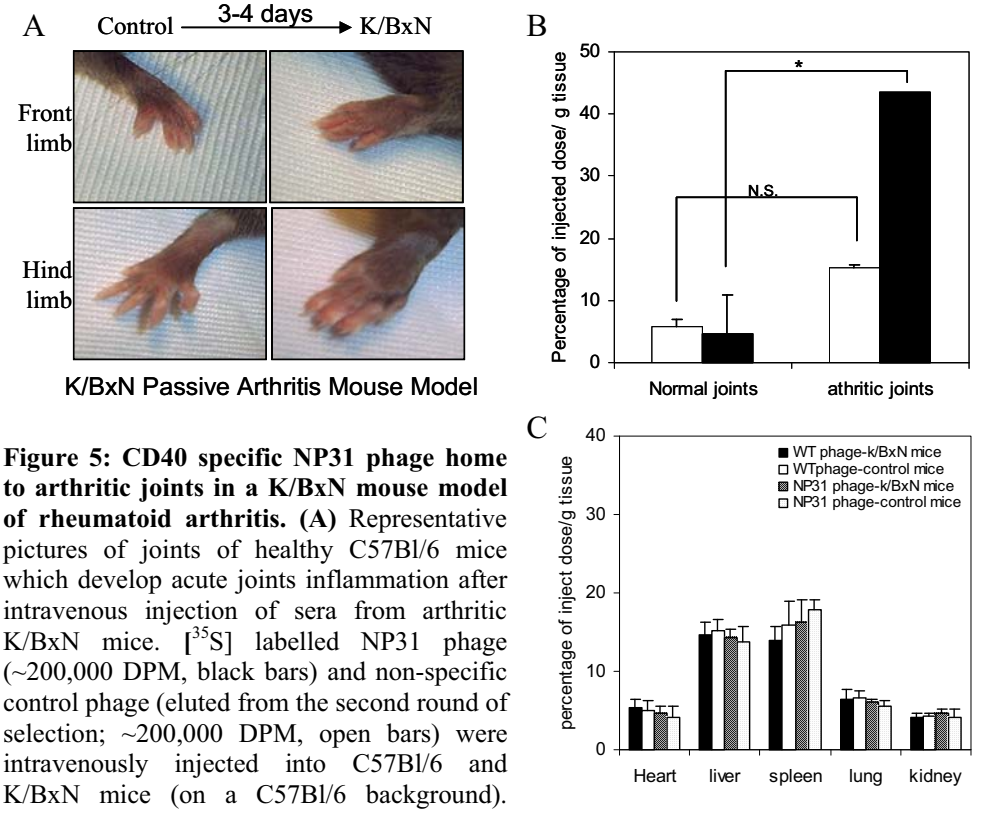

Figure 5: CD40 specific NP31 phage home to arthritic joints in a K/BxN mouse model of rheumatoid arthritis. (A) Representative pictures of joints of healthy C57Bl/6 mice which develop acute joints inflammation after intravenous injection of sera from arthritic K/BxN mice. [^{35}S] labelled NP31 phage (~200,000 DPM, black bars) and non-specific control phage (eluted from the second round of selection; ~200,000 DPM, open bars) were intravenously injected into C57Bl/6 and K/BxN mice (on a C57Bl/6 background). After 2h of circulation, mice were subsequently perfused with DMEM and PBS, organs were removed and the distribution pattern of organ associated radioactivity in mouse joints **(B)** and main organs **(C)** was determined as a percentage of the injected dose/g tissue. Values represent means ±SD of three mice/group (* $P<0.05$).

Unbiased combinatorial phage display technology was used to identify novel peptide ligands of CD40 with high specificity and potency. The selection based on recombinant human CD40 revealed a conserved sequence WRKWVMWG, which is relatively rich in tryptophans and basic amino acids. A synthetic peptide encompassing the WRKWVMWG core sequence (NP31) bound selectively and potently to CD40. In addition, NP31 showed a similar high affinity to human CD40 in competition studies of NP31 phage binding (ELISA) and of streptavidin-HRP docked NP31 binding (ELISA), with IC50 values of 440 and 215 nmol/L, respectively. FACS analysis confirmed that NP31 did bind to native cell expressed CD40 as well. Previously, homologues modeling studies have revealed that the interface of CD40/CD40L comprises a high density of charged residues, with CD40L presenting basic side chains (K143, R203, R207), and CD40 presenting acidic side chains (D84, E114, E117)[23]. It is tempting to assume that the basic amino acids of NP31 mimic the basic binding cleft of CD40L and are instrumental in CD40 binding. In agreement, truncation and alanine scan studies showed that a cyclic 11-mer GSWRKWVMWGG motif was required for selective CD40 binding, confirming the importance of the C-terminal basic amino acids in CD40 recognition.

StrepHRP conjugated NP31 tetrameric complex were used to set up the peptide competition ELISA. We first have synthesized the N-terminal biotinylated NP31 (biotin-KCMSYEGSWRKWVMWGGCG) which was found to display weak binding to human CD40 when conjugated with strepHRP (data not shown). As the phage displayed peptides were fused via their C-terminus to the phage capsid protein, we therefore decided to use NP31 (CMSYEGSWRKWVMWGGCGGGK(ε-biotin)), carrying a C-terminal biotin group that was linked via a triglycyl spacer, arguing that it would facilitate protrusion of the core motif from the streptavidin scaffold. Indeed the NP31 biotinylated at the C-terminal end showed potent binding to human CD40 when conjugated with strepHRP as a tetramer indicating the importance of secondary structure of peptide for CD40 recognition.

In conclusion, we describe in this paper the design by phage display of a novel specific peptide ligand of human CD40, NP31. NP31 displayed nanomolar affinity towards human CD40 and partially antagonized CD40L induced cytokine production. Truncation studies revealed a 11-mer core motif that sufficed for high affinity binding. Furthermore, we show that NP31 phage clone specifically homed to sites of inflammation in a mouse model of rheumatoid arthritis. We propose that CD40 specific peptides or peptidomimetics based on NP31 may through their partial antagonistic activity and potent binding capacities allow a more dedicated intervention and diagnosis in inflammatory disorders such as atherosclerosis and autoimmune disease than CD40 blocking antibodies which completely ablate immune responses.

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