

Materials and methods

1 Materials

1.1 Equipment

Pipette	Eppendorf-pipette 0.5-10 µl, 10-100 µl, 50-200 µl, 50-250 µl, 100-1000 µl, 1-10 ml (Eppendorf, Hamburg)
Sodium heparin tube	Becton Dickinson (Plymouth, UK)
96-well cell culture plate	NUNC (Denmark)
24-well cell culture plate	Costar (USA)
96-well immunoassay plate	Costar (Cambridge, MA)
Pipetting device	Pipetus akku (ICN Flow, Meckenheim)
Electropower supply	Typ BCN58-200L (Berlin Germany)
Centrifuges	Megafuge 1.0 R (Heraeus Sepatech) Biofuge pico, Heraeus (Osterode, Germany) Ttettich Rolanta (Osterode Germany) Eppendorf-Zentrifuge Model 3200, 5414 (Eppendorf, Hamburg)
CO ₂ -incubator	BB16, heraeus (Osterode, Germany)
Microscope	Diavert, Leitz (Wetzlar, Germany) BX60F5, Olympus (Berlin, Germany)
Precision balances	Type 2662, Sartorius (Göttingen, Germany) Ohaus (CT600-S)
Digital balance	Basic (Satorius, München)
Magnetic stirrer	MR 3001, Heidolph (Kehlheim, Germany)
Mixer	Thermomixer comfort, Eppendorf (Hamburg, Germany) Vortex IKA VF2, Jahnke und Kunkel (Staufen I. Br.D) Vortex Genie 2 TM (Bender & Hobein, Zürich, Schweiz)

Waterbath	Haake GH/D8 (Bender & Hobein, Zürich, Schweiz)
Spectrophotometer	GeneQuant II (Parnacia, Freiburg)
Hood	Biological Safely Cabinets (Nu-425-600)
Magnetic cell separators	MiniMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany)
Columns	MS ⁺ /RS ⁺ plus adaptor (Miltenyi Biotec, Bergisch-Gladbach, Germany)
FACSCalibur	Becton Dickinson (San Jose, CA)
Power Macintosh G3	(USA)
CellQuest™ software	Becton Dickinson (San Jose, CA)
ELISA reader	Dynatech MR5000 (Denkendorf, Germany)

1.2 General reagents

10 x PBS	GIBCO BRL (Eggenstein, Germany)
1 x PBS	Biochrom KG (Berlin, Germany)
Ficoll-paque™ Plus	Pharmacia (Uppsala, Sweden)
HBSS	GIBCO BRL (Eggenstein, Germany)
Trypan blue	Biochrom KG (Berlin, Germany)
Anti-CD28	Immunotech (Marseille, France)
SEB	Sigma (Munich, Germany)
PMA	Sigma (St. Louis, Mo)
Ionomycin	Sigma (St. Louis, Mo)
DMSO (Dimethyl Sulfoxide)	Serva Electrophoresis GmbH (Heidelberg, Germany) (used for cells freezing storage) Sigma Chemical CO. (Steinheim, Germany) (used for peptides solution)
Albumin Bovine Fraction V	Biomol Feinchemikalien (Hamburg)
Penicillin/Streptomycin	Biochrom KG (Berlin, Germany)
L-Glutamin	Biochrom KG (Berlin, Germany)
Fetal Calf Serum (FCS)	GIBCOBRL (Life Technologies, Paisley, Scotland)
RPM1 1640	GIBCOBRL (Life Technologies,

	Paisley, Scotland)
Infliximab	cA2 Remicade (Fa Essex/Centocor)
FACS flow	Becton Dickinson (Heidelberg, Germany)
FACSRinse	Becton Dickson (San Jose, CA)
FACSafe	Becton Dickson (San Jose, CA)
Cellwash	Becton Dickson (San Jose, CA)
Brefeldin A	Sigma (Munich, Germany)
EDTA	Merck KgaA (Darmstadt, Germany)
Ethanol	(BDH, Karlsruhe)
37% Formaldehyde Solution	J.T. Baker (Holland)
Sodium azid	Merck KgaA (Darmstadt, Germany)
FACS lysing solution	Becton Dickinson (San Jose, CA)
FACS Permebealizing Solution	Becton Dickinson (Heidelberg, Germany)
Beriglobin	Centeon Pharma (Berlin, Germany)
IFN γ Catch Reagen	Miltenyi Biotec (Bergisch Glodbach, Germany)
IFN γ Detection Antibody (PE)	Miltenyi Biotec (Bergisch Glodbach, Germany)
Anti-PE MicroBeads	Miltenyi Biotec (Bergisch Glodbach, Germany)
Propidium iodide (PI)	Becton Dickinson (Heidelberg, Germany)
CFDA-SE	Molecular Robes (Eugene, Oregon)
IL2	Boehringer Mannheim (Frankfurt, Germany)
ELISA kits	Pharmigen (Bergisch Gladback, Germany)

1.3 Special reagents and buffers

1). 10% Sodium Azid

10 g sodium azid

100 ml DH₂O

2). 2% Formaldehyde:

2.7ml 37% formaldehyde

47.3ml 1 x PBS

3). FACS buffer (PBS/BSA buffer)

0.5% BSA (2.5g/500ml Albumin Bovine Fraction V)

50 ml 10 x PBS

5 ml 10% Na azid

adding DH₂O → total volume 500ml

4). MACS buffer (sterile)

phosphate buffer saline pH 7.2
0.5% bovine serum albumin (BSA)
2 mM EDTA

5). 10% DMSO

DMSO was diluted 1:10 with FCS

6). Medium for cell culture (sterile)

A: for short term culture

RPMI 1640 containing 10% heat-inactivated fetal calf serum
2 mM L- Glutamine
Penicillin 100 units/ml
Streptomycin 100 µg/ml

B: for long term culture

RPMI 1640 containing 10% autologous serum
2 mM L- Glutamine
Penicillin 100 units/ml
Streptomycin 100 µg/ml

7). RCLS (Red cell lysing solution, sterile)

NH₄Cl 8.29g
KHCO₃ 1.0g
EDTA 0.372g
Adding DH₂O to volume 1 L

8). FACS flow

1000 ml 10 x PBS
9000 ml milliPore Water
20 ml 10% sodium azide (final concentration: 0.02%)

9). ELISA buffers

Coating buffer: 0.1 M Na₂HPO₄, pH>9.0
Blocking buffer: 10% inactivated fetal calf serum (FCS) dissolved in
 phosphate buffered saline (PBS)
Washing buffer: PBS with 0.1% Tween 20
Substrate buffer: 0.05M phosphate-citrate buffer with 0.03% sodium
 perborate capsules (Sigma, St.Louis, MO)
Stopping buffer: 2N HCl

1.4 Recombinant proteins as antigen:

Aggrecan G1 protein	(delivered by Y. Zhang from McGill University, Montreal, Canada.)
Glycoprotein 39	(kindly provided by M.Boots, Akzo Nobel, The Netherlands)
Collagen II	(kindly provided by Fibrogen, U.S.)
Human hsp60	(obtained from R. Lauster, DRFZ, Germany)
Yersenia 19KD	(obtained from R. Lauster, DRFZ, Germany)

1.5 Staining antibodies

Anti-CD3 PE	Becton Dickinson (San Jose, CA)
Anti-CD4 PerCP	Becton Dickinson (San Jose, CA)
Anti-CD4 Cy5	Pharmingen (San Jose, CA. Coupled in our lab.)
Anti-CD8 PerCP	Becton Dickinson (San Jose, CA)
Anti-CD8 PE	Becton Dickinson (San Jose, CA)
Anti-CD14 PE	Becton Dickinson (San Jose, CA)
Anti-CD19 FITC	Becton Dickinson (San Jose, CA)
Anti-CD69 PE	Becton Dickinson (San Jose, CA)
Anti-CD69 FITC	Becton Dickinson (San Jose, CA)
Anti-IFN γ	Pharmingen (San Jose, CA; Coupled to Cy5 in our Lab; 1:400 dilution)
Anti-IFN γ FITC	Pharmingen (San Jose, CA)
Anti-TNF α	Pharmingen (San Jose, CA; Coupled to FITC in our Lab. 1:800 dilution)
Anti-TNF α FITC	Pharmingen (San Jose, CA)
Anti-IL10 APC	Pharmingen (San Jose, CA)
Anti-IL4 PE	Pharmingen (San Jose, CA)
Anti-IL4 APC	Pharmingen (San Jose, CA)

2 Patients and donors

All patients were seen at the University Hospital Benjamin Franklin in Berlin, German. Aspiration of synovial fluid was performed for diagnostic or therapeutic reasons from knee joints, with approval from the Ethical Committee of the Benjamin Franklin

Hospital. AS patients fulfilled the modified 1984 New York criteria for a diagnosis of AS (108) and all RA patients the ACR-criteria for the diagnosis of RA (109).

Table 1. Characteristics of patients and controls

Diagnosis	Source	n	Age (years)	Disease duration
Ankylosing spondylitis*	PB	47	35.4 ± 8	6.7 ± 7
Ankylosing spondylitis	SF	7	43.3 ± 9	9.6 ± 1
Rheumatoid arthritis	PB	22	56.4 ± 5	6.2 ± 8
Rheumatoid arthritis	SF	4	51.2 ± 7	7.4 ± 9
Healthy control	PB	20	42.5 ± 7	

PB = peripheral blood *95% of patients were positive for HLA-B27.

SF = synovial fluid

The characteristics of the patients and donors are shown in Table 1. All patients were in an active state of disease which means that they had current inflammatory back pain, a joint effusion, an elevated CRP, with at least two of these three parameters being positive. However, the degree of activity varied and I did not attempt to quantify disease activity in more detail. Peripheral blood (PB) and synovial fluids (SF) of consecutive patients was collected.

For T cell response to candidate autoantigens G1, gp39 and collagen II, PB from 47 and SF from 7 AS-patients, PB from 22 and SF from 4 RA-patients and PB from 20 healthy controls (HC) were examined.

For analysis of T cell response to h-hsp60 and γ -19KD, 45 of 47 AS patients, with mean age(years) \pm SD: 35 \pm 7.5, were tested; the case number of RA and HC, that were detected, was the same as that for analysis of T cell response to G1.

3 Methods

3.1 Preparation of G1 protein

Molecular production of the G1 domain of aggrecan contains 19 exons ranging in size from 77 to 4224 base pairs. It was recombinantly product by the following method:

Human aggrecan G1 domain (AG1) proteins were expressed and purified in an adenovirus expression system. Briefly, a cDNA fragment encoding the N-terminal 431 amino acids of human aggrecan with a His-tag at its C-terminus was generated from a human chondrocyte RNA preparation by RT-PCR (5'-GCAGATCTACTA TGGCCACTTTACTCTGGGTTTTTCG -3 and 5'-CAGATCTCAATGGTGATGGTGAT GATGCTCAGCGAAGGCAGTGGC-3'). This PCR fragment was cloned into a pCR2.1 vector using a TA cloning kit (Invitrogen Inc., Carlsbad, CA). The construct, included human aggrecan G1 globular domain and a partial interglobular domain (IGD) plus 6 histidine residues at its C-terminal, was sub-cloned into pQBI-AdCMV5-IRES-GFP transfer vector from the ADENO-QUEST KIT (Quantum Biotechnologies Inc, Montreal, QC) at Bgl II site. After linearized by FseI restriction enzyme digestion, 1 mg of the recombinant transfer vector plasmid is co-transfected into 293 cells with 1mg of QBI-viral DNA from the same kit using Lipofectamine plus reagent (Lifetech Co. Burlington, ON). Screening and purification of recombinant adenovirus were performed according to Adeno-Quest application manual included in the kit. For recombinant AG1 production, 293 cells were split onto 150mm dishes in 1 to 10 dilution in DMEM plus 5% FCS, grown for two days till cells were ~90% confluent, then the media were changed into 293 serum-free media (Lifetech Co. Burlington, ON), meanwhile recombinant virus were added at 50 MOI. On day 3 after infection, the supernatant containing recombinant AG1 protein was collected. The supernatant was applied to a Sephadex G-25 column equilibrated with PBS, pH7.4: the protein-containing fractions were collected and applied to a Ni-NTA agarose column (Qiagen Inc., Mississauga, ON). The column was washed with 40 mM imidazole in PBS containing 0.3 M NaCl, pH 7.4. Recombinant VG1 and AG1 were eluted with 100 mM imidazole in PBS, pH7.4, containing 0.3 M NaCl.

3.2 Generation of recombinant h-hsp60 and Y-19KD

The complete open reading frames of the Yersinia 19kd was amplified by PCR. The **human hsp60** gene was amplified from a complementary DNA preparation derived from a patient's cartilage, using Pwo I polymerase (Boehringer Mannheim, Mannheim, Germany) and commercial oligonucleotides (Tib-MolBiol, Berlin, Germany). The amplified fragments were isolated from agarose gels with a Jet Scorb kit (Genome, Bad Oeynhausen, Germany), digested with appropriate

restriction enzymes, and ligated into 1 of the pQE vectors (Qiagen, Hilden, Germany).

In the construction of the pQE 90 vector, the polycloning site of pQE60 was modified such that Nde I and Nsi I restriction sites were introduced. The RO vectors contained the lac repressor gene cloned into the Xba I site of the pQE series. These plasmids are independent from cotransformed pREP 4 strains. A positive clone was selected after transformation of the electrocompetent E coli strain M15 (pREP 4). Fluorescence sequencing was performed on an Alf sequencer (Pharmacia LKB) by Replicon (Berlin, Germany). Expression of the cloned gene was induced by addition of 1 mM isopropyl- β -D-thiogalactopyranoside (sigma). Cells were harvested, and purification of the recombinant proteins with a His tag was performed on Ni-NTA resin affinity columns according to the manufacturer's protocol (Qiagen).

The procedure for preparation of Yersinia 19KD and h-hsp60 is briefly described as follows (110): cultured bacteria were disrupted in a French press in the presence of a proteinase inhibitor cocktail. High speed ultracentrifugation (150,000g) was applied for 10 hours in order to separate cytoplasmic proteins from a pellet containing membrane proteins as well as nucleo- and ribonucleo- proteins. This pellet was acid extracted and the solute dialyzed to neutrality. Further purification was performed on a fast protein liquid chromatography system (Pharmacia LKB, Freiburg, Germany) using a Mono-S cation exchange column with an appropriate salt gradient. The resulting peaks were then purified by reverse-phase high-performance liquid chromatography (HPLC) using a preparative C8 column (SuperPac Sephasil C8, 5 μ m, 4 x 250 mm; Pharmacia LKB), with an acetonitrile gradient (0-80%) in 0.1% trifluoroacetic acid in water. The resulting peaks were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dialyzed against RPMI 1640 medium.

SDS-PAGE (T=12.6%, C=2.7%) was performed without boiling under reducing and nonreducing conditions. The gels were stained with Coomassie blue R 250.

For western blotting, Immobilon polyvinylidene difluoride (PVDF) membranes (Milipore, Bedford, MA) with Yersinia 19kd and human hsp60 bands were incubated with patients' sera in a 1 hour with peroxidase-labeled goat anti-human IgG and anti-human IgA (Dianova, Hamburg, Germany) diluted 1:5000.

3.3 Preparation of Collagen II

Collagen II, also recombinantly produced, was kindly provided by Fibro Gen, San Francisco, U.S. It was expressed in yeast and lacks hydroxylysine and the glycosylated forms of hydroxylysine.

3.4 Preparation of cartilage Gp 39

HC gp39 was isolated from MG-63 osteosarcoma cell line (111). MG-63 cells (human osteosarcoma CRL 1427; ATCC) were cultured in cell factories in serum-free medium. HC gp39 was purified from the culture supernatant by affinity chromatography on heparin-Sepharose followed by gel filtration on Superdex 75 (Pharmacia, Roosendaal, The Netherlands). Purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and HPLC, the purity was >97%. N-terminal amino acid sequencing confirmed the identity of the protein.

3.5 G1 Peptides synthesis

Peptides were synthesized by a robotic multiple peptide synthesizer (SYRO, MultiSynTech, Bochum, Germany) using a Fmoc/tBu solid-phase synthesis strategy (112). Wang resin(p-benzyloxybenzylalcohol-polystyrene) (Novabiochem, Bad Soden, Germany) was used as solid support. Side chain protected Fmoc-amino acids were obtained from Senn Chemicals(Dielsdorf, Switzerland) and Novabiochem (Bad Soden, Germany). Peptides were characterized by reversed-phase HPLC (M480 pump, UVD-320 S diode-array UV-detector, GINA 160 autosampler, Gynkotech,

Table 2. Amino acid sequence of G1 protein

Position.	Amino acid sequence				
1-50	mtllwfvvt	lrvitaavtv	etsdhdnsls	vsipqpsplr	vllgtsltip
51-100	cyfidpmhvp	ttapstapla	prikwsrvsk	ekevllvat	egrvrnsay
101-150	qdkvslpnyp	aipsdatlev	qslrsndsgv	yrcevmhgje	dseatlevvv
151-200	kgivfhyrai	stryldfdr	aqraclqnsa	iiatpeqlqa	ayedgfhqcd
201-250	agwladqtr	ypihtpregc	ygdkdefpgv	rtygirdtne	tydvycaee
251-300	megevyats	pedftfqaee	necrrlgarl	attghvylaw	qagmdmcsag
301-309	wladrsvryp				

Germering/Munich, Germany) on Nucleosil C18, 100A, 5 μ m (Macherey-Nagel, Düren, Germany) and electrospray mass-spectrometry (ESI-Quattro II, Micromass Ltd., Altrincham, UK). 46 overlapping 18 mer peptides, which covered all 394 amino acid residues of G1 protein, were synthesized (table 2), and each peptide overlapped the next by 10 amino acid (table 3).

Table 3. Amino acid sequence for 46 overlapping 18 mer peptides

No.	Sequence
peptide 1	VETSD HDNSL SVSIP QPS
peptide 2	SLSVS IPQPS PLRVL LGT
peptide 3	PSPLR VLLGT SLTIP CYF
peptide 4	GTSLT IPCYF IDPMH PVT
peptide 5	YFIDP MHPVT TAPST APL
peptide 6	VTTAP STAPL APRIK WSR
peptide 7	PLAPR IKWSR VSKEK EVV
peptide 8	SRVSK EKEVV LLVAT EGR
peptide 9	VVLLV ATEGR VRVNS AYQ
peptide 10	GRVRV NSAYQ DKVSL PNY
peptide 11	YQDKV SLPNY PAIPS DAT
peptide 12	NYPAI PSDAT LEVQS LRS
peptide 13	ATLEV QSLRS NDSGV YRC
peptide 14	RSNDS GYVRC EVMHG IED
peptide 15	RCEVM HGIED SEATL EVV
peptide 16	EDSEA TLEVV VKGIV FHY
peptide 17	VVVKG IVFHY RAIST RYT
peptide 18	HYRAI STRYT LDFDR AQR
peptide 19	YTLDF DRAQR ACLQN SAI
peptide 20	QRACL QNSAI IATPE QLQ
peptide 21	AIIAT PEQLQ AAYED GFH
peptide 22	LQAAY EDGFH QCDAG WLA
peptide 23	FHQCD AGWLA DQTVR YPI

peptide 24	LADQT VRYPI HTPRE GCY
Table 3. continued.	
peptide 25	PIHTP REGCY GDKDE FPG
peptide 26	CYGDK DEFPG VRTYG IRD
peptide 27	PGVRT YGIRD TNETY DVY
peptide 28	RDTNE TYDVY CFAEE MEG
peptide 29	VYCFA EEMEG EVFYA TSP
peptide 30	EGEVF YATSP EKFTF QEA
peptide 31	SPEKF TFQEA ANECR RLG
peptide 32	EAANE CRRLG ARLAT TGH
peptide 33	LGARL ATTGH VYLAW QAG
peptide 34	GHVYL AWQAG MDMCS AGW
peptide 35	AGMDM CSAGW LADRS VRY
peptide 36	GWLAD RSVRY PISKA RPN
peptide 37	RYPIS KARNP CGGNL LGV
peptide 38	PNCGG NLLGV RTVYV HAN
peptide 39	GVRTV YVHAN QTGYP DPS
peptide 40	ANQTG YPDPS SRYDA ICY
peptide 41	PSSRY DAICY TGEDF VDI
peptide 42	CYTGE DFVDI PENFF GVG
peptide 43	DIPEN FFGVG GEEDI TVQ
peptide 44	VGEE DITVQ TVTWP DME
peptide 45	VQTVT WPDME LPLPR NIT
peptide 46	WPDME LPLPR NITEG EAR

3.6 Preparation of peptides solution

Peptides was first dissolved in Dimethyl Sulfoxide (DMSO), then diluted with DH₂O. The quantity of DMSO have to be controlled to a final dilution of 1/ 500-1000 if a peptide solution is used to stimulate the cells because high concentrations could lead to cell damage. Usually, 50-150 µl DMSO was added into the tube containing the peptide, mixed well until the peptide was completely dissolved, then an appropriate volume of DH₂O was added to a total volume of 2 ml and stored at -20°C.

3.7 Preparation of peripheral blood mononuclear (PBMC) and synovial fluid mononuclear (SFMC)

Peripheral blood mononuclear cells, consisting of lymphocytes and monocytes, were isolated from heparinized fresh blood samples from patients. Briefly, the samples were diluted 1:2 with HBSS, 35 ml of diluted blood were carefully layered over 15 ml Ficoll Paque (1.077 density) in a 50 ml conical tube and centrifuged at 2500rpm for 20 min at 20⁰C (without brake). The upper layer, which contains diluted 25-50% autologous serum, was aspirated and the mononuclear cell layer was left undisturbed at the interphase. The interface cells (lymphocytes, monocytes and thrombocytes) were carefully transferred to a 50 ml conical tube and the conical tube was filled with PBS and mixed well. The supernatant was carefully removed completely after the sample was centrifuged at 1500 rpm for 10 minutes at 4⁰C. The cell pellet was resuspended in 50 ml of HBSS and centrifuged at 1200 rpm (at such a slower speed to get rid of the platelets) for 10 minutes at 4⁰C. Carefully, the supernatant was completely removed and the cell pellet was resuspended in an appropriate volume of PBS containing 2 mM EDTA. Subsequently the cells were counted. The PBMC were sometimes stored in a refrigerator overnight in PBS containing 2 mM EDTA supplemented with 10% autologous serum after the last washing step. In most case, cells resuspended in RPMI 1640 medium (with FCS, Glutamin, Penicilin and Streptomycin) and stimulated with antigen immediately. At this point, sample can also be frozen in PBS with 1% BSA and 10% DMSO.

For SFMC, the SF was firstly filtered against 70 µm filter to get rid of tissue pellet and then diluted 1:5 with HBSS, then washed at 1500 rpm for 10 min at 4⁰C. The supernatant was decanted and the cells were resuspended with an appropriate volume of RPMI1640. 20-35 ml of resuspension was carefully layered over 15 ml Ficoll in a 50 ml conical tube and centrifuged at 2500rpm for 20 min at 20⁰C (without brake); The following steps were the same as for PBMC (see above).

3.8 Freezing and thawing of PBMC

After having been separated by Ficoll-paque density centrifugation and washed two time, PBMC were suspended in cold appropriate volume of FCS and mixed well with an equal volume of 20% DMSO. Then the cell solution was transferred into 2 ml Greiner tube (each tube contained 10-20 million cells in 1-2 ml

10% DMSO) and put on ice for 10-15 min. Finally, the cells were stored at -70°C until use.

When frozen cells needed to be used, they were thawed rapidly in a 37°C water bath, immediately transferred into RPMI or PBS or HBSS and washed two times (1500 rpm, 10 min, 4°C). After the supernatant was decanted, the cells were resuspended again in medium with 10% FCS, 2 mM L- Glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

3.9 Antigen stimulation and cell culture

T cells were stimulated in vitro in the presence of brefeldin A which inhibits intracellular transport. Thus cytokines produced during the activation are retained inside the cell. The unstimulated control sample also contained brefeldin A. Protein antigen (G1, Gp39, Collagen II, h-hsp60, Yersinia-19KD) were tested at a optimal final concentration which was previously found. Anti-CD28 was acted as a costimulator.

Briefly (106), 1ml whole heparinized peripheral blood and antigens were put in the 15 ml polypropylene tubes (with caps), tubes were loosely capped and vortexed for 10 sec. The cells were stimulated with antigen (20 $\mu\text{g}/\text{ml}$ G1 protein, 20 $\mu\text{g}/\text{ml}$ Gp39, 10 $\mu\text{g}/\text{ml}$ h-hsp60, 20 $\mu\text{g}/\text{ml}$ Y-19KD, 20 $\mu\text{g}/\text{ml}$ Collagen II) in the presence of anti-CD28 (1 $\mu\text{g}/\text{ml}$), in the presence of anti-CD28 alone as a negative control, or in the presence of SEB as a positive control for 6 hours. Brefeldin A (10 $\mu\text{g}/\text{ml}$) was added for the last 4 hours of the stimulation. The culture tubes were left at 5° slant at 37°C in a 5% CO_2 incubator. At 6 hours, 100 μl EDTA (2 mM final concentration) was added and vortexed for 10 sec, then incubated for 15 min at room temperature. Afterwards nine ml of 1 x FACS lysing solution (or RCLS) was added to 1 ml of blood and vortexed gently, incubated for 10 min at room temperature in the dark to lyse erythrocytes and fix cells (the cells must be fixed with 2% formaldehyde if erythrocytes were lysed with RCLS buffer). The pellet was washed again with 1 x PBS after having been centrifuged (1500rpm, 10 min, 4°C). Finally, the cells were put in the PBS/BSA buffer and stored at 4°C .

Synovial fluid was obtained by arthrocentesis which was necessary for therapeutic reasons. Whole synovial fluid (1ml containing 5×10^6 cells) were stimulated in the presence of anti-CD28 (1 $\mu\text{g}/\text{ml}$) for 6 hours with antigens (negative and positive control were the same as those of PB, see above). Brefeldin A (10 $\mu\text{g}/\text{ml}$)

was added after 2 hours. The culture tubes were left at 5° slant at 37°C in a 5% CO₂ incubator. At 6 hours, the sample were taken out of incubator, 100 µl EDTA (final concentration: 2 mM) was added, vortexed for 10 sec and incubated for 15 min at room temperature. The cells were fixed with 1ml 2% formaldehyde for 20 min at room temperature after having been washed with 1 x PBS (centrifuging at 1500rpm, 10 min, 4°C), then the pellet was washed again with 1 x PBS and the cells were kept in the PBS/BSA buffer at 4°C.

If there were less than 5×10^6 cells in 1 ml SF, the synovial fluid was diluted (1:5) with HBSS and washed (centrifuging 1500rpm, 10min, 4°C). The cell pellet was resuspended with RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 2 mM L- Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin) to a final concentration of 5×10^6 cells per milliliter, then cells were stimulated with antigens in the incubator.

3.10 Cytokine staining and analysis by flow cytometry

Activated PB or SF cells were transferred into 12 x 75 mm polystyrene tubes, labelled, washed again with 0.5% PBS/BSA, and centrifuged (300g, 10 min, 4°C); 500 µl of 1 x FACS permeabilizing solution was added into each tube, mixed well and incubated for 10 min at temperature in the dark, the cells were washed with 0.5% PBS/BSA at 1500 rpm for 10 min at 4°C.

The cells were quadruple stained for CD4-, CD69-surface markers and two intracellular cytokines. To avoid nonspecific binding of antibodies to Fc-receptors, all the staining was done in the presence of Beriglobin (3mg/ml). The following antibodies were used: anti-CD4 PerCP (clone Leu-3a), anti-CD69 FITC, anti-CD69 PE (Leu-23), anti-TNF α FITC, anti-IFN γ Cy5, anti-IL-4 PE (4D9), and anti-IL10 APC.

Staining solution was prepared at a volume of 50 µl/test, for some of the samples half of cells were stained for the cytokines IFN γ and TNF α , and the others were stained for IL4 and IL10. This means that two types of staining solution were prepared in this study: CD4 PerCP (1:10 dilution), CD69 PE (1:10), IFN γ cy5 (1:400), TNF α FITC (1:800); and CD4 PerC P (1:10), CD69 FITC (1:10), IL4 PE (1:100), IL10 APC (1:400).

After permeabilization and washing, 50 µl staining solution was added into each tube and the pellet was resuspended by pipette and incubated for 30 min at room temperature in the dark. The cells were washed at 300g for 10 min at 4°C and

resuspended with 300-500 ml 0.5% PBS/BSA. In some experiments, the samples can be stored at 4°C in the dark for up to 24 hours prior to analysis.

Flow cytometric analysis was performed on a FACSCalibur™ flow cytometer. Data were acquired using CELLQuest™ software. After proper instrument setup, CD4⁺ lymphocytes were gated. For this, typically 100,000 CD4⁺ events were collected using FL3 (PerCP) as a fluorescent trigger.

Since all specific cytokine expression should occur within the CD69⁺ (activated) cell subset, CD69 staining was included to exclude non-specific cytokine staining.. Forward scatter vs side scatter gating was employed in data analysis to exclude any CD4⁺ monocytes.

Data were displayed as four-color dot plots in PAINT-A-GATE^{plus} software to measure the proportion of the double-positive(cytokine⁺/CD69⁺) cells. In this study, they were displayed as dot plots of TNF α FITC/IFN γ Cy5 (x-axis) and CD69 PE (y-axis) or IL4 PE/IL10 APC (x-axis) and CD69 FITC fluorescence (four decade log scales). Quadrant markers were positioned to include >99% of control Ig staining cells in the lower left quadrant.

Positive cells were subsequently quantified. Only cytokine-positive T cells which were also positive for the early activation antigen CD69 were counted. To analyse whether cytokines were produced by the same or different cells, CD4⁺ T cells positive for 2 cytokines were also counted at the same time. An example of a FACS analysis is shown in Fig.3.

The FACS data were given in both, a qualitative and a quantitative manner. For the qualitative approach it was essential to determine a cutoff. This was done by 1. looking at the dot plots to judge whether a distinct population can be identified by eye (Fig.3); 2. seeing how many cells have to be eligible at least to allow for proper counting; 3. comparing the results between patients and controls; 4. adding two standard deviations to the mean percentage to be more certain that the differences measured are meaningful. With this approach I determined the minimally required cells to be at least 30 and set the cutoff to 0.02% of the cells gated.

3.11 Peptide-stimulation assay

Forty-six overlapping 18-mer G1 peptides, each overlapping by 12 AA, were put into 5 pools, with 8-10 in each pool. Pool 1 contained peptides 1-10, pool 2 peptides 11-19, pool 3 peptides 20-28, pool 4 peptides 29-38, pool 5 peptides 39-46.

For stimulation with peptides, mononuclear cell (MC) instead of whole blood was used. Immediately after the blood was drawn in heparinized syringes, MC of 13 AS patients were obtained by Ficoll-paque density centrifugation. The cells then were suspended in RPMI 1640 medium with 100 units/ml penicillin, 100µg/ml streptomycin 2mM L-glutamine, and 10% heat-inactivated fetal calf serum. At least 1×10^6 cells/ml were stimulated with G1 pool of peptides (each peptide 5µg/ml) in the presence of anti-CD28, in the presence alone as negative control, or with SEB as positive control. Finally, staining with monoclonal antibodies directed against the surface markers CD4 and CD69, and against intracellular cytokines IFN γ and TNF α was performed (see cytokine staining and analysis by cytometry). For fine epitope mapping, fresh blood was taken again from patients who responded to pools of peptides and were stimulated with single peptides.

3.12 Isolation of G1-specific T cells using IFN γ secretion assay

The IFN γ secretion upon antigen stimulation was designed for the detection, isolation and analysis of live IFN γ -secreting leucocytes (113). It is especially useful for the isolation of antigen-specific Th1 cells after stimulation with specific antigen in vitro to induce secretion of IFN γ . The mechanism (Fig.1) of this technique is that an affinity matrix for IFN γ is generated by attaching IFN γ catch reagent to CD45 on the cell surface. The secreted IFN γ binds to the catch reagent on the surface of secreting cells and can subsequently be stained with a second IFN γ specific antibody. This IFN γ detection antibody is conjugated to Phycoerythrin (PE), allowing sensitive analysis by flow cytometry. Subsequently, cells can be magnetically labeled with anti-PE microbeads and enriched on a column which is placed in the magnetic field of a MACS separator. The magnetically labeled IFN γ secreting cells are retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the magnetically retained IFN γ secreting cells can be eluted as positively selected cell fraction, highly enriched with antigen-specific T cells. The cells can now be used for analysis or cell culture.

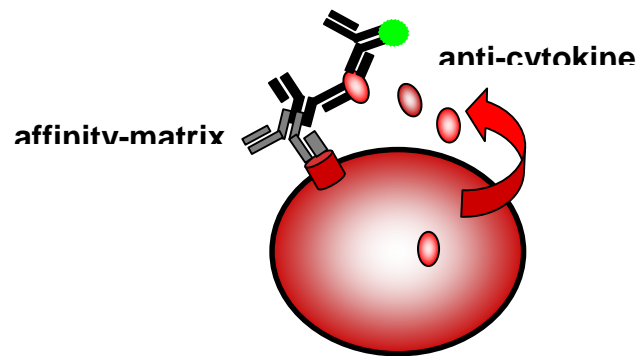


Figure 1. **Model of IFN γ secretion assay.** Firstly, IFN γ catch reagent attached on the surface of T cells by anti-CD45. Secondly, the secreted IFN γ binds to the Catch Reagent on secreting cells and can subsequently be stained with a second IFN γ specific antibody (IFN γ Detection antibody). Finally, MicroBeads were connected by anti-PE which make the cells magnetic and retained when they pass through column of separator.

In this study, IFN γ secreting cells were isolated from PB of AS patients. Two fractions (each contains 20 ml) of fresh heparin peripheral blood were incubated for 12 hours (at 37 $^{\circ}$ C, 5% CO $_2$) with G1 protein (20 μ g/ml) in the presence of anti-CD28 (1 μ g/ml) or without G1 protein (as control). At 12 hours, EDTA (2mM) was added, the cells were vortexed and incubated for 15 min at room temperature. Then, the blood was diluted by 1:1 with RPMI (without FCS, penicillin and streptomycin), each 10 ml diluted blood was mixed well with 40 ml RCLS and incubated for 15 min in ice water (was shaken every other 3-5 min) to lyse the red cells. The cells were spun down (1200 rpm, 10 min, 4 $^{\circ}$ C) and decanted, washed again by adding cold PBS/BSA buffer (without azid), and centrifuged at 1500 rpm for 10 min at 4 $^{\circ}$ C.

Subsequently, the cell pellet was resuspended in 160 μ l of cold buffer (80 μ l per 10 7 cells), 40 μ l of IFN γ catch reagent (20 μ l/10 7 total cells) was added (as 10 7 estimated cells in 10 ml blood), mixed well and incubated for 7 min on ice. After incubation, the cell suspension of 20 ml blood was directly aliquated into two 50 ml test tubes, 45 ml warm (37 $^{\circ}$ C) medium was added to dilute the cells, then incubated for 45 min (37 $^{\circ}$ C, 5% CO $_2$), and mixed well every other 5 min.

The samples were centrifuged at 1500 rpm for 10 min at 4 $^{\circ}$ C, supernatant was removed completely and washed again. The cell pellet was resuspended again in 160 μ l of cold buffer, 40 μ l of IFN γ detection antibody (PE) (20 μ l for 10 7 cells, 1:5 dilution) was added and additional the staining reagents CD4 Cy5 and Beriglobin were put in, mixed well and incubated for 10 min on ice. Cells were washed with cold buffer, centrifuged at 1500 rpm for 10 min at 4 $^{\circ}$ C.

For magnetic labeling, cell pellet was resuspended in 160 μ l cold buffer, 40 μ l of anti-PE microbeads (1:5 dilution) was added, mixed well, and incubated for 15 min at 4⁰C followed by washing of the cells (1500rpm, 10 min, 4⁰C) and removing the supernatant completely. Finally, the cells were resuspended in 1 ml of cold buffer.

For magnetic separation, MS⁺/RS⁺ column was put in the magnetic field of the MACS separator and rinsed 3 times with 1 ml of cold buffer. Magnetically labeled cells were applied and pass through the column. The column was washed with 3 x 1 ml cold buffer, and the effluent was collected as negative fraction. Then, the column was removed from separator and placed in a suitable collection tube. Three ml of cold buffer was pipetted on top of the column, retained cells were flashed out by using the plunger supplied with the column. This step was repeated to increased the purity. Finally, the retained cells were eluted in buffer and used for analysis and further cell cultures.

3.13 Flow cytometry analysis of G1-specific T cells

Flow cytometric analysis was performed with the original fractions (before enrichment, A.) and with the enriched fractions (after enrichment, B.). Propidium iodide was added just prior to flow cytometric analysis at a final concentration of 0.5 μ g/ml. A lymphocyte gate based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to improve the sensitivity of the analysis. Upon activation of the lymphocyte gate, dead cells were gated out according to PI fluorescence in a fluorescence 2 versus fluorescence 3 plot. Then antigen-specific T cells were analysed according to staining with CD4 Cy5 and anti-IFN γ PE (Fig.15).

3.14 Culture of G1-specific T cells

IFN γ ⁺ cells were put in a 96-well plate and cultured in the presence of recombinant IL2 (20 units/ml) in tissue culture medium, which contained RPMI 1640, 10% autologous serum, 2 mM L- Glutamine, Penicillin 100 units/ml, Streptomycin 100 μ g/ml. IL2 was added every 3-4 day, the cells were frequently observed under microscope and the expanded cells were transferred to other wells if necessary.

3.15 Restimulation of G1-specific T cells

For re-analysis of G1-specific T cells, the cells had to be left for at least 5 days without IL2. The cells were harvested, passed through 70 μ m mesh filter to remove

clumps and washed with HBSS or RPMI (1500rpm, 10 min, 4⁰C). Subsequently, autologous PBMC (as APCs) were labeled with CFDA-SE (procedure: cells were washed several times in PBS to remove protein-containing buffer, 5 μ M CFDA-SE staining solution was prepared in PBS, the cells were resuspended at a concentration of 10⁷/ml in staining solution and incubated for 4 min at room temperature, the reaction was stopped by washing the cells in medium containing 10% FCS or PBS/BSA at 1500rpm for 10 min at 4⁰C, then washed again). Next, T cells were mixed with autologous PBMC as a 1:5 dilution and transferred into 15 ml test tubes, each tube contained at least 0.2 million T cells and adequate PBMC in 1 ml of tissue medium with RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 units penicillin and 100 μ g streptomycin. Finally, G1 protein and other antigens of interest were added to different tubes, vortexed for 10 seconds and incubated for 6 hours in the presence of anti-CD28. Brefeldin A was added after 2 hours, the cells were harvested at 6 hours, fixed with 2% formaldehyde and resuspended in PBS/BSA (see 2.8 of methods).

3.16 Staining and analysis of G1-specific T cells by flow cytometry

Stimulated cells were transferred into tubes for staining. After permeabilization, cells were washed once and staining was performed for 30 min at RT in the dark using a titrated mixture of fluorescent conjugated mAbs (anti-CD4 PerCP, anti-CD69 PE and anti-IFN γ Cy5).

For flow cytometric analysis, after activation of the lymphocyte gate, APCs were gated out according to CFDA-SE fluorescence in a fluorescence 2 versus fluorescence 1 plot. Then a gate for CD4⁺ T cells was set, the percentage of IFN γ /CD69 double positive cells of the CD4⁺ T cell subpopulation was indicated (see Fig.17 of results).

3.17 Clinical experiment about anti-TNF α treatment (infliximab)

twenty active AS patients were randomly selected into two groups as part of double-blind, placebo-controlled, multicenter trial of treatment of AS patients with infliximab. Patients of the first group underwent three infusions of 5 mg/kg infliximab at 0, 2, and 6 weeks, patients of the second group were treated with placebo at the same time points. After 6 weeks, patients from the placebo group were treated in a similar way with 5mg/KG infliximab at week 0, 2 and 6 weeks. Cytokine were

investigated before, 6 weeks and 12 weeks after treatment in the infliximab group, and before, 6 weeks during placebo treatment and 6 weeks after patients in the placebo group were switched to infliximab treatment. Peripheral blood from participants was taken at baseline, at 6 and 12 weeks, PBMC were isolated and stored at -80°C until further analysis.

For analysis, cells were thawed (see 2.8 of methods) and put in the incubator overnight to come to life. Then 1×10^6 cells were cultured for 6 hours in the presence of 5 ng/ml PMA and 1 ng/ml ionomycin, or with a pool of 46 overlapping 18 mer peptides (5 $\mu\text{g}/\text{ml}$ for each peptide) derived from the G1 domain of aggrecan in the presence of anti-CD28, and in the presence of anti-CD28 alone as negative control.

T cells were stained after in vitro stimulation as described before (106). Briefly, cells from PBMC were washed with PBS/BSA, centrifuged (300g, 10min, 4°C), and cells were quadruple stained for CD3, CD8-surface markers and two intracellular cytokines, either $\text{IFN}\gamma$ / IL4 or $\text{TNF}\alpha$ /IL-10. All stainings were performed in FACS™ Permeabilizing Solution. To avoid nonspecific binding of antibodies to Fc-receptors, all the staining was done in the presence of Beriglobin (3mg/ml).

Because stimulation with PMA/ionomycin induces a reduction of CD4-expression on the cell surface (36), CD4+ T cells were identified indirectly by gating on CD3-positive but CD8-negative lymphocytes. After gating either on CD3+/CD8+ or CD3+/CD8- (CD4) lymphocytes, data were analysed using CELLQuest software and displayed as dot plots of FITC (x-axis) and APC (y-axis) fluorescence. Quadrant markers were positioned to include > 99% of control Ig staining cells in the lower left quadrant.

3.18 Separation of cells by MACS and detection of ratio of CD4/CD8 and CD14/CD19 by flow cytometry

For cell separation (Fig.2), around 4×10^6 PBMC were taken and washed at 1500rpm for 10 min at 4°C , cells were resuspended with 200 μl PBS/BSA buffer, 50 μl CD3 microbeads (1:5 dilution) were added and incubated for 15 min at 4°C . The cells were put through column that was placed in the magnetic field of MACS separator. The magnetically labeled CD3⁺ cells were retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the magnetically retained CD3⁺ cells were eluted with 3 x 1 ml cold PBS/BSA as positively selected cell fraction. The cells were washed at 1500rpm for 10 min at 4°C .

Subsequently, the CD3⁺ cells were stained with anti-CD4 Cy5 (1:400) and anti-CD8 PE (1:10), and the CD3⁻ cells were stained with anti-CD14 PE (1:10) and anti-CD19 FITC (1:10) in the presence of Beriglobin (3mg/ml). After washing, cells were resuspended with 1 ml PBS/BSA, Propidium iodide (PI) was added (0.5 µg/ml). Then, CD4, CD8, CD14 and CD19 cells were checked by flow cytometry after the dead cells were gated out according to PI fluorescence in a fluorescence 2 versus fluorescence 3 plot. At last, calculated the ratio of CD4/CD8 and CD14/CD19.

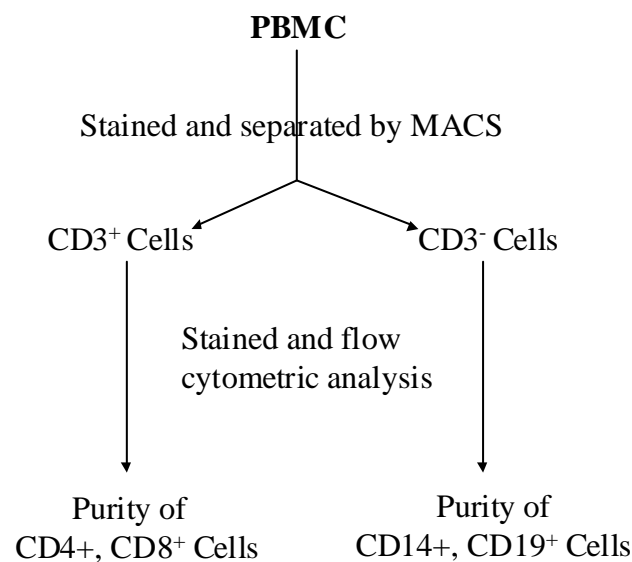


Figure 2. Model of separation and purity detection for CD4+, CD8, CD14+ and CD19+ cells.

3.19 In vitro stimulation of macrophages With LPS and cytokine detection by ELISA

To test the effects of infliximab on cytokine production by macrophages, 1×10^6 PBMC from different time points were cultured with purified Lipopolysaccharide (LPS) at a concentration of 10 µg/ml for 18 hours, then cell-free supernatants were collected and frozen at -70°C until the cytokine assay was performed.

Secretion of $\text{TNF}\alpha$ and IL10 was analysed in the supernatant using a sandwich ELISA with purified anticytokine Mab (capture) and biotinylated anticytokine antibodies (detecting), with a detection level of 20 pg/ml for both

cytokines using an ELISA reader from Dynatech. The ELISA protocol is briefly described as following:

The capture antibodies were coated in 96-well immunoassay plate with binding buffer (50 μ l/well) at a concentration of 1-4 μ g/ml at 37⁰C for 1 hour. The plate was blocked with PBS supplemented with 10% heat-inactivated FCS at 37⁰C for 1 hour. After washing with washing buffer, supernatants from samples and standards with known amounts of the appropriate cytokines were pipetted into the wells and incubated at 4⁰C overnight. After washing, the detecting antibodies specific for the respective cytokines were added to wells and incubated at 37⁰C for 1 hour. After washing, a horseradish peroxidase (HRP) conjugated streptavidin were added to the plates which were then incubated at 37⁰C for 45 minutes. Finally, the substrate solution [substrate buffer + o-phenylenediamine (OPD) Dihydrochloride] was added. After 10 minutes, the color development was stopped by 2N HCl and the absorbance was read at 490 nm using an ELISA reader (Dynatech MR5000). The amount of cytokines was determined by comparing the absorbance of the samples with standard proteins.

3.20 Statistical analysis

χ^2 test was used to compare frequency in different groups; Student's T test was used to analyse differences among medians of percentage of positive patients and to compare cytokine frequency induced by G1 between PB and SF; Wilcoxon-test was used to compare cytokine production between before and after placebo or anti-TNF α treatment. Differences were considered to be significant in case of a the two-tailed p-value of less than 0.05.