Full title:

Targeted delivery of anti-inflammatory therapy to rheumatoid tissue by fusion proteins containing an IL-4-linked synovial targeting peptide

Running Title:

Targeted therapy of synovial tissue in rheumatoid arthritis

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Abstract

We provide first-time evidence that the synovial endothelium-targeting peptide (SyETP) CKSTHDRLC successfully delivers conjugated IL4 to human rheumatoid synovium transplanted into SCID mice. SyETP, previously isolated by *in vivo* phage display and shown to preferentially localize to synovial xenografts, was linked by recombinant technology to hIL-4 via an MMP-cleavable sequence. Both IL-4 and the MMP-cleavable sequence were shown to be functional. IL-4-SyETP augmented production of IL-1ra by synoviocytes stimulated with IL-1β in a dose-dependent manner. *In vivo* imaging confirmed increased retention of SyETP-linked-IL-4 in synovial grafts which was enhanced by increasing number of copies (one to three) in the constructs. Strikingly, SyETP delivered bioactive IL-4 *in vivo* as demonstrated by increased pSTAT6 in synovial grafts. Thus, this study provides proof of concept for peptide-tissue-specific targeted immunotherapy in rheumatoid arthritis. This technology is potentially applicable to other biological therapies providing enhanced potency to inflammatory sites and reducing systemic toxicity.

Introduction

Rheumatoid arthritis (RA) is a systemic, inflammatory autoimmune disorder that presents as a symmetric arthritis associated with swelling and pain in multiple joints. Articular inflammation causes activation and proliferation of the synovial tissue with hypertrophy of the lining layer, expression of inflammatory cytokines, chemokine-mediated recruitment of inflammatory cells, as well as B cell activation with autoantibody production[1-3]. Cytokines such as interleukin (IL)-1, Tumour Necrosis Factor (TNF) and IL-6 are found in great abundance[4]. These cytokines mediate cartilage and bone degradation by augmenting matrix degrading enzymes such as aggrecanases and matrix metalloproteinases and the activation of osteoclasts, which cause, bone resorption.

Antagonists to these cytokines now play a fundamental role in the treatment of RA, most notably anti-TNF. These treatments result in clinical benefits for the majority of patients[3], however, up to 30-40% of patients do not respond. In addition, due to systemic immunosuppression, there is a risk of reactivation of latent infections such as tuberculosis. There is a great need for the development of new targeted therapies, as they have the potential for diminishing systemic toxicity while increasing pharmacological drug concentrations at the disease site.

One therapeutic approach to decrease pro-inflammatory cytokine expression is to administer anti-inflammatory cytokines, several of which have been shown to be effective in models of RA[4-11]. These include IFNβ[5], IL-10[6] and IL-4, which has been shown to reduce cartilage destruction and inhibit neoangiogenesis[7,8] as well as sharing some of the anti-inflammatory properties of IL-10[4, 9-11]. Clinical trials with IL-4 however reported a lack of efficacy, and it was

speculated that to achieve an efficacious dose in the synovium, the administered dose would not be tolerated systemically. This cytokine pleiotropism is a limitation to their clinical use.

One approach to overcome these problems is to target the delivery of cytokine to the specific diseased tissue. Linking cytokines to targeting peptides or antibodies are progressing to clinical trials for the treatment of cancer[12-14]. However, this therapeutic modality in chronic inflammatory conditions such as RA has so far been relatively unexplored.

Neoangiogenesis in RA, similarly to cancer, leads to an enlarged vascular bed and leukocyte infiltration within the synovial tissues and ultimately accelerates disease progression[15]. These new vessels are discontinuous, leaky and present a dysregulated expression of a number of molecules such as integrins, cell surface proteoglycans, proteases, and extracellular matrix components as well as endothelial cell growth factor receptors which are virtually absent or barely detectable in established blood vessels[16]. The differences between these new vessels in RA and normal vessels provide a good opportunity for targeted therapy.

Neoangiogenesis is also observed when RA synovial tissue is transplanted into SCID mice. We have used this xenograft model for identification of tissue-specific synovial homing motifs[17]. This has been facilitated by the application of phage display of random peptides[18,19] and antibody fragment libraries[20] to target microvasculature endothelium (MVE) in various tissues[21,22]. Homing peptide sequences have been generated for numerous organs in mice and human tumour vasculature[23,24] and have been shown to target and concentrate drugs to tumours engrafted in nude mice[21,23]. Notably, conjugation of the NGR homing peptide to TNF improved the

therapeutic index of TNF in preclinical studies[25] and NGR fused to human TNF is now entering Phase III clinical trials in cancer.

Treatment of mice with collagen-induced arthritis with RGD-containing cyclic peptide (RGD-4C), linked to a proapoptotic peptide dimer [D(KLAKLAK)2], decreased clinical arthritis and increased apoptosis of synovial blood vessels[26]. Recently, an unconjugated phage-encoded (CLDNQRPKC) peptide suppressed adjuvant-induced arthritis, attributed at least in part to peptide-mediated reduction of T-cell trafficking and the inhibition of angiogenesis[27,28].

However, to our knowledge, there are no studies in the literature demonstrating specific targeting to human arthritic tissue. We have previously identified a synovial endothelium targeting peptide (SyETP) that targets endothelial cells within vessels of human inflamed synovial tissue grafted into SCID mice[29]. Here we present evidence that fusion proteins consisting of the SyETP (CKSTHDRLC) linked to the anti-inflammatory cytokine IL-4 led to specific accumulation of the cytokine in synovial tissue transplanted into SCID mice and demonstrated local delivery by the increased STAT-6 phosphorylation detected within synovial but not to skin grafts. Thus this study provides proof of concept that homing peptides are a viable means of targeting therapeutics to the MVE of human synovial tissue, opening up a new avenue for translating these findings into novel treatment strategies for patients with inflammatory arthritis.

Results

Design, expression and characterization of peptide-cytokine fusion proteins

Fusion proteins were designed to allow genetic fusion of SyETP (CKSTHDRLC[29]) to hIL-4. The first fusion protein was constructed by adding a single copy of SyETP to the IL-4 C terminal (IL-4 single peptide: IL-4SP) [Fig. 1a (i)]. To increase avidity of binding, a second fusion protein was constructed by adding three copies of the synovial homing peptide (IL-4 triple peptide: IL-4TP) with the SyETPs separated from each other by a rigid spacer peptide[30] [Fig. 1a (ii)]. A 6xHis tag was added to each fusion protein to enable purification by affinity chromatography (*Supplementary Methods*). In addition, as the inflammatory microenvironment is enriched in matrix metalloproteinases [31], an MMP-cleavable sequence[32] was inserted between the hIL-4 and synovial homing peptides, with the aim of enabling release of the hIL-4 from the homing peptide at the disease site. This is expected to perform two functions: firstly, enable the hIL-4 to diffuse and interact with target cells physically separate from those cells recognizing the SyETP, and secondly, remove any inhibition of hIL-4 activity which might be conferred by attachment of the SyETP. In addition, as a short peptide, we would expect the proteolytically released SyETP to be degraded rapidly, thus avoiding local saturation of the SyETP binding sites and potentially enabling increased accumulation of the therapeutic payload. A control construct (IL-4 triple scrambled peptide: IL-4TS), containing 3 copies of a scrambled peptide (CRKLHTSDC; Fig. 1a) was also generated [Fig.1a (iii)].

Fusion proteins were expressed in insect cells using the baculovirus system and purified by immobilized metal ion affinity chromatography (*Supplementary Methods*). Incubation with MMP1 *in vitro* confirmed the susceptibility of the proteins to cleavage, shown by reduction in

molecular weight (anti-IL-4 antibody) and loss of reactivity with anti-His antibody, which is expected upon loss of the C-terminal peptide containing the 6xHis tag [Fig. 1b]. To confirm the IL-4 in the fusion proteins was bioactive, we employed the TF-1 human erythroleukemia cell line which proliferates in response to IL-4. Bioactivity of the IL-4 fusion proteins was confirmed by measuring intracellular ATP accumulation as a surrogate marker for TF-1 cell proliferation (*Supplementary Methods*). IL-4TP and the scrambled control (IL-4TS) stimulated TF1 cell proliferation to the same degree [Fig 1c]. Upon digestion with MMP1 IL-4 bioactivity was increased in both proteins. Importantly, IL-4 bioactivities of IL-4TP and IL-4TS did not differ significantly from each other (EC50s within 2-fold) pre- and post-cleavage with MMP1 [Fig.1c].

IL-4 synergises with IL-1β to enhance IL-1ra production from synoviocytes *in vitro*.

To assess whether SyETP-linked IL-4 retained the capacity of activating anti-inflammatory pathways, primary RA- and OA-SF were stimulated with rIL-4 in the presence of the pro-inflammatory cytokine IL-1β (*Supplementary Methods*). Figure 2 shows that there was no constitutive expression of IL-1ra from resting synoviocytes isolated from patients with both RA and OA. Upon stimulation with IL-1β (10ng/ml), the concentration of IL-1ra in the culture supernatant was measured at 300pg/ml. Such IL-1ra induction was enhanced in a dose dependent fashion with increasing concentrations of rhIL-4 (5-50ng/ml) ranging from 1000-1500pg/ml. Importantly, rhIL-4 alone did not induce IL-1ra production. The synergistic effect of IL-4 and IL-1β on IL-1ra production was also observed for all three fusion proteins, albeit to a different degree. Importantly, however, there were no significant differences in the capability of the three fusion proteins to enhance IL-1ra production. In all cases, pre-incubation with IL-4 induced a highly significant ($p<0.001$) increase in the production of IL-1ra over IL-1β alone

(Fig. 2). This indicates that SyETP-linked IL-4 retained the capacity of stimulating the production of an anti-inflammatory cytokine in the presence of IL-1β.

The synovial specific peptide (CKSTHDRLC) retains targeting capability of fusion proteins to synovial grafts *in vivo*.

Having confirmed *in vitro* that the IL-4 in the constructs was bioactive and could activate anti-inflammatory pathways, we next wanted to confirm that the SyETP in the fusion proteins had retained the synovial targeting capability *in vivo*. To assess this we radiolabeled the fusion proteins with ^{125}I and administered them intravenously into grafted SCID mice (Fig 3 a,b). Prior to that experiment we confirmed that radiolabeling did not affect the integrity of the fusion proteins (*Supplementary Figure 1*). *In vivo* imaging by NanoSPECT-CT allowed quantification of the level of radioactivity in the grafts per mm^3 of tissue. To allow direct comparison of the results from individual mice and to normalise for the levels of radioactivity administered, the data are expressed as a ratio of level of radioactivity retained by synovium over that in skin control per mm^3 of tissue at each time point. Figure 3a shows a two-fold increase in the level of activity in the synovium versus the skin for IL-4SP during the first 30 minutes post-injection. Importantly, by exploiting the increased avidity of the triple peptide (IL-4-TP) the higher activity could be extended to 180 minutes, peaking between 90-120 minutes post-injection. In contrast, the scrambled IL-4-TS showed no enhanced retention by the synovial graft and dissipated from both synovial and skin xenografts at the same rate. These data confirm that SyETP (CKSTHDRLC) leads to preferential accumulation of fusion proteins in synovial compared to control skin grafts.

Systemic administration of SyETP-, but not scrambled-peptide-linked-IL-4, results in functional bioactivity in synovial but not skin grafts *in vivo*.

In order to assess whether the IL-4 linked to the SyETP and delivered to synovial grafts maintains functional bioactivity *in vivo*, we measured STAT6 phosphorylation (pSTAT6) in the targeted synovial and skin control grafts. STAT6 is a Th2-associated transcription factor that is activated by IL-4 through phosphorylation in the cytoplasm, which is followed by translocation to the nucleus. Thus, we measured the level of STAT6 phosphorylation in the cytoplasmic and nuclear fractions of the grafts by Western blot following intravenous (i.v.) administration of SyETP-fusion proteins. As a positive control, rIL-4 was injected intra-graft (i.g.). Prior to the *in vivo* experiments, we confirmed the ability of all three fusion proteins to induce STAT6 phosphorylation by stimulating fragments of synovial tissue (organ culture) *in vitro*. Without stimulation, no phosphorylated STAT6 could be detected in the synovial tissue in either the cytoplasmic or the nuclear fractions (data not shown). However, following stimulation with both the fusion proteins and rIL-4, phosphorylated STAT6 was clearly detected. The level of phosphorylation was similar with each of the proteins [Fig.4a]. Having confirmed the ability of the fusion proteins to induce STAT6 phosphorylation in synovial tissue, we then assessed to what degree functional bioactivity was maintained *in vivo*. As seen in Figure 4b, rIL-4 administered intra-graft (i.g.) induced hyperphosphorylation of STAT6 in both the synovial and skin grafts, with an average increase of 13 and 17 fold over the PBS control. These data confirm that both synovial and skin grafted human tissues could respond well to rIL-4. Conversely, and of critical importance, upon intravenous (i.v.) administration of fusion proteins, only low levels of pSTAT6 were detected in the cytoplasmic fractions of the skin grafts. In contrast, IL-4SP and IL-4TP induced an average of 8 and 11 fold increases respectively in the synovial grafts, approximately 5 fold greater than the scrambled control peptide ($p=0.004$ and $p=0.001$),

emphasizing the crucial role of the SyETP sequence in delivery of bioactive molecules to synovial tissue [Fig.4 b,c]. The same pattern was observed in the nuclear fractions (data not shown).

Discussion

In this study we have demonstrated that recombinant fusion proteins containing one or three copies of a SyETP can target and retain IL-4 in synovial tissue grafts and that the length of time the fusion proteins are retained increases with increased copies of the peptide. In addition, we have shown that the conjugated rIL-4 is delivered in a bioactive form *in vivo*. These experiments were preceded by *in vitro* confirmation that IL-4 in the fusion proteins was bioactive and the MMP cleavage site accessible and susceptible to cleavage with MMP1. *In vitro* the fusion proteins were shown to have approximately equal IL-4 bioactivity both pre- and post-cleavage with MMP1. We were also able to demonstrate the capacity of these constructs to exert anti-inflammatory activity by measuring the induction of an anti-inflammatory mediator: IL-1ra.

It is well know that in RA, pro- and anti- inflammatory cytokine networks are operational both at local and systemic levels and that IL-1β and TNF are pivotal for the development of chronicity and tissue damage[33,34]. The physiological activity of IL-1 is controlled by its naturally occurring inhibitor, IL-1ra which binds to IL-1 receptors without activating the target cell. It is thought that dysregulation of IL-1ra may be responsible for the predominance of IL-1 in RA[35]. The main source of IL-1ra in the joint are synovial macrophages, although RA synovial fibroblasts (RASF) also produce it. Both cell types are known to contribute significantly to the perpetuation of disease[36,37]. Notably, IL-4 has been shown to reduce IL-1β production by increasing IL-1Ra in these cells[38,39]. In our study we have shown that IL-4 augments secretion of IL-1ra by RASF in the presence of IL-1β *in vitro* both alone and when conjugated to SyETP thereby validating the capacity of our constructs to deliver powerful anti-inflammatory signals.

Importantly, these *in vitro* experiments provided the evidence to support the rationale for testing the therapeutic properties of the constructs *in vivo*, on the premise that, if SyETP conjugation delivers IL-4 preferentially to synovial tissue, this would increase the therapeutic index of the cytokine as has been shown for TNF when conjugated to the tumour neovasculature by the homing peptide NGR in a pre-clinical model[25]. Clinical use of TNF has been limited due to systemic toxicity [40], however NGR-humanTNF is now entering Phase III clinical trials as a systemic agent [http://clinicaltrials.gov, trial identifier NCT01098266]. In the case of inflammatory disease, targeting has proved effective in animal models such as collagen-induced arthritis, where the effects of IL-10 on paw swelling were significantly enhanced when coupled to an antibody designed to target inflamed joints[41, 42]. However to our knowledge few studies have explored the concept of targeted therapy in human RA.

Our fusion proteins have half lives in the range of 30-60 minutes (*Supplementary Methods and data not shown*), comparable to that of free IL- 4 (12-19 mins[43, 44]). Targeting extends retention specifically in the target tissue and we reason that non-retained protein would be quickly cleared from the circulation, reducing the potential for unwanted systemic effects.

Previously, it has been problematic to isolate targeting peptides that bind their ligands with high affinity. The results of this study confirm that it is possible to use multiples of cyclic peptides to enhance binding avidity and increase the period of retention in the microvasculature of synovial tissue. Identifying the molecule that binds to the target peptide will be important in order to further develop other targeting moieties and also to improve our understanding at the molecular level of what makes the synovial microvasculature different from that at other tissues or organs.

Of extreme relevance and importance is the confirmation in this paper of the capacity of the SyETP constructs to deliver biologically active IL-4 *in vivo* in the target tissue, demonstrated by the enhanced phosphorylation of STAT6 in nuclear and cytoplasmic fractions of synovial graft tissue homogenate, providing strong functional evidence in support of the selective effectiveness of this therapeutic modality. Physiologically, IL-4 acts rapidly and primarily through phosphorylation of STAT6 via binding to IL-4 receptors type I or II. Both receptors are expressed on a wide range of cell types including macrophages, RASF and B cells which constitute a large percentage of the cells in RA synovial tissue. Our data, therefore, demonstrate that not only are the peptides retaining IL-4 in the synovial tissue, but also that such retention results in enhanced local activation of IL-4 signaling pathways *in vivo*. Importantly, we demonstrated that the preferential activation in synovial grafts was not due to "hypo-responsiveness" of skin grafts, as rIL-4 injected intra-graft induced STAT6 phosphorylation in both skin and synovial grafts, with an average increase slightly higher in skin grafts: 17 versus 13 fold over PBS control respectively.

In summary, we have shown that conjugation of multiple copies of a SyETP improves retention of IL-4 in transplanted, vascularised arthritic tissue and maintains bioactivity *in vivo*. RA is a heterogenous disease and a diverse array of therapies may be required for effective treatment of this patient population. As demonstrated here with the anti-inflammatory cytokine IL-4, targeting methodologies may help to improve the therapeutic window for treatment of RA when applied to currently used therapeutics as well as yet un-trialed novel conventional and/or biologic agents. Moreover, this work further confirms the unique role of the synovium/SCID chimera model for drug development in the context of target validation in human tissues prior to early

phase studies in patients. The opportunity to test mechanistic functionality in this model provides a solution to expensive and ethically problematic trials in humans.

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Author contributions:

SW: produced IL-4-fusion proteins, confirmed their ability to activate anti-inflammatory pathways *in vitro*, designed and performed the *in vivo* studies and wrote majority of the paper. DD: built the fusion protein DNA constructs, designed the control protein, produced the fusion proteins, validated their bioactivity *in vitro* and contributed to the writing of the paper. TT: performed one *in vivo* experiment and the Western blot analysis for STAT6 and Phospho-STAT6. CF and SM: provided technology, technical support and expertise for the *in vivo* imaging studies. RJ: Provided technical assistance in grafting human tissue onto SCID mice. YKSM: Provided technical advice on the baculovirus system. MB and AN contributed to discussions. YC and CP supervised all aspects of the study. SW and DD contributed equally to this work.

Figure legends

Figure 1: Biochemical characterization of the baculovirus expressed fusion proteins

(a) Schematic representation of the three fusion proteins consisting of IL-4 linked via an MMP cleavage site to (i) one (IL-4SP), (ii) three (IL-4TP) copies of a synovial endothelial targeting peptide (SyETP) or (iii) three copies of a scrambled peptide (IL-4-TS). Multiple copies of SyETP or scrambled peptide were connected by helical linkers. Histidine tags were added to each fusion protein for the purpose of purification; (b) To confirm the MMP cleavage site was accessible and susceptible to cleavage, IL-4SP, IL-4TP and IL-4TS were incubated overnight with or without rMMP1 and immunoblotted for (i) IL-4 and (ii) anti-4xhis using appropriate antibodies. (c) IL-4 bioactivity pre- and post-incubation with MMP1 was determined by assessing the proliferative response of the IL-4 responsive cell line (TF-1) to increasing concentrations of IL-4TP and IL-4TS. Data represent mean and standard deviation of triplicate wells and are given in arbitrary luminescence units (RLU). The data is representative of two independent experiments.

Figure 2 Synergistic effect of IL-lβ-and IL-4 on IL-lra secretion on human synoviocytes.

Synoviocytes were incubated for 60 min with or without rhIL-4 or IL-4-fusion proteins: IL-4TP, IL-4TS or IL-4SP (as described in Fig 1a) before adding 10ng/ml IL-lβ and incubated for a further 72 h. The concentration of IL-1ra in culture supernatant was detected by ELISA. Data represent the mean +/- SE obtained with synoviocytes from three patients. The data is representative of three independent experiments.

Figure 3: Preferential accumulation of SyETP-IL-4 constructs in synovial but not in skin xenografts.

SCID mice (seven per group) were grafted with both human synovium and skin on either side of the animal subcutaneously in a dorsal position distal to the shoulder joints. A period of ten days was allowed for the grafts to vascularise. Mice were then injected with iodinated IL-4 fusion proteins and imaged by nanoSPECT-CT for up to six hours. (a) The levels of radioactivity per mm^3 of tissue in the two grafts were determined by Region of Interest analysis of the images and the ratio between the uptake in synovium and skin transplants calculated. The ratios are shown for IL-4-TP, IL-4-TS and IL-4-SP fusion proteins as described in Fig 1a. (b) Representative image highlighting the graft on the back of the mouse. Data represent mean.± SE.

Figure 4: Synovial endothelial targeting peptide (SyETP)-linked IL-4 maintains functional bioactivity when delivered to synovial grafts in vivo

SCID mice (2 per group) were grafted with two fragments (3-5 mm^3) of human synovium (n=4) and two fragments (3-5 mm^3) of human skin (n=4) subcutaneously. The mice were left for ten days to allow for the grafts to establish and vascularise. The mice were then injected i.v. with IL-4SP, IL-4TP, IL-4TS (as described in Fig 1a) or PBS. An additional control group was represented by mice injected intra-graft (i.g.) with rIL-4. The mice were sacrificed 45 minutes post-injection, the grafts harvested and the level of pSTAT6 in the nuclear and cytoplasmic fractions of the grafts determined by Western blot. (a) Immunoblotting for pSTAT6 or total STAT6 in the cytoplasmic fractions of synovial tissue stimulated *in vitro* by IL-4SP, IL-4TP, IL-4TS (as described in Fig 1a) or rIL-4. (b) Immunoblotting for pSTAT6 or total STAT6 in the cytoplasmic fractions of the grafts. (c) Fold increase in the ratio of pSTAT6: total STAT6 over the PBS

control. Data represent mean ±SE and are representative of two independent experiments. Statistics are shown for IL-4SP vs IL-TS vs IL-4TP.

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Materials and Methods

Cells & Reagents

Details in Supplementary Methods.

Human tissue transplantation into SCID animals

Human synovial tissue was obtained from patients undergoing joint replacement surgery. Control human skin was obtained from plastic surgery procedure. Informed consent was obtained prior to the use of these tissues: ethical approval was obtained from the local ethics committee. Five-week old Beige SCID CB-17 mice (Charles River City, country) were maintained under sterile conditions in individually ventilated cages. All procedures were carried out in a sterile environment. Synovium and skin were transplanted as previously described [29, 45]

Construction of fusion proteins

Human IL-4 (hIL-4) cDNA was amplified by PCR (from a plasmid kindly provided by DNAX Corp, USA), using the primers CCCAAGCTTATGGGTCTCACCTCCCAACTGC and ATCTTTTCAGGAATTCGCTCGAACACTTTGAATATTTCTCTC to add HindIII and EcoRI sites to the 5' and 3' ends respectively. After digestion with HindIII and partial digestion with EcoRI due to an endogenous EcoRI site, DNA of the appropriate size was purified by gel extraction and inserted into a pcDNA3 vector encoding an MMP-cleavable site[25] flanked by EcoRI and NotI restriction sites. Novel DNA sequences encoding one or three copies of a synovial homing peptide [22] and a C-terminal His-tag followed by an ApaI restriction site were synthesised by oligonucleotide annealing (*Supplementary Methods*) and inserted 3' of the MMP-cleavable sequence. Full-length IL-4-SP (single peptide), IL4-TP (triple peptide) and IL-4-TS

(triple scrambled) cassettes flanked by HindIII and ApaI sites were then inserted into a pFASTBAC1 (Invitrogen) vector that had been modified by removal of the multiple cloning site (MCS) at the BamHI and HindIII sites and replacement with annealed oligonucleotides GATCCAAGGTACCACCGCCAAAGCTTACTAAGTTGGGCCCG (forward) and AGCTCGGGCCCAACTTAGTAAGCTTTGGCGGTGGTACCTTG (reverse). Constructs were verified by DNA sequencing.

Targeting-specificity of recombinant fusion proteins – imaging by NanoSPECT-CT

All three fusion proteins were labelled with ^{125}I (*Supplementary Methods*). Mice were injected i.v. with 100µl of the iodinated construct (100µg/ml) with a starting activity of approximately 10MBq. At 0, 40, 90, 180 and 300 mins post-injection the mice were imaged using a NanoSPECT-CT animal scanner (Bioscan Inc.Washington DC, USA) as previously described [46]. The animals were kept warm and were anaesthetised using 2% isoflurane for the duration of the scan. Helical SPECT images of the transplants were acquired in 20 projections over 30 minutes using the 4-headed camera with 4 x 9 (1.4mm) pinhole collimators. CT images were acquired in 180 projections and 1000ms exposure time using a 45kVP X-ray source over 3 minutes. Radionuclide images were reconstructed using HiSPECT (Scivis GmbH) iterative reconstruction software and fused with CT images using proprietary InVivoScope (Bioscan) software. A three dimensional volume-of-interest was defined around each graft to calculate the volume and level of activity (MBq) within. Uptake was expressed as activity (MBq) per mm^3 tissue. Equipment and methodology used have been previously validated in this model [46].

Detection of pSTAT6 in human tissue

Detection of phosphorylated-STAT6 in human tissue. Five-week-old Beige SCID CB-17 mice (Charles River laboratories), 2 per group, were grafted with two pieces of human synovial tissue and two pieces of human skin as previously described[29, 45]. Once the grafts had established, 100µl of each of the fusion proteins (70µg/ml) or 100µl PBS were administered i.v. As a positive control, an additional group of mice was injected intra-graft with 50µl of rIL-4 (100ng/ml). The proteins were allowed to circulate for 45 minutes. The mice were then sacrificed, the grafts removed and the nuclear and cytoplasmic fractions were extracted using a NE-PER nuclear and cytoplasmic extraction reagents (ThermoScientific, Southend-on Sea, UK). The proteins were quantified by BCA assay (Pierce, Cramlington, UK). Whole cell lysates were normalized for their protein content, resolved by SDS-PAGE, and then transferred to nitrocellulose filters and immunoblotted with the indicated antibody: total STAT6; (s-20) and pSTAT6 (Tyr641), both at 1µg/ml (Santa Cruz (Insight Biotechnology, Wembly UK). The gels were scanned and band intensity calculated using TotalLap v1. 10. The pSTAT6/STAT6 ratios were derived from the band intensities and then divided by the PBS value to obtain the fold increase over the PBS control. The above method of detecting total STAT6 and pSTAT6 was also applied to synovial tissue samples stimulated *in vitro*.

Statistical analysis

All statistical analysis was performed using SPSS Statistics 17.0. One-way analysis of variance (ANOVA) was used when comparing more than two groups (non-parametric data underwent normal transformation). If the groups differed significantly from each other, a Tukey post-test was applied to determine where those differences lie. P values of less than 0.05 were considered significant.

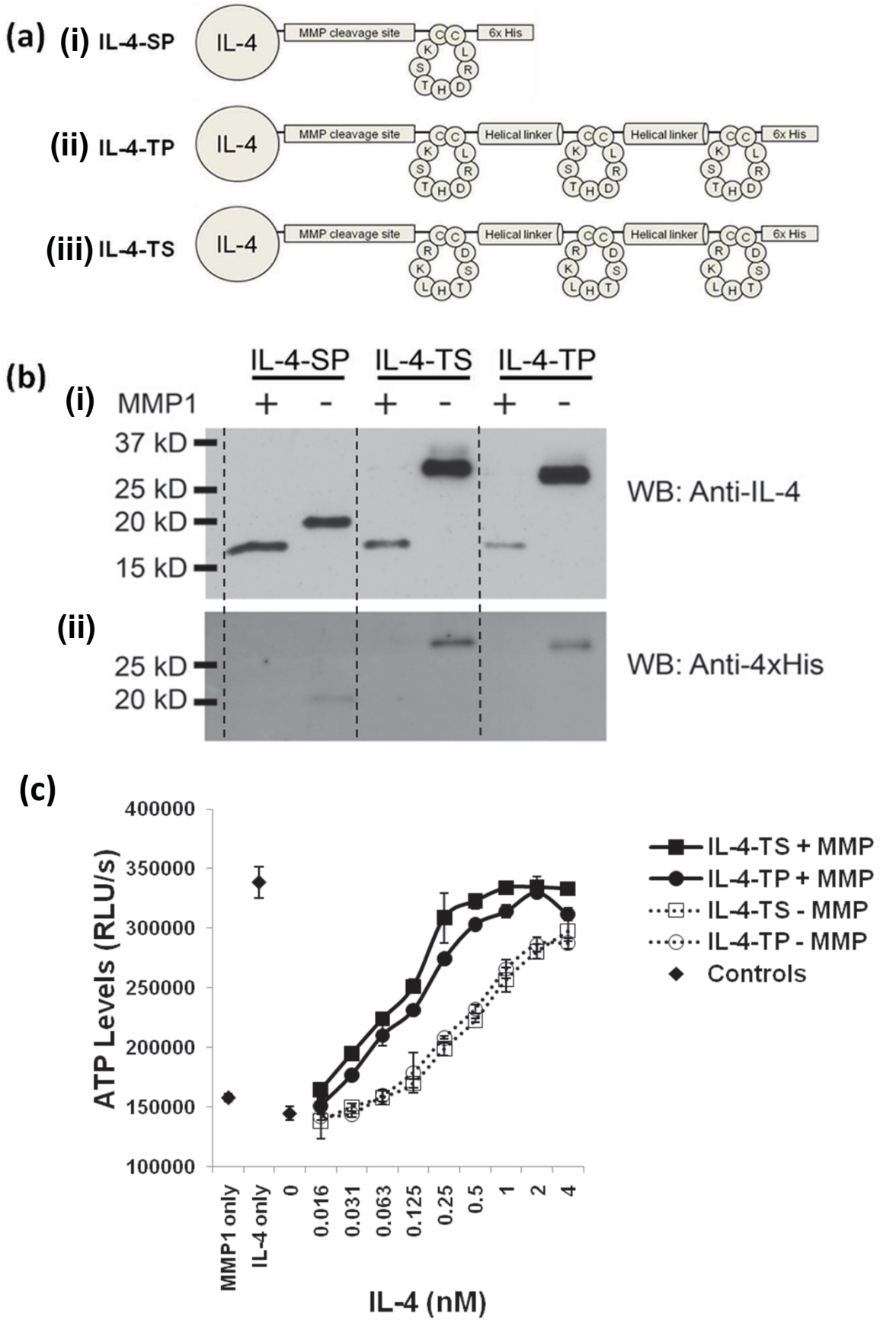


Figure-1 (Wythe)

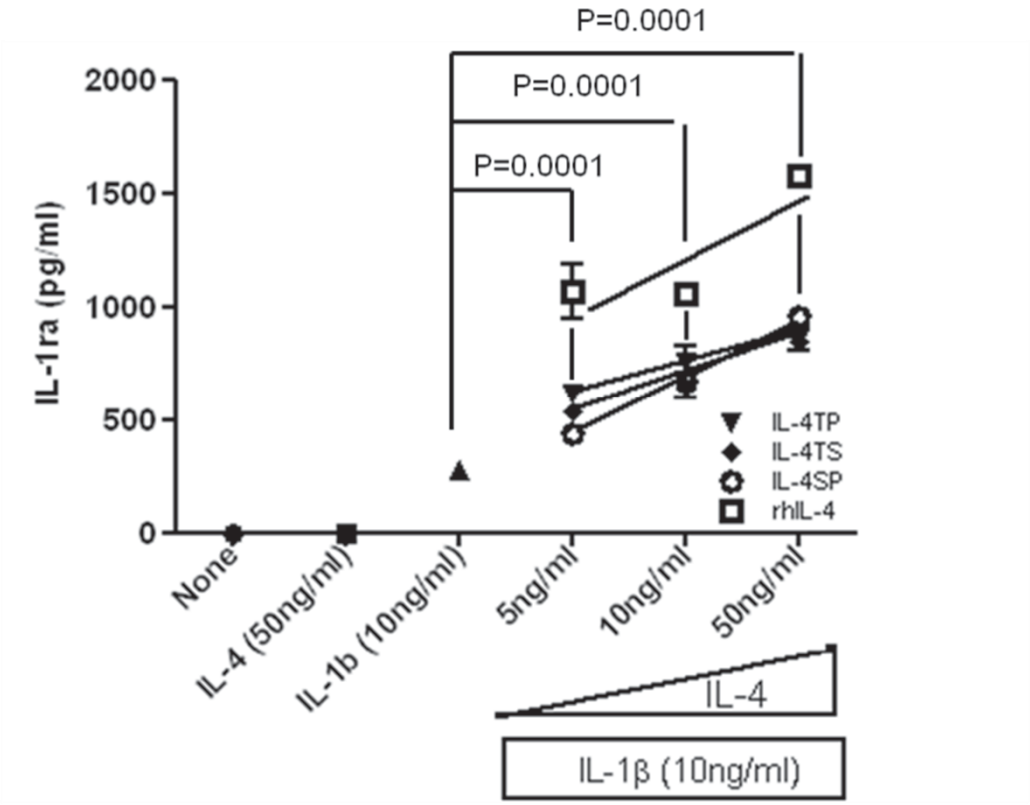
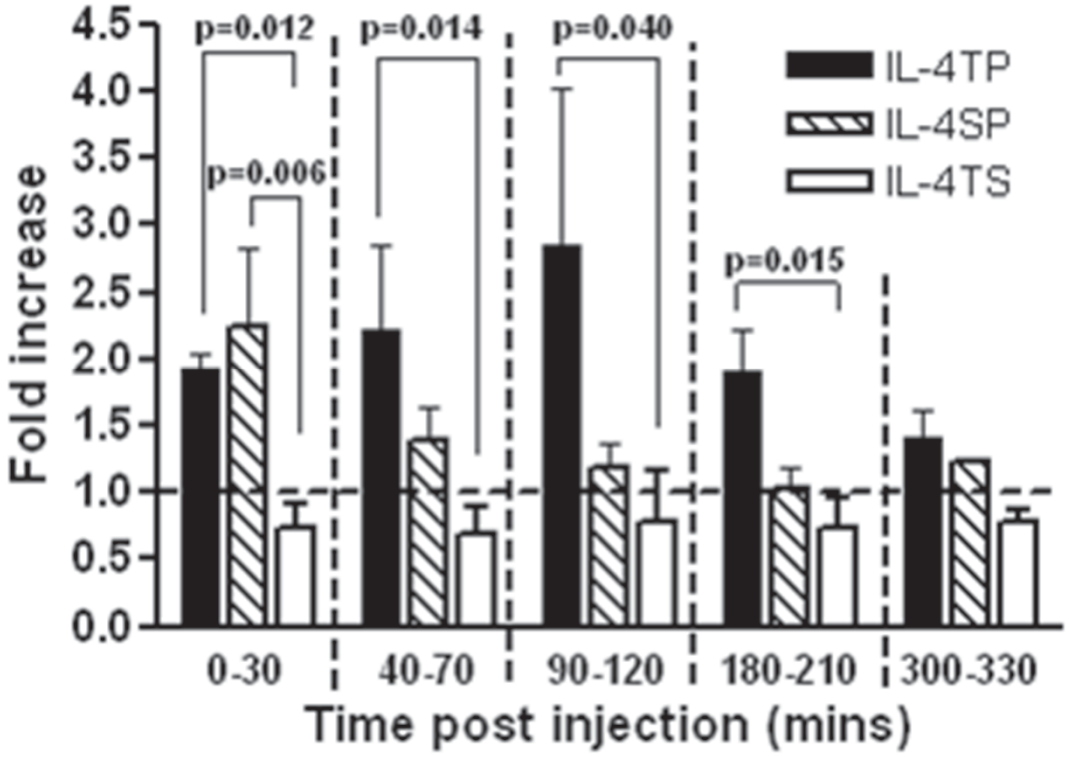


Figure-2 (Wythe)

(a)



(b)

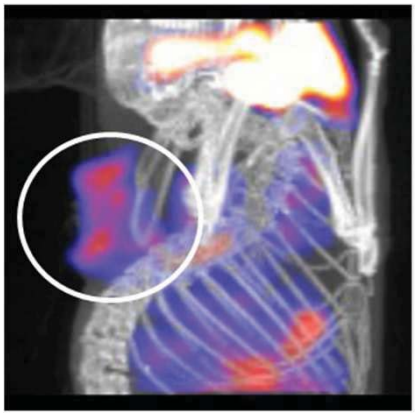


Figure-3 (Wythe)

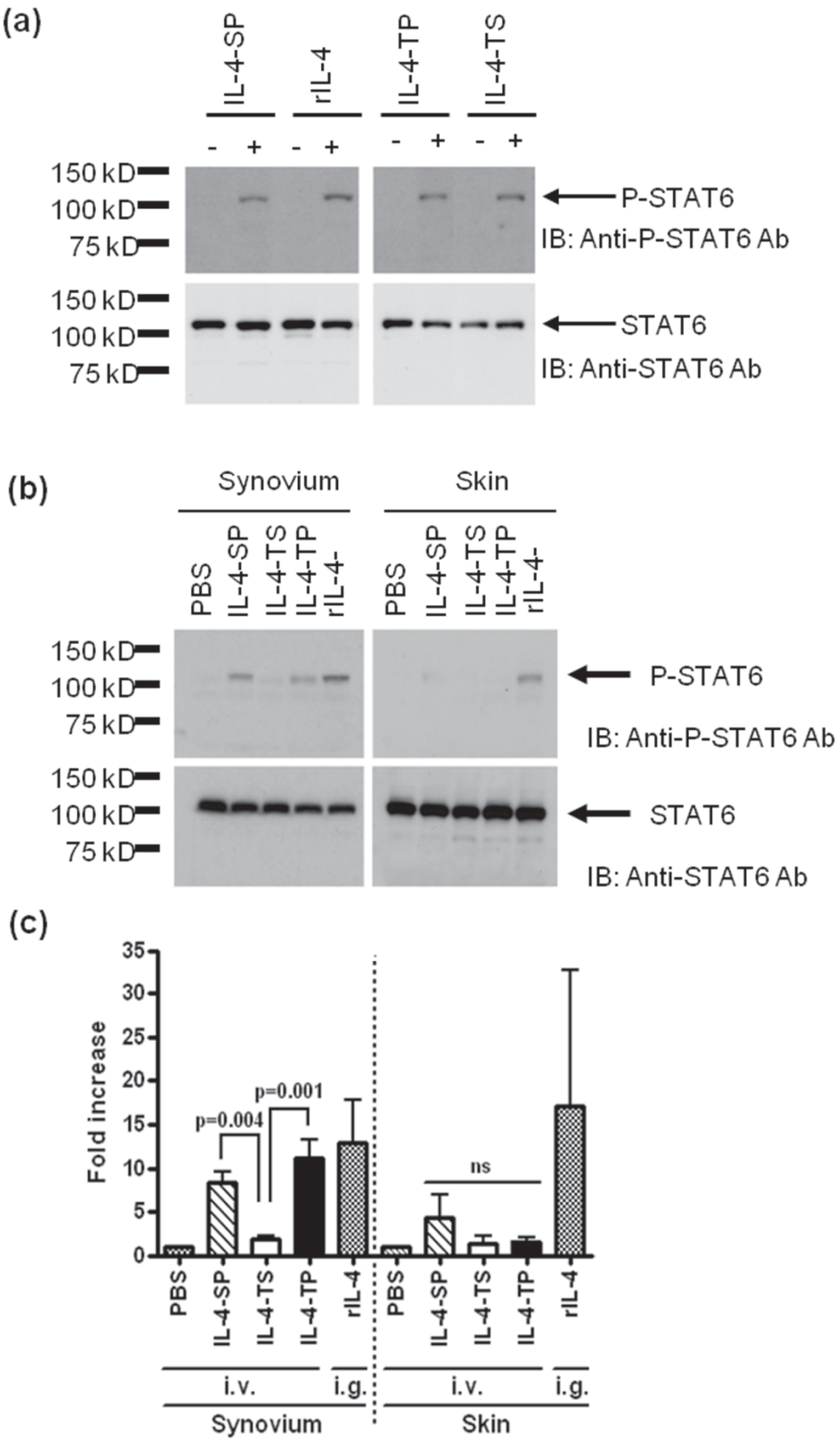


Figure-4 (Wythe)