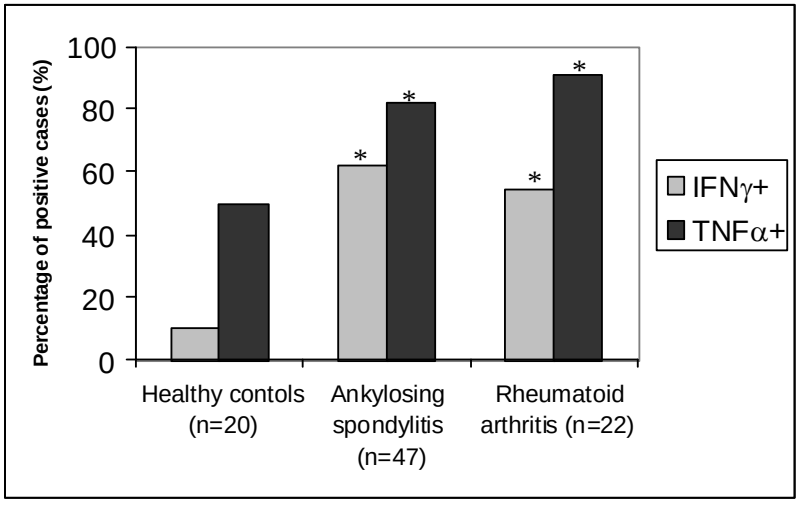
Results

1. Antigen (cartilage-derived) -specific cytokine secretion in ankylosing spondylitis and rheumatoid arthritis compared to healthy controls

As shown in Figure 3, there is an increased frequency of IFNγ positive T cells in PB specific for the G1-protein in AS and RA: 61.7% (29/47) of the AS patients and 54.5% (12/22) of the RA patients compared to only 10% (2/20) HC had increased percentages of IFNγ positive T cells in response to G1. TNFα positive CD4 T cells responding to G1-stimulation were even detected in a higher percentage in AS and RA patients but also in controls: 91.5% (43/47), 81.8% (18/22), 50% (10/20), respectively (Fig. 3). This difference between AS and RA compared to HC is significant for both cytokines ($p<0.05$). Most (26 out of 29 patients) of the IFNγ-positive CD4+ T cells were double positive for TNFα (not shown). There was no T cell response to gp39 and collagen II. An example for IFNγ secretion of PB $CD4^+$ T cells in response to stimulation with cartilage-derived antigens (G1, gp39, collagen II) is shown for one AS patient in Fig. 4.



*$p<0.05$, comparing with healthy controls.

Figure 3. T cell response to the G1-domainof aggrcan in PB. Percentage of patients with ankylosing spondylitis, with rheumatoid arthritis and of controls responding to the in vitro stimulation with the G1-domain of the proteoglycan aggrecan. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole peripheral blood**.

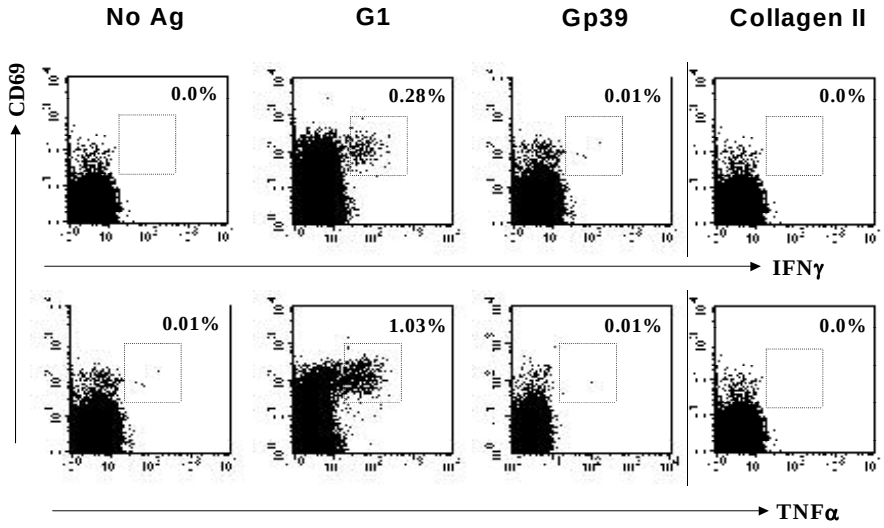
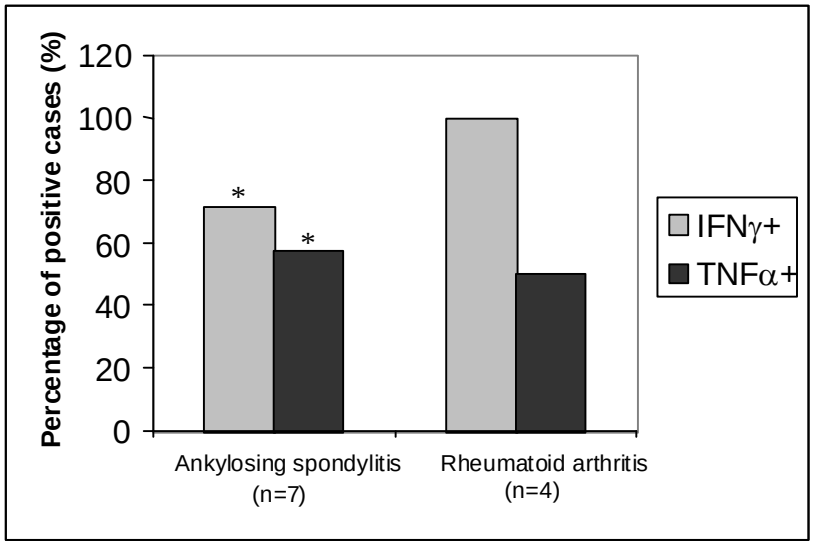


Figure 4. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag) or with the human cartilage derived antigens glycoprotein (gp) 39 or collagen II in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.



*p>0.05, comparing with rhematoid arthritis.

Figure 5. T cell response to the G1-domain in SF. Percentage of patients with ankylosing spondylitis and with rheumatoid arthritis responding to the in vitro stimulation with the G1-domain of the proteoglycan aggrecan. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole synovial fluid**.

In SF, 71.5% (5 out of 7) of the AS patients responded to in vitro stimulation with G1 by IFNγ–secretion and 57.2% (4 out of 7) by TNFα (Fig. 5). In SF from RA patients, a response to G1 was detectable in all 4 patients (100%) as judged by IFNγ-production and in 50% by TNFα-production (Fig. 5). Interestingly, in SF the percentage of patients responding by IFNγ-secretion was higher than the percentage responding by TNFα-secretion while it was the other way around in PB (Fig. 3 and 5).

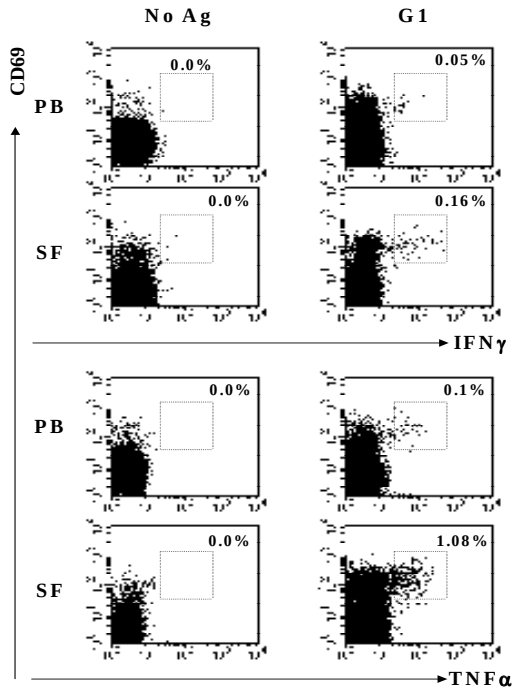


Figure 6. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag) in a patient with **ankylosing spondylitis**. **The T cell response in synovial fluid (SF) is higher compared to peripheral blood (PB).** After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T cell subpopulation is indicated.

An example for IFNγ secretion of SF CD4+ T cells in response to stimulation with these antigens is shown for one AS patient in Fig. 6. None of the AS patients showed a T cell response to gp39 or to collagen II (an example is shown in Fig. 4) and none of the RA patients showed a T cell response to collagen II (an example is shown in Fig.7; gp39 was not tested in RA). The G1 specific T cell response in SF (mean ± SD: 0.26 ± 0.23% for IFNγ; 0.43 ± 0.39 % for TNFα) was significantly ($p=0.01$ for IFNγ, $p=0.007$ for TNFα) stronger than that in PB (mean ± SD: 0.06 ± 0.05% for IFNγ; 0.07 ± 0.03% for TNFα). An example is shown in Fig. 6 for one AS patient.

No increased percentages of IL4- or IL10-positive CD4+ T cells were observed after stimulation with G1, gp-39 or collagen II in any of the three groups (data not shown).

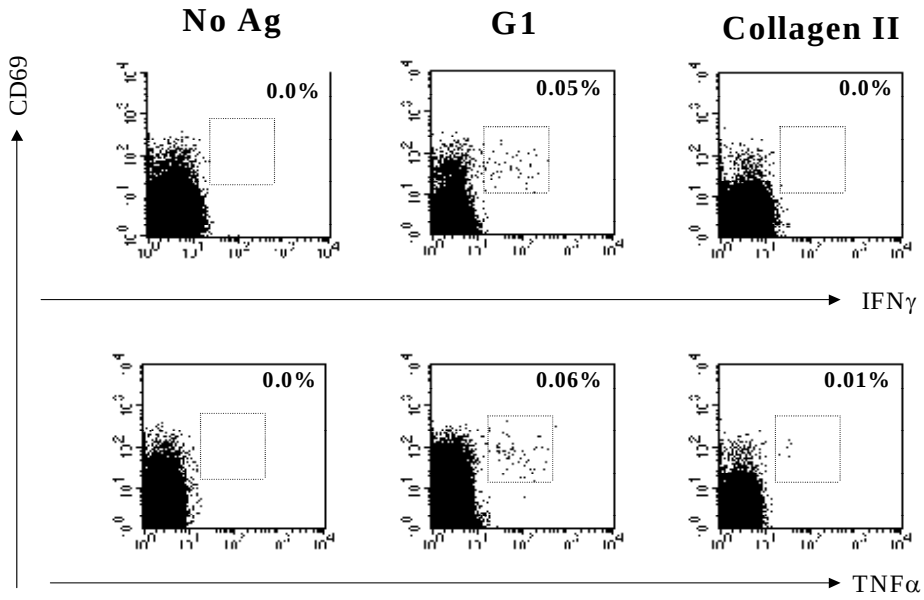


Figure 7. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag) or with the human cartilage derived antigen collagen II in a patient with **rheumatoid arthritis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T cell subpopulation is indicated.

2. Characterization of immunodominant G1- epitopes

When the response of CD4+ T cells derived from peripheral blood after stimulation with G1-pools of peptides was investigated in 13 of the AS patients, in 6 out of 13 (46.2%) patients a response to pool 4, containing peptides 29-38, was observed, but not to any of the other 4 pools (Table 4, Fig 8). No pool-specific response was detectable in the other 7 AS patients. Restimulation of peripheral blood T cells from the 5 responding patients with single peptides (all out of pool 4) indicated that peptide 35 (in 4 patients) and peptide 30 (in one patient) (Table 4, Fig. 8) were stimulatory. Peptide 35 is located within residues 292 to 309 of the G1-domain (AGMDMCSAGWLADRSVRY) while peptide 30 is located within residues 252 to 269 (EGEVFYATSPEKFTFQEA).

Table 4. T cell response to G1 pools of peptides and single peptide in ankylosing spondylitis patients.

Patient	Positive pool[#]	Positive peptide*
Pat.1	Pool 4 (peptides 29-38)	Peptide 35
Pat.2	Pool 4 (peptides 29-38)	Peptide 35
Pat.3	Pool 4 (peptides 29-38)	Peptide 30
Pat.4	Pool 4 (peptides 29-38)	Peptide 35
Pat.5	Pool 4 (peptides 29-38)	Peptide 35
Pat.6	Pool 4 (peptides 29-38)	not tested

[#] None of the other 4 pools of peptides was stimulatory.

* None of the other 9 peptides out of pool 4 was stimulatory.

Peptide 35 (AA-sequence): AGMDM CSAGW LADRS VRY

Peptide 30 (AA-sequence): EGEVF YATSP EKFTF QEA

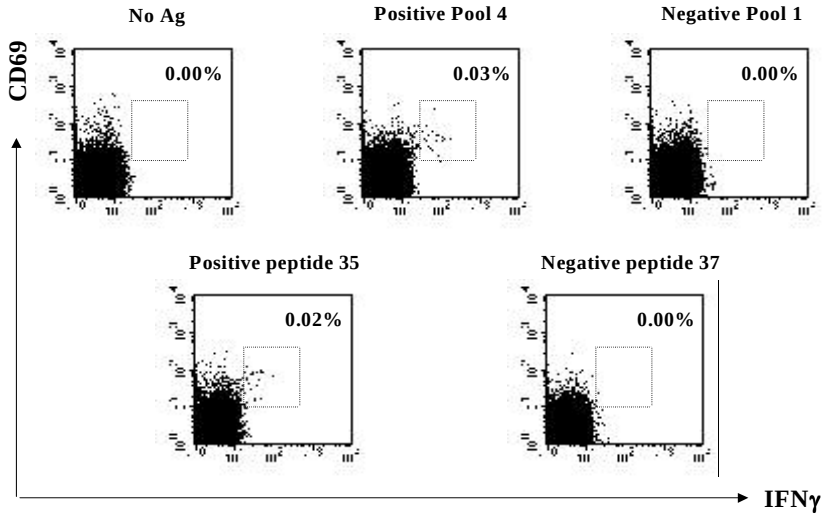
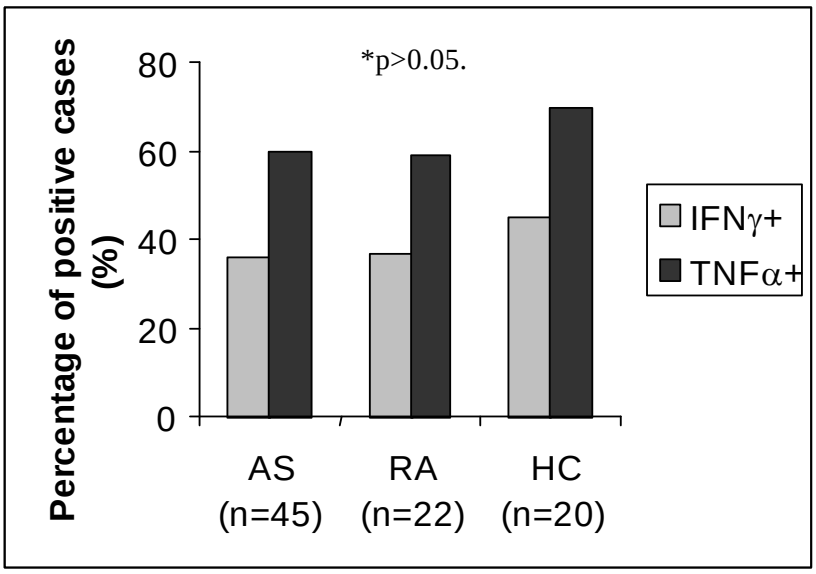


Figure 8. Example of an **ankylosing spondylitis patient** with an antigen-specific response to one of the **pools of peptides** (Pool 4), but not to Pool 1, derived from G1-domain of the proteoglycan aggrecan compared to stimulation without antigen (Ag). Out of Pool 4, containing peptides 29-38, only the single peptide 35 but not peptide 37 was recognized by this patient's CD4+ T cells. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-positive cells of the CD4+ T cell subpopulation is indicated.

3. T cells response to human hsp60 in patients and healthy controls

IFNγ^+ CD4 T cells responsive to h-hsp60 were detected in the AS, RA patients and HC, with an incidence of 36% (16/45), 36.5% (8/22), and 45% (9/20), respectively; The corresponding percentage of TNFα^+ CD4 T cells was 60% (27/45), 59% (13/22) and 70% (14/20). There was no significant difference between AS or RA and HC ($p>0.05$) (Fig.9). Fig.10 shows an example for an h-hsp60-specific IFNγ^+ or TNFα^+ response of PB CD4$^+$ T cells in AS, RA and HC.

For SF, 71.4% (5 out of 7 patients) AS- and 75% (3 out of 4 patients) of RA-cases showed a T cell response to h-hsp60 by IFNγ-secretion. 85% (6/7) of AS- and 75% (3/4) of RA-cases showed a TNFα-secretion in response to h-hsp60. In comparison with PB, hsp60-specific IFNγ/TNFα-response were stronger (mean ± SD: 0.18 ± 0.3 in SF vs 0.09 ± 0.08 in PB for IFNγ; 0.35 ± 0.64 vs 0.15 ± 0.05 in PB for TNFα), but the difference was not significant ($p>0.05$ for both cytokines).



*There is no significant difference among all groups for both cytokines.

Figure 9. T cell response to h-hsp60 in PB. Percentage of patients with ankylosing spondylitis, with rheumatoid arthritis and of controls responding to the in vitro stimulation with the h-hsp60. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole peripheral blood**.

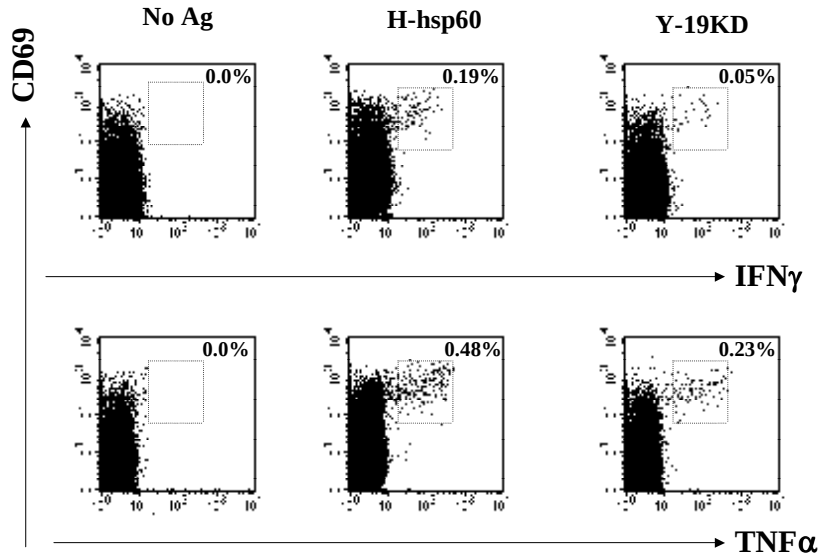
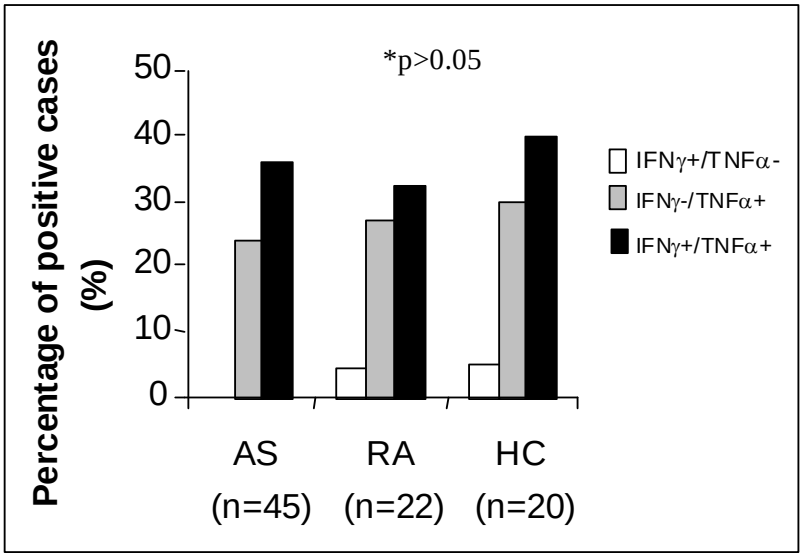


Figure 10. Example of the antigen-specific response to the human heat shock protein 60 (h-hsp60) and Yersinia 19KD (Y-19KD) compared to stimulation without antigen (Ag), in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.



*There is no big difference among all groups for combination analysis.

Figure 11. Combining analysis of h-hsp60-specific IFNγ and TNFα in PB. Percentage of cases with IFNγ/TNFα single or double positive responding to the in vitro stimulation with h-hsp60 in patients with ankylosing spondylitis, with rheumatoid arthritis and of controls. Analysis was done with **whole peripheral blood**.

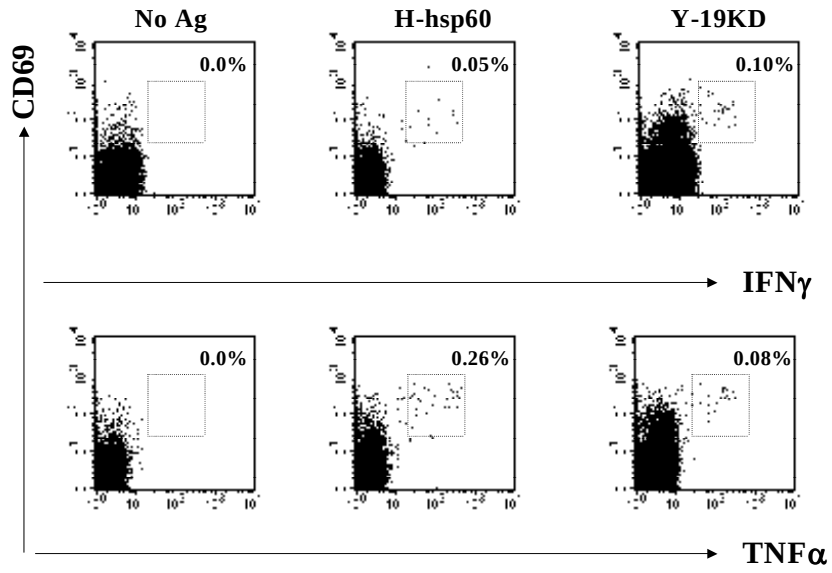


Figure 12. Example of the antigen-specific response to the human heat shock protein 60 (h-hsp60) and Yersinia 19KD (Y-19KD) compared to stimulation without antigen (Ag), in a patient with **rheumatoid arthritis**. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.

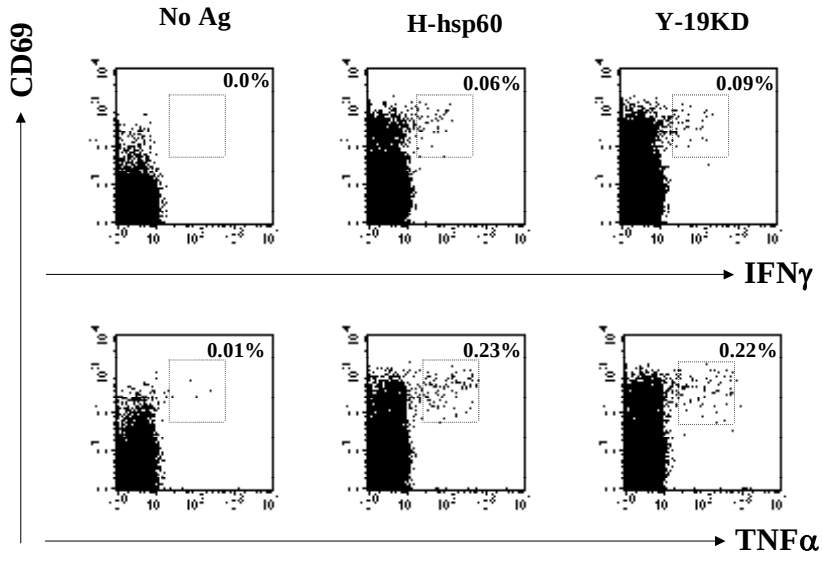


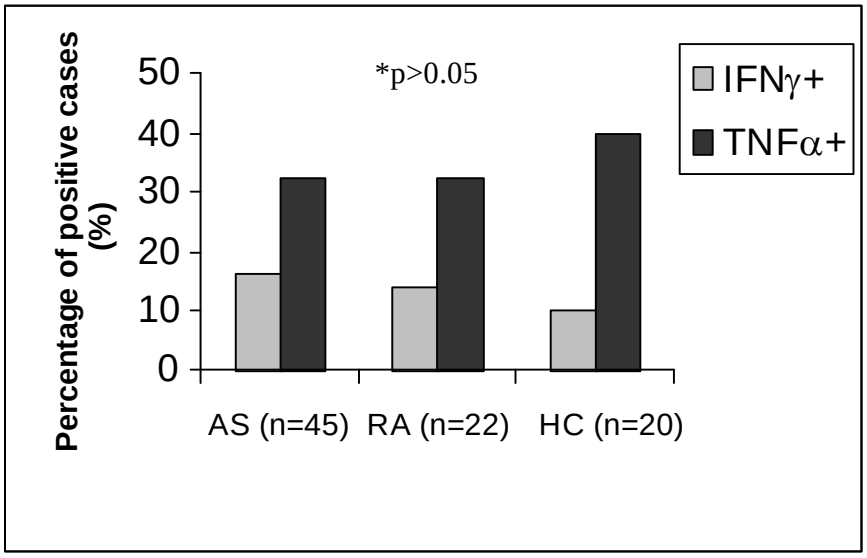
Figure 13. Example of the antigen-specific response to the human heat shock protein 60 (h-hsp60) and Yersinia 19KD (Y-19KD) compared to stimulation without antigen (Ag), in a healthy control. After staining for T cell surface markers and intracellular cytokines a gate for CD4+ T cells was set. The percentage of IFNγ/CD69- or TNFα/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.

A combined analysis for IFNγ and TNFα secretion indicates that 0% (0/45) of $IFN\gamma^+/TNF\alpha^-$ (IFNγ single positive), 24% (11/45) of $IFN\gamma^-/TNF\alpha^+$ (TNFα single positive) and 36% (16/45) of $IFN\gamma^+/TNF\alpha^+$ (double positive) were detected in AS patients after stimulation with h-hsp60 (Fig. 11); The percentages of $IFN\gamma^+/TNF\alpha^-$, $IFN\gamma^-/TNF\alpha^+$ and $IFN\gamma^-/TNF\alpha^+$, in RA, were observed with an incidence of 4.5% (1/22), 27% (6/22) and 32% (7/22) (Fig. 11), respectively (an example of T cell response to h-hsp60, in one patient with RA, was shown in figure 12); The corresponding frequency in HC is 5% (1/20), 30% (6/20) and 40% (8/20) (Fig.11). No significant difference was shown among different groups ($p>0.05$). An example of antigen-specific response to h-hsp60, in a healthy control, was shown in figure 13.

4. T cells response to yersinia-19KD in patients and healthy controls

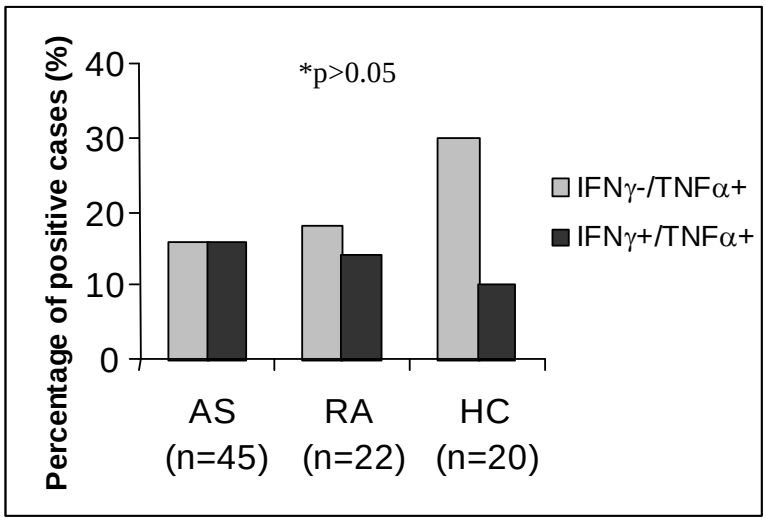
A Y-19KD-specific T cell response could be detected in all 3 groups (Fig. 10, 12, 13). 16% (7/45), 14% (3/22), 10% (2/20) of samples with $IFN\gamma^+$ $CD4^+$ T cells induced by yersinia-19KD were observed in AS, RA patients and controls, respectively (Fig. 14). For y-19KD-specific $TNF\alpha^+$ $CD4^+$ T cells, the percentage was 32% (14/45), 32% (7/22), 40% (8/20), respectively, in the corresponding groups (Fig. 14). There was no significant difference among different groups. The incidence of $IFN\gamma^-/TNF\alpha^+$ was detected with a percentage of 16% (7/45), 18% (4/45), 30% (6/20) in AS, RA and HC, respectively; The $IFN\gamma^+/TNF\alpha^+$ frequency showed an incidence of 16% (7/45), 14% (3/22) and 10% (2/20), respectively, in the corresponding groups. No single $IFN\gamma^+$ ($IFN\gamma^+/TNF\alpha^-$) positive cells were found in any of the 3 groups (Fig.15). There was no difference between any of groups ($p>0.05$).

Although a higher percentage of AS patients (42.8%, 3 out of 7 patients for IFNγ; 71.4%, 5 out of 7 patients for TNFα) compared to RA (25%, 1 out of 4 patients for IFNγ; 50%, 2 out of 4 patients for TNFα) showed a synovial T cell response to the 19KD protein, this difference was non-significant, possibly due to the small number of patients.



*There is no big difference among different groups for both cytokines.

Figure 14. T cell response to y-19KD in peripharal blood. Percentage of patients with ankylosing spondylitis, with rheumatoid arthritis and of controls responding to the in vitro stimulation with the y-19KD. Response was measured either by IFNγ- or TNFα-production of CD4+ T cells after antigen-specific stimulation in comparison to stimulation without antigen. For more details see Methods section. Analysis was done with **whole peripheral blood**.



*There is no big difference among different groups for combination analysis.

Figure 15. Combining analysis of y-19KD-specific IFNγ– and TNFα–secretion in peripharal blood. Percentage of cases with single TNFα or IFNγ and TNFα double positive responding to the in vitro stimulation with y-19KD in patients with ankylosing spondylitis, with rheumatoid arthritis and of controls. No single IFNγ+ case, responding to the in vitro y-19KD stimulation, was observed in any of the groups. Analysis was done with **whole peripheral blood**.

5. Relationship of cytokine producion by h-hsp60 and Y-19KD

In this study, PB of 67 patients (AS 45, RA 22) underwent cytokine secretion analysis. Almost all Y-19KD specific IFNγ^+ samples were accompanied by h-hsp60 specific IFNγ^+ production (just 1 exception). As table 5 shows, IFNγ^+ section was observed in 24 samples including 9 responses to both h-hsp60 and Y-19KD, 14 single responses to h-hsp60, 1 only response to Y-19KD. There is a significant relationship of IFNγ producing between by h-hsp60 and by 19KD ($p<0.05$).

Furthermore, all Y-19KD specific TNFα^+ samples were accompanied by h-hsp60 specific TNFα^+ secretion. There were 40 samples out of 67 samples responding by TNFα-secretion, of which the cases with a TNFα response to both h-hsp60 and 19KD were 21, 19 patients only responded to h-hsp60 alone; none of them responded only to Y-19KD (Table 6). A significant correlation could be observed in TNFα response between h-hsp60 and 19KD ($p<0.05$).

6. Non-specific cytokine secretion in AS and RA

By intracellular cytokine staining and flow cytometric analysis, I investigated whether a difference in cytokine secretion, induced by SEB, could be detected at single cell level in different groups. Of which, only IFNγ showed a significant difference among different groups when measured. Levels of IFNγ were lower in AS patients (mean ± SD 2.28 ± 1.87%) than those both in HC (4.09 ± 3.57%, $p=0.005$) and in RA (3.43 ± 3.24%, $p=0.049$); TNFα secretion was a little bit lower in AS patients (8.30 ± 5.51%) than those both in RA patients (9.41 ± 5.58%) and in HC (10.88 ± 6.35%), but the difference was not significant. IL10 secretion was similar in AS and RA patients (0.06 ± 0.05% versus 0.05 ± 0.04%). Also no marked difference of IL4 producing CD4+ T cells was observed in any of the patients' groups (AS: 0.82 ± 0.70% versus RA: 0.96 ± 0.65%) (not detected in HC).

7. Enrichment of G1-specific T cell

For further epitope analysis, G1-specific T cells were enriched by the IFNγ secretion assay, as described in Methods (see 3.12 of materials and methods). As Fig. 16 shows, a high frequency of G1-specific IFNγ^+ CD4 T cell (61.28%, cellular

Table 5. Relationship between h-hsp60 and Y-19KD for IFNγ producing cells*.

	$IFN\gamma^+$ CD4+ T cells after stimulation with h-hsp60	$IFN\gamma^-$ CD4+ T cells after stimulation with h-hsp60
$IFN\gamma^+$ CD4+ T cells after stimulation with Y-19KD	11	1
$IFN\gamma^-$ CD4+ T cells after stimulation with Y-19KD	22	53

*$p<0.05$, χ^2 test

Table 6. Relationship between h-hsp60 and Y-19KD for TNFα producing cells.

	$TNF\alpha^+$ CD4+ T cells after stimulation with h-hsp60	$TNF\alpha^-$ CD4+ T cells after stimulation with h-hsp60
$TNF\alpha^+$ CD4+ T cells after stimulation with Y-19KD	29	0
$TNF\alpha^-$ CD4+ T cells after stimulation with Y-19KD	25	33

$p<0.05$, χ^2 test

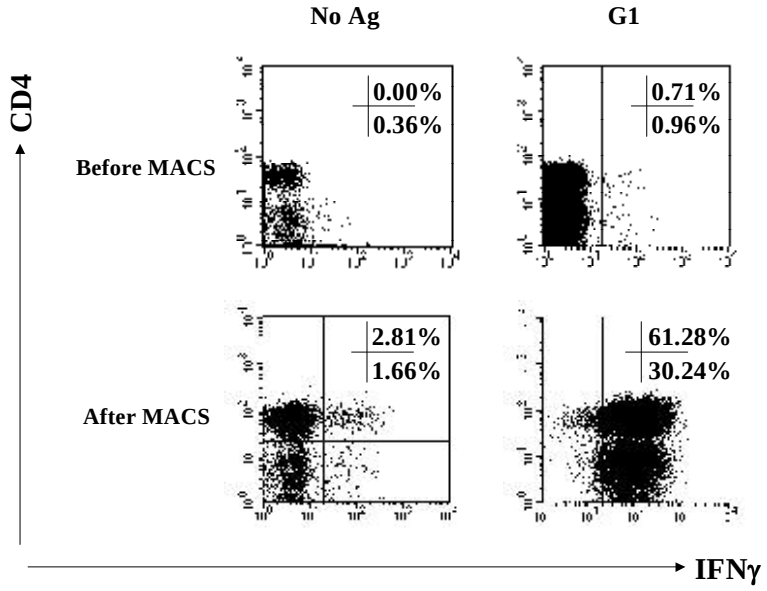


Figure 16. Example of G1-specific $CD4^+$ T cell enrichment compared to stimulation without antigen (Ag), in a patient with **ankylosing spondylitis**. After staining for T cell surface marker and cytokine IFNγ bound on the cellular surface, dead cells were gated out according to PI fluorescence in fluorescence 2 versus fluorescence 3 plot and a gate for CD4+ T cells was set. The percentage of IFNγ positive cells of the CD4+ T cell subpopulation is indicated. For more details see Methods section.

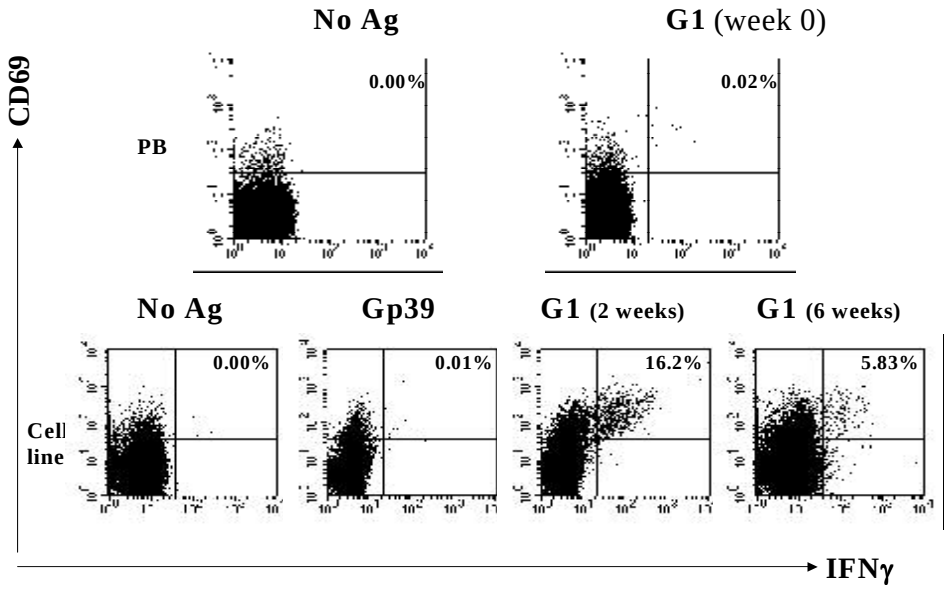


Figure 17. Example of an antigen-specific response to the G1-domain of the proteoglycan aggrecan at different time points (2 or 6 weeks in culture) from the same **ankylosing spondylitis**. For the cell line, after staining for T cell surface markers and intracellular cytokines and after gating out antigen presenting cells according to CFDA fluorescence in a fluorescence 2 versus fluorescence 1 plot, a gate for CD4+ T cells was set. The percentage of IFNγ/CD69-double-positive cells of the CD4+ T cell subpopulation is indicated.

surface staining) was present after second MACS in the G1 stimulated fraction, but in the CD4 T cell fraction without stimulation just few of IFNγ$^+$ CD4 T cells were obtained (2.81%). After several days of culture of G1-specific T cells isolated by MACS, a good antigen specificity was shown upon restimulation of this cell line with the G1 protein. An example is shown in Fig.17 indicating that 16.2% of G1-triggerred IFNγ$^+$ CD4 T cell could be observed in the G1-specific cell line at 2 weeks of culture. This frequency of IFNγ-positive T cells was much higher than that (0.02%) obtained by direct stimulation of PB of the same patient at baseline. What is more, another antigen (gp39) is not stimulatory to this cell line (Fig.17). However, it seems that the antigen specificity of the cell line became weaker after a 6 weeks in culture. Figure 17 shows that just 5.83% of G1-triggerred IFNγ$^+$ T cells was detected in the same cell line after 6 weeks of culture.

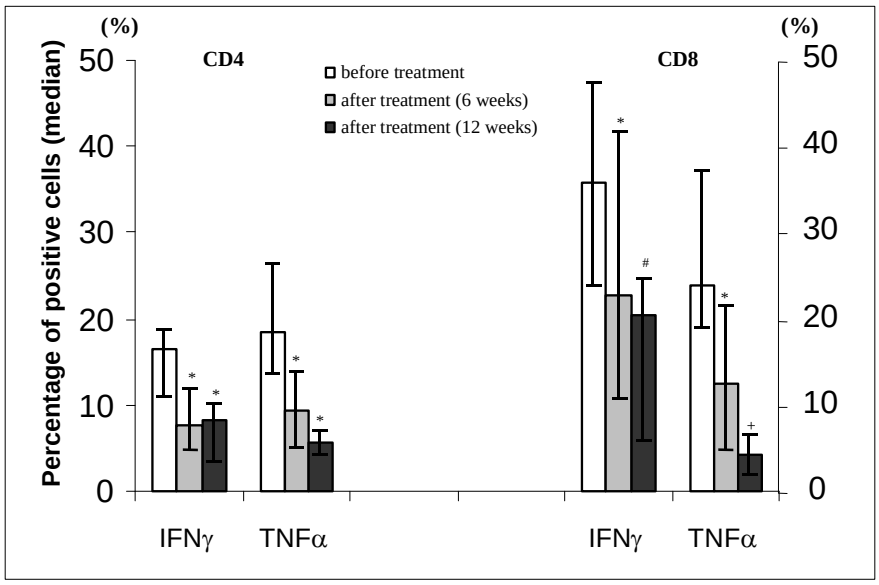
8. Monitoring of cytokine productions of CD4$^+$ and CD8$^+$ T cells after infliximab treatment

8.1 Infliximab treatment induces a decrease in the number of IFNγ and TNFα positive T cells after non-specific in vitro-stimulation.

For the infliximab group, treatment with 2 infusions of infliximab resulted in a significant decrease in the number of IFNγ- and of TNFα- positive T cells upon PMA/ionomycin stimulation after 6 weeks compared to before treatment both in the CD4+ subpopulation [median (25^{th} – 75^{th} percentile) IFNγ: 16.5% (11.1 – 18.8%) vs 7.8% (4.9 – 12%), p<0.005; TNFα: 18.4% (13.5 – 26.4%) vs 9.3% (5.2 – 14%), p<0.005.] and in CD8$^+$ subpopulation [IFNγ: 35.7% (23.8 – 47.4%) vs 22.8% (10.7 – 41.9%), p<0.005; TNFα: 23.8% (18.9 – 37.2%) vs 12.4% (4.7 –21.6%), p<0.005] (Fig.18). An example for the downregulation of IFNγ and TNFα production by infliximab is shown in Fig.19.

After patients had received the third infliximab infusion at week 6, no further decrease in the production of IFNγ [8.12% (3.4 – 10.2%)], but a non-significant decrease in the production of TNFα [5.62% (4.1 – 7.2%)] by CD4+ T cells could be observed at week 12 after non-specific stimulation in vitro. The difference was significant when the numbers before and after treatment at 12 weeks were compared

(p<0.005 for both cytokines) (Fig.18). For the cytokine production of $CD8^+$ T cells, a further significant decrease in the frequency of IFNγ and TNFα secreting cells was shown after 12 weeks [IFNγ: 20.36% (5.6 – 24.6%) , p<0.005 compared to the value before treatment and p=0.037 compared to that after 6 weeks; TNFα: 4.34% (2.12 – 6.6%), p<0.005 compared both to the value before treatment and to that after 6 weeks] (Fig.18) .



*p<0.005, comparing with that before treatment
#p<0.005, comparing with those both before treatment and at weeks
+p<0.005, comparing with that before treatment and p=0.037, comparing with that at weeks

Figure 18. Comparison of non-specific cytokine production between before and after infliximab treatment (at 6 weeks and 12 weeks)**.** A significant decrease of production of IFNγ and of TNFα by $CD4^+$ and $CD8^+$ T cells, upon PMA/ionomycin stimulation, was observed after infliximab treatment. The medians and range of non-specific cytokine production are indicated in the figure.

In contrast, no change of IFNγ or TNFα-secretion was observed during treatment with placebo upon PMA/IONO stimulation (Fig.20) [(before treatment vs placebo treatment at 6 weeks) IFNγ: 12.99% (8.55 – 14.04%**)** vs 11.25% (7.51 – 17.82%) and TNFα: 9.03% (6.18 – 10.14%**)** vs 9.93% (7.11 – 12.97%) by CD4+ T cells, p>0.05 for both cytokines]; The correspondent number of cytokine-positive CD8+ T cells was: IFNγ: 23.67% (14.99 – 36.74%**)** vs 29.38% (13.38 – 38.78%); TNFα: 8.59 % (5.71 – 18.18%) vs 9.27% (7.24 – 20.41%), p>0.05 for both cytokines.

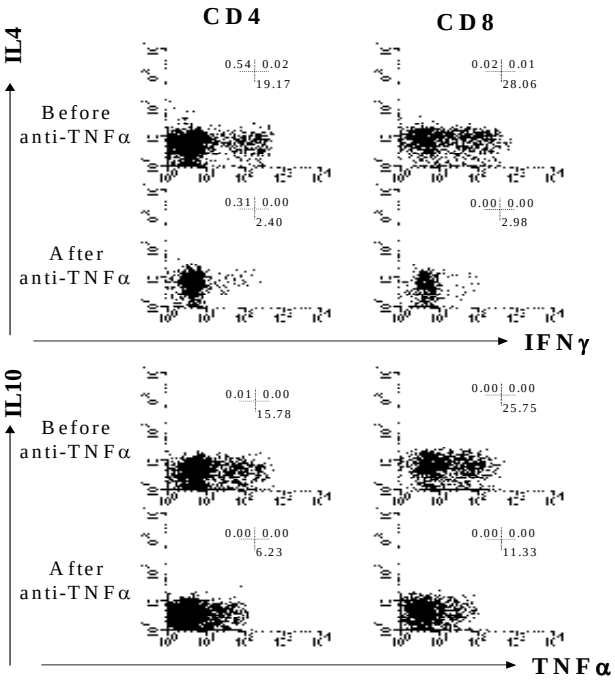
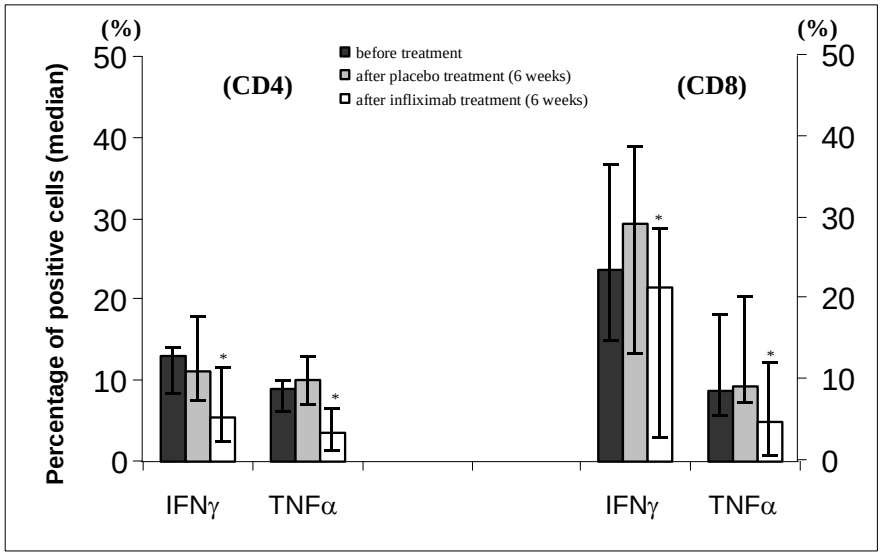


Figure 19. Example of the decrease of non-specific cytokine production by infliximab upon PMA/ionomycin stimulation in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines the gate for $CD8^+$ T cell and indirect gate for $CD4^+$ T cell were set. The percentage of IFNγ, IL4, TNFα, IL10, IFNγ/IL4- or TNFα/IL10-double-positive cells of the $CD4^+$ or $CD8^+$ T cell subpopulation is indicated.



*p<0.02, comparing with those before treatment and after placebo treatment.

Figure 20. Comparison of non-specific cytokine production between before and after placebo/infliximab treatment. A similar non-specific cytokine production was detected in time points of without and with placebo treatment, but a significant decrease of production of IFNγ and of TNFα by $CD4^+$ and $CD8^+$ T cells, upon PMA/ionomycin stimulation, was observed again after the patients accepted infliximab treatment. The medians and range of non-specific cytokine production are indicated in the figure.

After patients from placebo group were treated with infliximab, again a significant downregulation of antigen non-specific cytokine production by CD4 and CD8 was observed after 6 weeks [IFNγ: 5.36% (2.52 – 11.78%) by CD4 and 21.4% (2.9 – 28.9%) by CD8; TNFα: 3.53% (1.44 – 6.65%) by CD4 and 4.95% (0.81 – 12.24%) by CD8, $p<0.05$ in comparison with those before treatment for both cytokines] (Fig.20)

8.2 Infliximab treatment induces a decrease of IFNγ and TNFα by T cells after antigen-specific in vitro-stimulation.

A higher number of IFNγ- and TNFα- positive CD8+ T cells was detected than those of CD4+ T cells upon G1 peptides stimulation in all 20 AS patients before treatment (IFNγ: mean ± SD, 0.62% ± 0.46% vs 0.12% ± 0.11%, $p<0.005$; TNFα: 0.52% ± 0.4% vs 0.18% ± 0.2%, $p<0.05$). An example is shown in Fig.21.

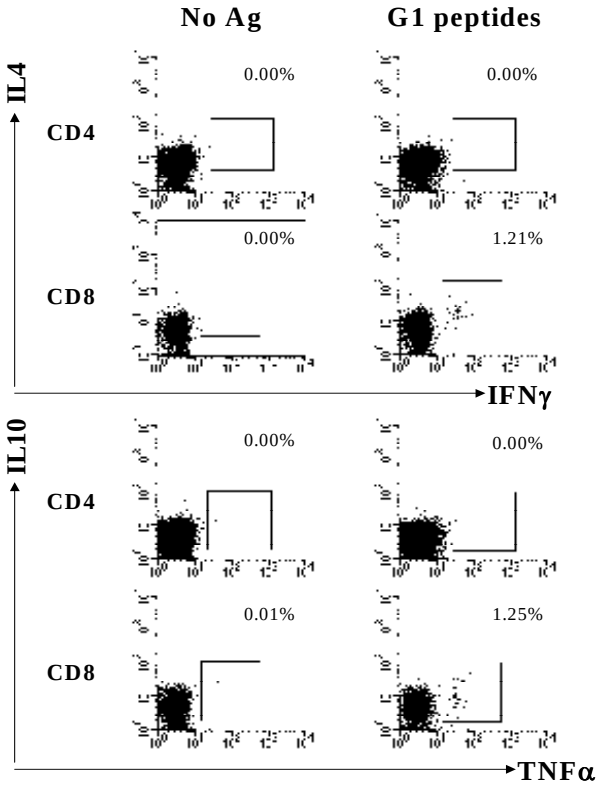
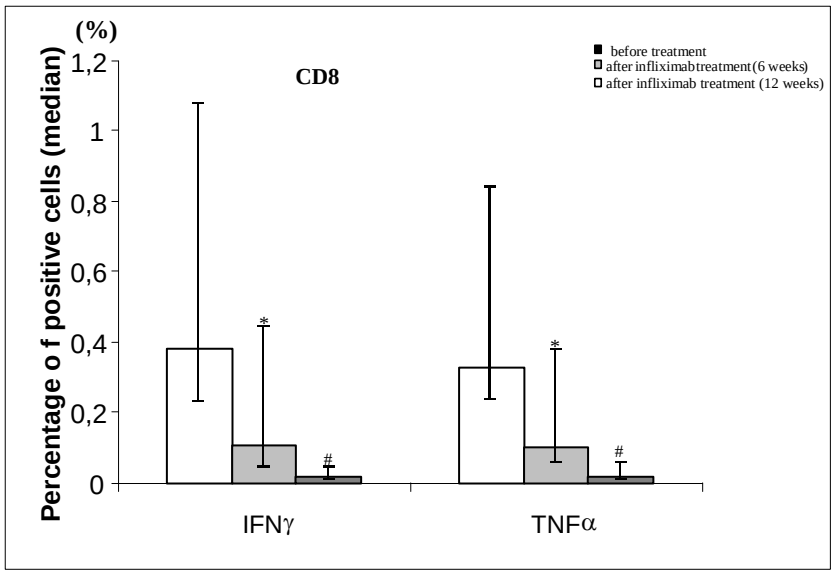


Figure 21. Example of an antigen-specific response to the G1 peptides compared to stimulation without antigen (Ag) in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines the gate for $CD8^+$ T cell and the indirect gate for $CD4^+$ T cell were set. The percentage of IFNγ and TNFα positive cells of the CD4+ or CD8+ T cell subpopulation is indicated.

Upon specific stimulation with G1-peptides, there was a significant reduction after 6 weeks of treatment for IFNγ positive CD8+ T cells (before treatment vs after treatment) [0.38% (0.23 – 1.08%) vs 0.11% (0.05 – 0.45%), p<0.02) and for TNFα positive CD8+ T cells [0.33% (0.24 – 0.84%) vs 0.1% (0.06 – 0.38%), p<0.02) (Fig.22). An example for the decrease in IFNγ- and TNFα-positive CD8+ T cells after G1-specific stimulation is shown in Fig.23. In comparison to $CD8^+$ T cells, however, the decrease in the numbers of IFNγ- or TNFα-positive $CD4^+$ T cells [IFNγ: 0.08% (0.03 – 0.17%) vs 0.05% (0.017 – 0.1%), p>0.05; TNFα: 0.08% (0.06 – 0.3%) vs 0.04% (0.017 – 0.1%), p>0.05] during treatment was not significant.

I also found a further decrease in the G1-specific IFNγ- and TNFα-positive $CD8^+$ T cells after 12 weeks of treatment [IFNγ: 0.015% (0.01 –0.05%); TNFα: 0.02% (0.01 – 0.06%), p<0.002 in comparision with that before treatment and p<0.05 in comparision with that at 6 weeks] (Fig.22). However, again no significant change in the number of cytokine-producing CD4+ T cells was detected.



*p<0.02, comparing with that before tretment .
#p<0.002, comparing with that before treatment and p<0.005, comparing with that at 6 weeks.

Figure 22. Comparison of the antigen-specific cytokine production between before and after infliximab treatment (at 6 weeks and 12 weeks). A significant decrease of the production of IFNγ and of TNFα by $CD8^+$ T cells, upon G1 peptides stimulation, was observed after infliximab treatment. The medians and range of antigen-specific cytokine production are indicated in the figure.

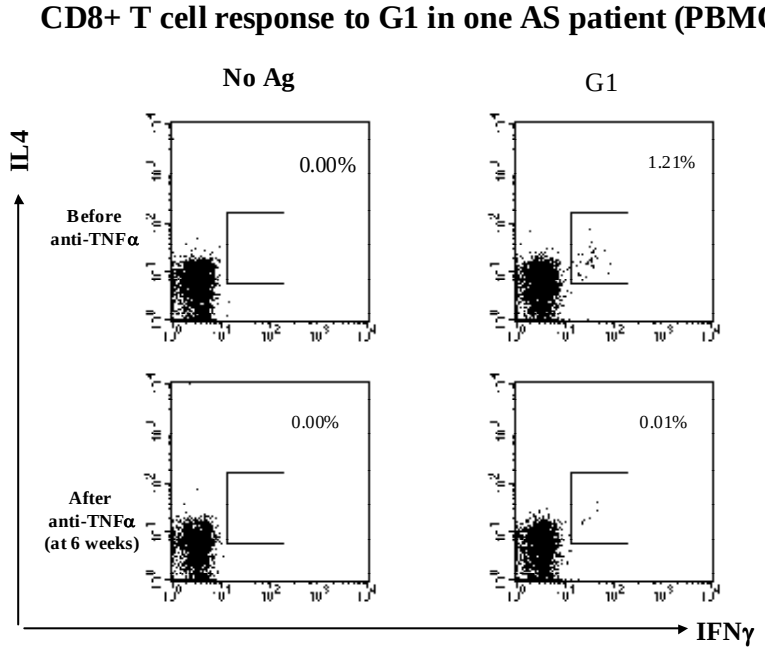
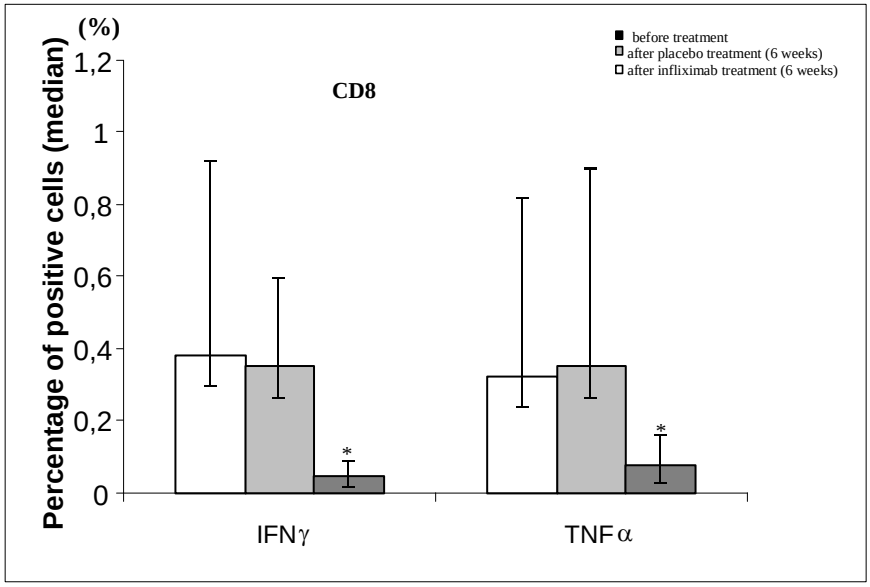


Figure 23. Example of decrease of antigen-specific cytokine production during infliximab treatment upon stimulation with G1 peptides in a patient with **ankylosing spondylitis**. After staining for T cell surface markers and intracellular cytokines the gate for $CD8^+$ T cells and indirect gate for $CD4^+$ T cells were set. The percentage of IFNγ and TNFα positive cells of the $CD8^+$ T cell subpopulation is indicated.



*p<0.02, comparing with those before treatment and after placebo treatment .

Figure 24. Comparison of antigen-specific cytokine production between before and after 6 weeks of placebo/infliximab**.** A similar antigen-specific cytokine production was detected before and after 6 weeks of placebo treatment, but a significant decrease of the production of IFNγ and of TNFα by $CD8^+$ T cells, upon G1 peptides stimulation, was observed again after the treatment was switched to infliximab (at 6 weeks of infliximab treatment). The medians and range of non-specific cytokine production are indicated in the figure.

In patients treated with placebo, no significant difference in the antigen-specific cytokine production between before and after placebo treatment was observed. For $CD4^+$ T cells, IFNγ: 0.07% (0.02 –0.14%) vs 0.06% (0.015 – 0.14%); TNFα: 0.08% (0.03 – 0.2% vs 0.09% (0.03 – 0.23%), p>0.05; for $CD8^+$ T cells, IFNγ: 0.38% (0.30 – 0.92%) vs 0.35% (0.26 – 1.0%); TNFα: 0.32% (0.24 – 0.82%) vs 0.35% (0.26 – 0.9%), p>0.05 for both cytokiens (Fig.24). After the patiens were switched to infliximab treatment, an significant decrease of antigen specific cytokine production by CD8 upon G1-peptides stimulation was observed [IFNγ: 0.05% (0.02 – 0.09%) and TNFα: 0.08% (0.03 – 0.16%), p<0.02 comparing with those at baseline for both cytokines (Fig.24)].

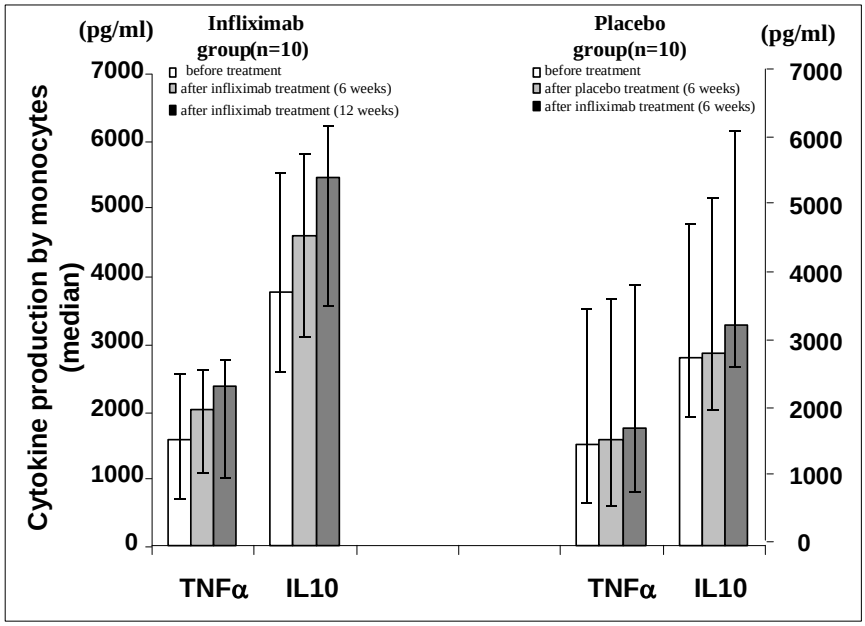
8.3 No change in IL-4- or IL-10-positive T cells during treatment

In both the infliximab and the placebo group, no significant change of antigen non-specific IL4 and IL10-secretion was observed during treatment (data not shown, p>0.05). G1-peptides-specific stimulation of T cells did not induce IL-4 or IL-10-secretion before or after treatment.

9. Effect of infliximab treatment on cytokine production by monocytes

No significant change in the numbers of cytokine-production by monocytes was observed in the infliximab group after 6 weeks upon in vitro stimulation with LPS (Fig.25) [median (25^{th} – 75^{th} percentile), before vs after treatment, TNFα: 1581.28 pg/ml (694.14 – 2531.85 pg/ml) vs 2024.42 pg/ml (1086.02 – 2610.05 pg/ml); IL10: 3765 pg/ml (2570 – 5525 pg/ml) vs 4605 pg/ml (3082.5 – 5815pg/ml) (p>0.05 for both cytokines)]. Also after 12 weeks of treatment, no significant change of cytokine production by monocytes was observed: TNFα: 2368 pg/ml (1011.5 – 2765.52 pg/ml); IL10: 5460.2 pg/ml (3542.1 – 6230.8 pg/ml), p>0.05 comparing with that before treatment for both cytokines. For the placebo group, a similar amount of cytokine production upon LPS stimulation was detected before and after placebo treatment [median (25^{th} – 75^{th} percentile), before vs after placebo treatment, TNFα: 1483.96 pg/ml (610.72 – 3500.67 pg/ml) vs 1575.18 pg/ml (608.77 – 3666.32 pg/ml); IL10: 2785 pg/ml (1917.5 – 4782.5 pg/ml) vs 2869.32 pg/ml (2032.5 – 5167.5pg/ml) (p>0.05

for both cytokines) (Fig.25)]. After the patients of the placebo group were switched to infliximab, again, there was still no marked change in the cytokine secretion (Fig.25) [median (25^{th} – 75^{th} percentile), TNFα: 1751.58 pg/ml (757 – 3866.35 pg/ml); IL10: 3280 pg/ml (2632.5 – 6167.5pg/ml) (p>0.05, in comparison with those before and after 6 weeks of placebo treatment for both cytokines)].



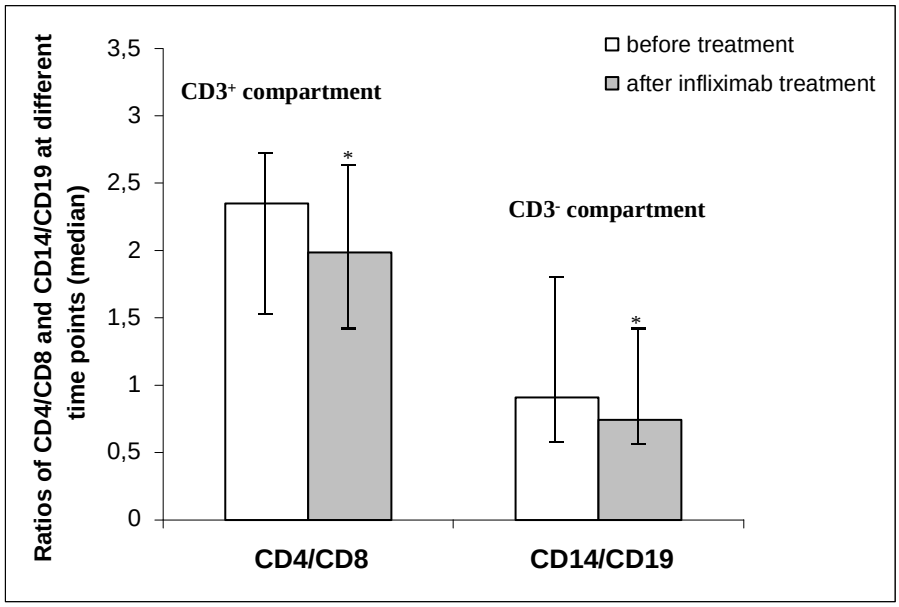
*p>0.05, there is no significant difference between before and after treatment for both cytokines in two groups.

Figure 25. Comparison of non-specific cytokine production by ,monocytes between before and after placebo/infliximab treatment. A similar non-specific cytokine production upon LPS stimulation was detected between before and after infliximab or between before and after placebo. The medians and range of non-specific cytokine production are indicated in the figure.

10. Change of ratio of CD4/CD8 in $CD3^+$ cells and of CD14/CD19 in $CD3^-$ cells after infliximab treatment

The cells at week 0 and week 6 after infliximab treatment from all 20 patients were investigated for the percentage of lymphocytes and macrophages. After the cells were separated into $CD3^+$ and $CD3^-$ fractions by MACS, the relative numbers of CD4 and CD8 cells were detected inside the $CD3^+$ fraction, and of CD14 and CD19 cells inside the $CD3^-$ fraction by flow cytometry. Subsequently, the ratio of CD4/CD8 cells was calculated in the $CD3^+$ population and CD14/CD19 cells in the $CD3^-$ population. The results indicated that 10 out of 20 patients had a decrease of CD4/CD8 ratio and the others had an increase after infliximab treatment. The general

ratio [median (25^{th} – 75^{th} percentile), before vs after treatment with infliximab]: 2.35 (1.53 – 2.72) (mean 2.23) vs 1.98 (1.42 – 2.63) (mean 2.13) (Fig.26), thus there was no clear difference (p>0.05). For $CD3^-$ cells, a decrease of CD14/CD19 ratio was observed in 13 out of 20 patients, an increase in 7 out 20 patients; again the general ratio did not show a significant difference [0.91 (0.57 – 1.81) vs 0.74 (0.56 – 1.4), p>0.05, Fig.26].



*p>0.05, comparing with that before infliximab treatment.

Figure 26. Comparison for ratios of CD4/CD8 and CD14/CD19 between before and after infliximab treatment. After $CD3^+$ and $CD3^-$ T cells were separated by MACS, a similar ratios of CD4/CD8 in the $CD3^+$ compartment and of CD14/CD19 in the $CD3^-$ compartment was detected by flow cytometric analysis between before and after infliximab, The medians and range of CD4/CD8 and CD14/CD19 ratios are indicated in the figure. For more details see methods.