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Interleukin-17 synergizes with IFNγ or TNFα to promote inflammatory mediator release and intercellular adhesion molecule-1 (ICAM-1) expression in human intervertebral disc cells

Mostafa A. Gabr M.D.[2]
Liufang Jing M.Sc.[1]
Antonia R. Helbling B.S.[1]
S. Michael Sinclair B.S.[1]
Kyle D. Allen Ph.D. [2]
Mohammed F. Shamji M.D., Ph.D.[1,3]
William J. Richardson M.D.[2]
Robert D. Fitch M.D.[2]
Lori A. Setton Ph.D.[1,2]
Jun Chen Ph.D.[1]

[1]Department of Biomedical Engineering, Duke University, Durham, NC

[2]Division of Orthopaedic Surgery, Duke University Medical Center, Durham, NC

[3]Division of Neurosurgery, The Ottawa Hospital, Ottawa, ON

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Running Title:
Effects of interleukin-17 on intervertebral disc cells *in vitro*

Corresponding Author
Jun Chen Ph.D.
Department of Biomedical Engineering
Duke University
Box 90281, 136 Hudson Hall
Durham, NC 27708-0281
Ph: 919-660-5131
FAX: 919-681-8490
Email: junchen@duke.edu

Abstract

Interleukin-17 (IL-17) is a cytokine recently shown to be elevated, along with interferon-γ (IFNγ) and tumor necrosis factor (TNFα), in degenerated and herniated intervertebral disc (IVD) tissues, suggesting a role for these cytokines in intervertebral disc disease. The objective of our study was to investigate the involvement of IL-17 and costimulants IFNγ and TNFα in intervertebral disc pathology. Cells were isolated from anulus fibrosus and nucleus pulposus tissues of patients undergoing surgery for intervertebral disc degeneration or scoliosis. The production of inflammatory mediators, nitric oxide (NOx), prostaglandin E2 (PGE2) and interleukin-6 (IL-6), as well as intercellular adhesion molecule (ICAM-1) expression, were quantified for cultured cells following exposure to IL-17, IFNγ and TNFα. Intervertebral disc cells exposed to IL-17, IFNγ or TNFα showed a remarkable increase in inflammatory mediator release and ICAM-1 expression (GLM and ANOVA, $p<0.05$). Addition of IFNγ or TNFα to IL-17 demonstrated a synergistic increase in inflammatory mediator release, and a marked increase in ICAM-1 expression. These findings suggest that IVD cells not only respond with a catabolic phenotype to IL-17 and costimulants IFNγ and TNFα, but also express surface ligands with consequent potential to recruit additional lymphocytes and immune cells to the IVD microenvironment. IL-17 may be an important regulator of inflammation in the IVD pathologies.

Introduction

Annually, 5.7 million individuals develop an intervertebral disc (IVD) related disease in the U.S. alone [1]. Low back pain (LBP) is an increasingly common and costly health problem, and as many as 40% of these cases are the result of IVD pathologies [2-6]. IVDs contribute to load support and flexibility in the spine, with a collagen-proteoglycan extracellular matrix that is maintained by a sparse population of fibrochondrocyte-like and nucleus pulposus cells [7-8]. Intervertebral disc degeneration is characterized by pronounced anatomic and biologic changes including loss of cellularity, decreased matrix hydration and loss of disc height, in a process that is strongly associated with aging [8-9]. Elevated inflammatory mediators and pro-inflammatory cytokines have been documented in tissues of the degenerated and herniated IVD such as interleukin-6 (IL-6), interleukin-8 (IL-8), prostaglandin E2 (PGE2) and nitric oxide (NOx) [10]; as well as key pathology regulatory cytokines including tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and interleukin-1beta (IL-1β) [11-15]. In the same context, histological evaluation of herniated disc tissue revealed elevated inflammatory cell infiltrations with abundant levels of macrophages- producers of majority of the previously mentioned cytokines [16-19]. When cultured in vitro, tissues of the degenerated and herniated discs have been shown to express higher levels of pro-inflammatory mediators including IL-6, PGE2 and NOx [15-20]. Intercellular adhesion molecule-1 (ICAM-1, aka CD-54) [21-22] has also been found in herniated IVD tissues [23], suggesting that inflammation-associated products in the herniated IVD may use ICAM-1 to attract effector cells of the immune system. Together these studies reveal a role for inflammation in mediating the degeneration of IVD in a process that is not yet fully understood.

Recent work by our group has revealed an elevated expression level for interleukin-17 (IL-17) in tissues obtained from degenerated and herniated IVD [11], suggesting a role for IL-17 in contributing to IVD pathology. IL-17 is a cytokine [24], produced by a newly identified arm of the T_H effector cell family termed T_H17 [25-26]. Historically, T-lymphocyte populations have been dichotomized between T_H1 and T_H2 phenotypes; T_H1 cells produce mainly IFNγ [27] and T_H2 cells produce mainly IL-4 (also IL-5, IL-13 and IL-25) [28]. A dysregulated T_H1 response has been identified with tissue inflammation and organ-specific autoimmunity [29]. Questions have emerged, however, when studies discovered that IL-17-producing $CD4^+$ T_H17 cells, not IFNγ –producing T_H1 cells, were responsible for inflammation in two autoimmune disease mouse models, experimental autoimmune encephalomyelitis and type II collagen induced arthritis [26,30-31]. These findings have suggested a potential role for T_H17 cells in regulation of multiple inflammatory processes [26,32-34]. The presence of the T_H17 cell product, IL-17, in IVD tissues suggests that this inflammatory pathway may also play a role in IVD pathology. Cells of other fibrocartilaginous tissues and articular cartilage, including fibroblasts and chondrocytes, as well as macrophages and synoviocytes, have increased inflammatory phenotypes in response to IL-17 alone or IL-17 combined with IFNγ or TNFα [24,35-39], suggesting that IVD cells may exhibit a similar response to these pro-inflammatory cytokines.

In our current study, we hypothesized that IL-17 can induce an inflammatory phenotype in IVD cells. Human cells isolated from anulus fibrosus and nucleus pulposus regions of the IVD were exposed to IL-17 and costimulants IFNγ and TNFα in culture, and their production of the inflammatory mediators NOx, PGE2 and IL-6, was quantified. In addition, the effects of IL-17 on inflammatory cell chemotaxis through ICAM-1 molecule expression were quantified for cells in separate cultures. The findings of this work reveal a significant role for IL-17 in

upregulating IVD cell inflammatory mediator release and ICAM-1 and suggest that IFNγ and TNFα act synergistically to elevate an inflammatory phenotype in the presence of IL-17.

Materials and Methods

IVD Tissue and Cell Isolation

Human lumbar IVD tissues were obtained as to-be-discarded surgical waste from patients undergoing surgery for degenerative disc disease (total n=5 patients, age 37-62 yo) or scoliosis (total n=2 patients, age 14 & 19 yo); following procedures classified as non-human subjects research, these tissues were de-anonymized and only data for patient age, gender and race were recorded. Disc tissues were well rinsed with wash medium (DMEM-high glucose (Gibco BRL, Grand Island, NY) with gentamycin, kanamycin and Fungizone) and grossly separated into anulus fibrosus (AF) and nucleus pulposus (NP) according to the anatomic appearance. Any tissues containing endplate bone or cartilage were discarded prior to cell isolation. Cells were isolated via a sequential pronase-collagenase digestion as described previously [40], and cultured on gelatin-coated tissue culture plastic with Ham's F12 media (Gibco) with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (culture medium) at 5% CO_2 and 37°C. All cells were seeded at a density of approximately 25,000 cells per cm^2 and passaged when 90% confluence was reached. Cells for all experiments were used after two passages.

Inflammatory Mediator Release

Separate AF and NP cells were plated at a density of 50,000 cells per well (approximately 66,000 cells per cm^2) in 48-well plates (n=4 replicates) and overlaid with 150 μL of culture medium (F12 with 10% FBS and other supplements as described above) for overnight culture. In order to determine an optimal IL-17 dose for all experiments, the responsiveness of a single patient IVD

cell production of nitric oxide (NOx) was first evaluated over a broad range of IL-17 doses (Figure 1). After overnight culture, cells were overlaid with 300 µL of fresh culture media containing IL-17 (0-300 ng/mL) supplemented with IFNγ co-stimulant (200 U/mL) [41], and cultured for an additional 72 hours. Nitric oxide (NOx) was measured in the collected supernatant using the Griess reaction [42]. Both AF and NP cells were found to increase their production of NOx over the 72 hour culture period in the presence of IL-17, with a maximal effect noted for IL-17 doses higher than 10 ng/ml (Figure 1). For this reason, additional studies were performed at a single dose for IL-17 of 10 ng/ml.

For all experiments, separate AF and NP cells from each patient (50,000 cells per well in 48-well plates, n=4 replicates) were cultured overnight in culture medium (F12 with 10% FBS and other supplements as described above) prior to the start of the experiment. Cells were then overlaid with 300 µL of fresh culture media containing one of the following final cytokine concentrations: no exogenous cytokine (control), TNFα (25 ng/mL), IL-17 (10 ng/mL), IFNγ (200 U/mL), a combination of IL-17 (10 ng/mL) and IFNγ (200 U/mL), or a combination of IL-17 (10 ng/mL) and TNFα (25 ng/mL). After 72 hours of cytokine treatment, supernatant was collected and evaluated for release of NOx as described above, prostaglandin E2 (PGE2) (Parameter™, PGE2 ELISA Assay, R&D Systems, Minneapolis, MN) and interleukin-6 (IL-6) (Quantikine®, human IL-6 immunoassay ELISA, R&D Systems).

Flow Cytometry Analysis for ICAM-1 Expression

A subset of AF and NP cells were cultured for flow cytometry analysis ($0.4\text{-}0.5 \times 10^6$ cells/well at a density of approximately 52,000 cells per cm^2 in 6-well plates) at 5% CO_2 and 37°C overnight. After overnight culture, FBS was withdrawn by replacing the overlying media with fresh serum-free medium (F12 media with 1x Insulin-Transferrin-Selenium, Gibco) for 16 hours, after which

the media was replaced with 2 mL of fresh serum-free media containing cytokine supplements as described above. Following 72 hours of culture in cytokine-containing media, cells were detached using 0.025% Trypsin/EDTA (Lonza, Switzerland) and allowed to recover in culture medium (F12 media with 10% FBS) for 30 minutes at 37°C. Cells ($5x10^5$) were then incubated in suspension with a mouse anti-human ICAM-1 (CD54) monoclonal antibody (AbD Serotec, Raleigh, NC) and appropriate isotype control followed by incubation with secondary antibodies (AlexaFluor 488, Molecular Probes, Eugene, OR). Cells from each group were analyzed for fluorescence (FACscan; Becton Dickinson, Franklin Lakes, NJ) to quantify the percentage of cells with positive (+) surface proteins and mean fluorescence intensity (MFI).

Statistical Analysis

A generalized linear model (GLM) approach was used to test for differences between treatment groups in NOx, PGE2 and IL-6 production for AF and NP cells separately. The GLM was designed to consider the individual effects of IL-17, TNFα, and IFNγ individually or through two interaction terms (IL-17+IFNγ or IL-17+TNFα). Synergism was detected as a significant effect in one of the two individual terms plus a significant and positive effect in the interaction term. Providing significance within the GLM, a post-hoc Tukey's HSD test was performed to investigate differences between groups. The values for percentage of cells positive (+) for ICAM-1 and MFI of ICAM-1 labeling were analyzed using one way ANOVA with a Post-Hoc Tukey's HSD test separately for AF and NP cells. Statistical significance is reported at a level of 0.05.

Results

Effect of IL-17 on Inflammatory Mediator Production.

When exposed to different cytokine treatments, both AF and NP cells responded to IFNγ, TNFα and IL-17, with a significant increase in the production of NOx compared to no cytokine controls (Figure 2, GLM, $P<0.05$, n=7 patient samples). In the presence of IFNγ costimulant, IL-17 induced significant increases in NOx production that were between 6 and 10-fold higher than that of IL-17 alone (Figure 2, GLM, $P<0.05$, n=7 patient samples, post-hoc Tukey's HSD). There was evidence of a synergistic increase of NOx release in both AF and NP cells when IFNγ was added to IL-17 as a costimulant (Figure 2, GLM, $P<0.05$, post-hoc Tukey's HSD). In the presence of TNFα costimulant, IL-17 induced statistically significant increases in NOx production above that of IL-17 or TNFα alone for NP cells only (Figure 2, GLM, $P<0.05$, n=3 patient samples, post-hoc Tukey's HSD). Finding of an increase in NOx levels with culture in IL-17 and TNFα was observed for AF cells as well, although this finding was not statistically significant (Figure 2, GLM, $p<0.05$, post-hoc Tukey's HSD). As for the IFNγ costimulant, there was evidence that IL-17 and TNFα co-stimulation was synergistic but for NP cells only (Figure 2).

Both AF and NP cells responded to TNFα or IL-17 alone, but not IFNγ, with a significant increase in the production of PGE2 compared to unstimulated controls (Figure 3a, GLM, $P<0.05$, n=7 patient samples). In the presence of IFNγ costimulant, IL-17 induced statistically significant increases in PGE2 production above that of IFNγ alone, but not above that of IL-17 alone for both AF and NP cells (Figure 3a, GLM, $P<0.05$, n=7 patient samples, post-hoc Tukey's HSD). In the presence of TNFα costimulant, however, IL-17 did not induce PGE2 production above that of IL-17 or TNFα alone.

The responsiveness of AF and NP cell production of IL-6 had a similar pattern to that observed for PGE2 (except IFNγ). AF and NP cells responded to IFNγ (NP only), TNFα or IL-17 alone, with a statistically significant increase in IL-6 production in comparison to unstimulated controls (Figure 3b, GLM, $P<0.05$, n=7 patients samples). In the presence of IFNγ costimulant, IL-17 induced statistically significant increases in IL-6 production above that of IFNγ alone, but not above that of IL-17 alone for both AF and NP cells (Figure 3b, GLM, $P<0.05$, n=7 patient samples, post-hoc Tukey's HSD). In the presence of TNFα costimulant, however, IL-17 did not induce IL-6 production above that of IL-17 or TNFα alone.

Effect of IL-17 on Cell Surface ICAM-1 Expression.

Treatment with TNFα or IFNγ alone, but not IL-17, significantly elevated ICAM-1 expression in both AF and NP cells, as noted by increased percentage of positive (+) cells (Figure 4b and Table 1) and MFI for ICAM-1 (Figure 4a and c, and Table 1). The effect of TNFα on MFI was greater than that of IFNγ in both cell types (Figure 4a and c, and Table 1). In the presence of IFNγ costimulant, but not TNFα, IL-17 significantly increased MFI above values induced by IL-17 alone in AF cells, and IL-17 or IFNγ alone in NP cells (Figure 4a, c and Table 1).

Discussion

The results of this study demonstrate that human IVD cells respond to IL-17 and pro-inflammatory costimulants, TNFα and IFNγ, by increasing production of inflammatory mediators (NOx, PGE2 and IL6) and ICAM-1 expression on the cell surface. The pro-inflammatory action of IL-17 considerably depends on its ability to trigger the expression of

inducible nitric oxide synthase (iNOS), which is responsible for the production of the free gaseous radical, NOx [10,43]. NOx has the ability at lower concentrations to generate or modify intracellular signals affecting the function of immune cells and resident cells of tissues and organs; excessive NOx release, however, often results in inflammatory destruction of the target tissues. In previous studies of chondrocytes and fibrocartilaginous cells and tissues, the expression of iNOS and subsequent NOx release was variably found to be triggered by IL-17 alone and/or additive or synergistic collaboration of the macrophage and T_H1 products, TNFα and IFNγ, respectively [10]. Some studies have reported that IL-17 alone induced NOx production in human cartilage and chondrocytes, osteoarthritic human and mice cartilage; still other studies of similar cell types have demonstrated that costimulation with TNFα, IFNγ or IL-1β appeared to be necessary for promoting IL-17-mediated increases in NOx [10,43]. In our study AF and NP cells exposed to IL-17 alone exhibited a significant increase in the levels of NOx production not unlike that reported previously for human cartilage and chondrocytes. Cells also exhibited a significant increase in the production of the pro-inflammatory cytokines, PGE2 and IL-6. The observed NOx, PGE2 and IL-6 release in AF and NP cells by IL-17 alone without costimulation suggests an important role for IL-17 in regulation of inflammatory processes in IVDs.

TNFα treated cells also exhibited a significant increase in all of the tested inflammatory mediators and cytokines, as has been reported previously for human NP cells [44]. Of interest was the observation that IFNγ treatment alone resulted in minimal effects on inflammatory mediator release in AF and NP cells. Together, these findings suggest the involvement of IL-17, TNFα, and IFNγ to a lesser extent, in regulating IVD inflammation, through induction of release of inflammatory mediators and cytokines in treated cells.

The possibility of an additive or synergistic effect when TNFα or IFNγ are combined with IL-17 during IVD cell treatment was also investigated. Results suggest that costimulation of both AF and NP cells with IL-17 and IFNγ, and costimulation of NP cells only with IL-17 and TNFα may act synergistically to increase NOx release, but not PGE2 or IL-6 production. An increase that was synergistic in NOx release, and additive in PGE2 and IL-6, was observed upon addition of IFNγ costimulant to IL-17 in both AF and NP cells; although the IFNγ alone treatment was unable to trigger any noticeable mediator release. On the other hand, a synergistic increase in production of NOx in NP cells was noticed upon addition of the TNFα costimulant to IL-17; but only additive in AF cells in NOx production and in AF and NP cells in PGE2 and IL-6 production. Suggestions have been made in previous literature that IL-17 receptors (IL-17R) may signal through a JAK/STAT1, MAP kinases or NF-KB pathways that clearly have overlap with the IFNγ and TNFα signaling pathways [10,25]. Other possible explanations for the observed synergism would be an induced stabilization, by the costimulants IFNγ or TNFα, to the cellular transcription process or mRNA; or possibly an increase in the cell surface expression of IL-17R. Future studies of pathway inhibitors will be useful for illustrating which pathways are engaged by IL-17 stimulation in AF and NP cells, and how they are affected by costimulation with IFNγ and TNFα.

Of similar importance to cellular production of inflammatory cytokines, the ability of IL-17 to stimulate cellular expression of surface adhesion molecules for immune cell attraction was also investigated. In our study, cells tested for ICAM-1 expression were FBS starved and a replacement supplement, ITS, was used instead to halt any possible stimulatory effects of FBS on IVD cells surface molecule expression. ICAM-1 expression has been previously reported to be elevated in human corneal epithelial cells exposed to TNFα and IFNγ in vitro [45]. Combined

IL-17 and IFNγ treatment has been shown to up-regulate ICAM-1 expression in human gingival fibroblasts[46] and keratinocytes [47]. In our study, although IL-17 alone resulted in an increase in IVD cell ICAM-1 expression that was lesser in extent than the induced TNFα or IFNγ effect, a major increase in the expression of ICAM-1 was noted when IFNγ was added as a co-stimulant to IL-17. This rise in ICAM-1 expression due to combined IL-17 and IFNγ costimulant treatment was noticed to be higher than the added values for the single IL-17 and IFNγ treatments. These findings hence suggest a presence of common signaling pathways between IL-17 and IFNγ for ICAM-1 expression [47]. In contrast, TNFα treatment alone seemed to induce such a potent increase in ICAM-1 expression that may have obscured any further effects of IL-17 on ICAM-1 expression levels. Although similar doses of TNFα have been used previously to study cytokine effects on IVD cells [48], use of lower TNFα doses may facilitate studies of IL-17 co-stimulation for IVD cells in future studies. Alternatively, the lack of noticeable effect upon addition of IL-17 to TNFα may be explained by an absence of co-stimulator effects for these two cytokines on ICAM-1 expression in IVD cells.

Previous studies by our group have revealed elevated levels of IL-17 in herniated and degenerated human surgical IVD specimens [11]; as well as in rat radiculopathy model [49]. Also, IL-17 was found to be present in non-degenerative specimens; but in lower levels than in the degenerated specimens and the latter having lower levels than the herniated specimens [11]. Such findings can be correlated to the degree of exposure of the tissue to inflammatory processes, with herniated tissue being most exposed to inflammation followed by degenerative tissue followed by the non-degenerate. Although our current study has tested degenerate and non-degenerate samples only; we assume that herniated in vitro samples exposed to IL-17 and costimulatory cytokines IFNγ and TNFα, would demonstrate a higher inflammatory response

than the degenerate and non-degenerate. Also, in spite of the suggestion by our previous studies that IL-17 levels in NP non-degenerate tissue was higher than that in the AF non-degenerate tissue [11], our current study demonstrates similarity in response between AF and NP cells in most of the tested conditions. This finding can be explained either by a possible similarity between AF and NP cell responses to inflammatory mediators, or a potential loss of unique AF and NP phenotypic responses caused by degeneration or in vitro plating and passaging. The pattern of cellular response to the cytokine stimulation was noted to differ between parameters of ICAM-1 expression and inflammatory mediator release. This may be due to differences in signaling events in IVD cells for the cytokines IL-17, IFNγ and TNFα that differentially regulate the inflammatory mediators and the ICAM-1 molecules. Furthermore, the response patterns were similar for cells obtained from both scoliotic and degenerate IVD tissues, although of different baseline magnitudes (cells from 14 and 19 years old scoliotic patients demonstrated higher responses for expression of ICAM-1 as shown in Table 1). Accordingly, the data obtained from both tissue types was combined in order to gain an increased statistical power of analysis.

Finally, the tested IL-17 dose in our current study -based on our dose-response toxicity curve- was noticed to be lower than that used in other reports with different experimenting conditions such as in synoviocytes and cartilage explants [24,50-51]. Also, IFNγ co-stimulant was found necessary in order to obtain a dose-response curve for IL-17. Nonetheless, the relatively low IL-17 dose utilized in this study can be explained by normal variability in physiological characteristics between different cell lines and tissues.

The results of this study demonstrate that human IVD cells respond to IL-17 and costimulants, IFNγ or TNFα, by increasing production of inflammatory mediators and cytokines; as well as demonstrate a chemotactic profile via up-regulation of the inflammatory surface

adhesion molecules, ICAM-1 expression. Together these findings indicate that not only do the cells respond with a catabolic phenotype to the IL-17 and costimulants stimulation, they also express surface ligands with consequent potential to recruit additional lymphocytes and immune cells to the IVD microenvironment. IL-17, as shown by our study, may be an important regulator of inflammatory pathology in the IVD herniation, degeneration or multiple pathologies deserving of further study. Future studies need to isolate the mechanisms by which IL-17 and related cytokines induce the upregulated inflammatory phenotype in AF and NP cells, as well as determine potential effects of IL-17 inhibition on IVD pathology.

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Figure legends

Figure 1. Dose-response curves for nitric oxide (NOx) production by AF and NP cells from human tissue stimulated by varying doses of IL-17, and costimulated with IFNγ (200 U/mL). Mean ± SE (n=4 replicates). Regression analysis yields an estimate of 1 ng/mL for 50% of maximal effect (r^2=0.98).

Figure 2. Values for nitric oxide (NOx) release from cultured human AF and NP cells exposed to IL-17 and costimulants IFNγ and TNFα (mean + SE, GLM, $P<0.05$, n=7 patient samples except for IL-17+TNFα treatment group n=3 patient samples). All treatments (except IFNγ alone) were statistically different from control (* = different from IL-17 alone, # = different from IFNγ alone, ¶ = different from TNFα alone, post-hoc Tukey's HSD; S = statistically significant synergism, a generalized linear model (GLM) with two interaction terms).

Figure 3. Values for (a) prostaglandin E2 (PGE2) and (b) interleukin-6 (IL-6) release from cultured human AF and NP cells exposed to IL-17 and costimulants IFNγ and TNFα (mean + SE, GLM, $P< 0.05$, n=7 patient samples except for IL-17+TNFα treatment group n=3 patient samples). All treatments (except IFNγ alone) were statistically different from control (# = different from IFNγ alone, post-hoc Tukey's HSD).

Figure 4. Intercellular adhesion molecule-1 (ICAM-1) expression levels in cultured AF and NP cells exposed to IL-17 and costimulants IFNγ and TNFα. (a) Representative histograms illustrate the relative fluorescence intensity on x-axis, cell number on y-axis for NP cells. The number appearing in each histogram indicates MFI of the treatment group (shaded area: cytokine stimulation, solid line: unstimulated control). (b) The percentage of cells with positive (+) surface proteins. (c) The mean fluorescence intensity (MFI) of all positive cells. All treatments (except IL-17 alone) were statistically different from control (mean + SE, ANOVA, $p< 0.05$, n=4 patient samples except for TNFα and IL-17+TNFα treatment group n=3 patient samples.* = different from IL-17 alone, # = different from IFNγ alone, post-hoc Tukey's HSD).

Figures

Figure 1.

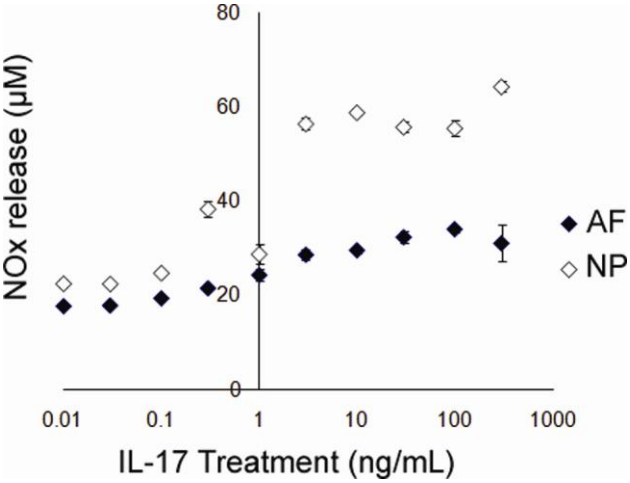

Figure 2.

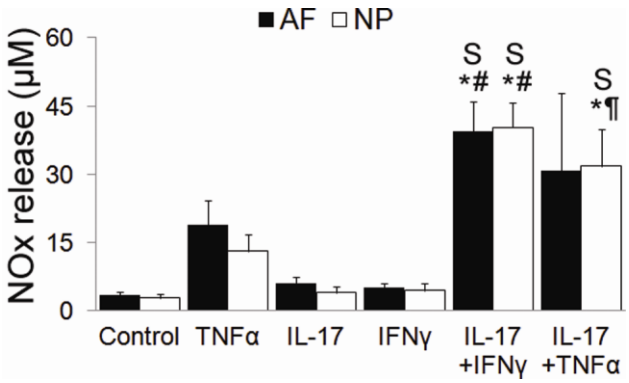

Figure 3.

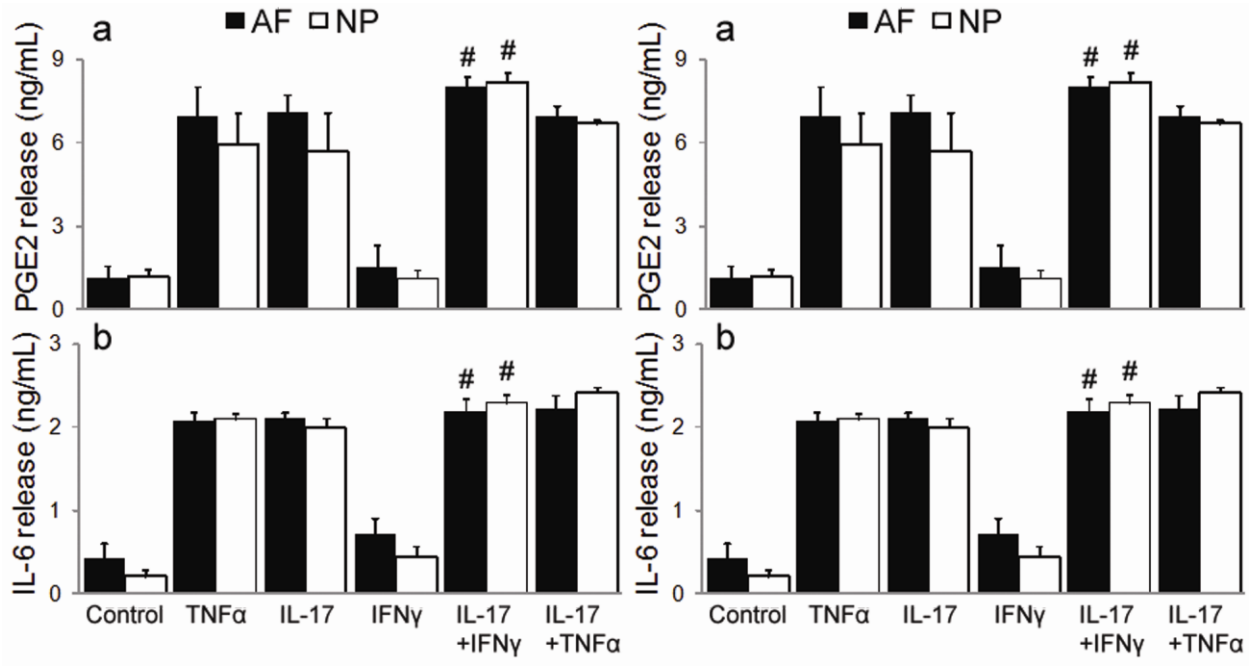

Figure 4.

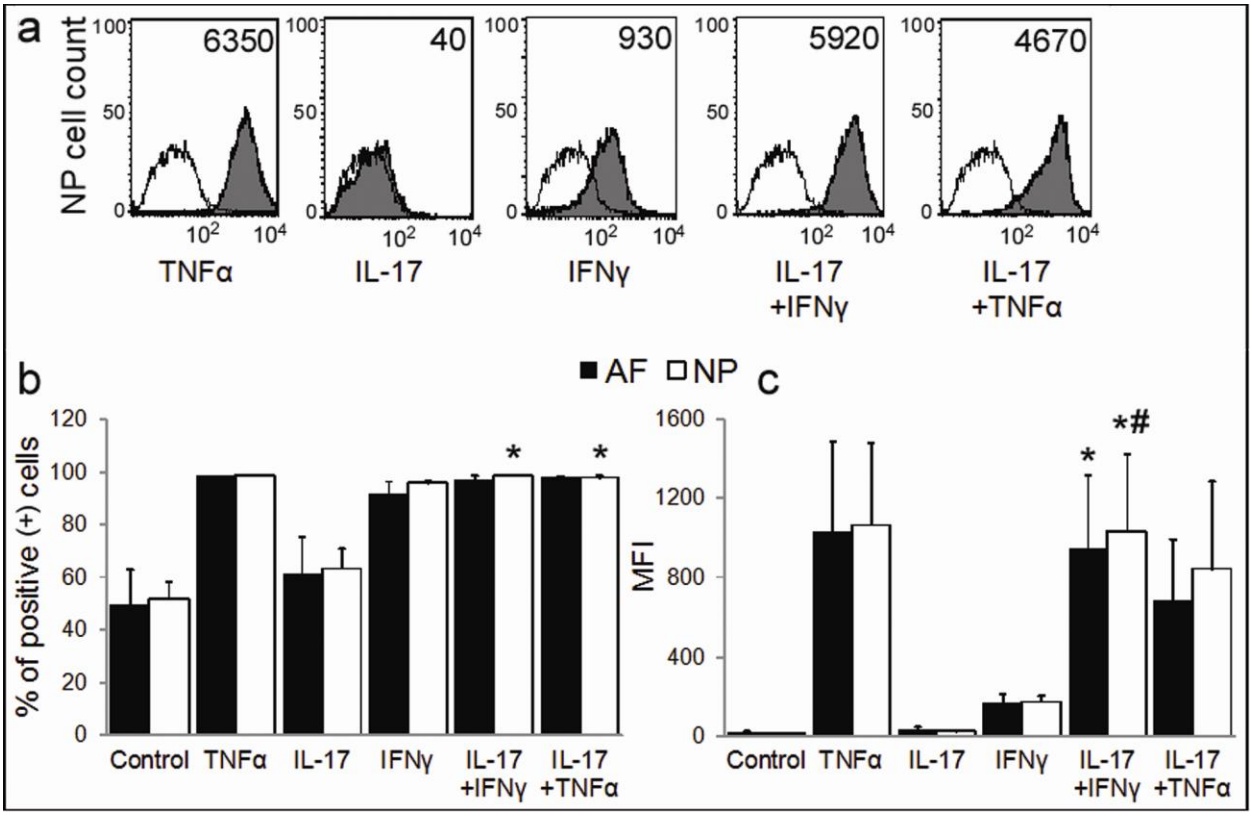

Table 1.

Table 1. Percentage of positive (+) AF and NP cells for ICAM-1 expression and mean fluorescence intensity (MFI) value by flow cytometry analysis in different cytokine treatment groups for individual patients (n=4 patient samples, TNFα and IL-17+TNFα n=3 patient samples). (Y= years old, M=male, F=female, SE= standard error, NT= not tested)

Patient	61Y/F		46Y/F		19Y/M		14Y/F		Average	SE	Average	SE
% (+) cells	*AF*	*NP*	*AF*	*NP*	*AF*	*NP*	*AF*	*NP*	*AF*		*NP*	
Control	64	39	20	59	38	42	77	67	49	13	45	7
TNFα	99	98	NT	NT	99	99	99	99	99	0	85	0
IL-17	70	47	24	74	61	53	91	79	60	14	56	8
IFNγ	98	94	78	97	92	94	98	98	93	5	83	1
IL-17 +IFNγ	99	98	92	98	99	99	99	99	98	2	87	0
IL-17 +TNFα	97	96	NT	NT	98	99	99	99	98	1	84	1
MFI	*AF*	*NP*	*AF*	*NP*	*AF*	*NP*	*AF*	*NP*	*AF*		*NP*	
Control	19	15	25	17	14	11	36	25	20	5	19	3
TNFα	310	329	NT	NT	937	1118	1849	1747	909	455	1049	418
IL-17	23	19	28	26	25	16	69	34	29	11	29	4
IFNγ	153	119	144	233	107	115	284	231	165	39	160	33
IL-17 +IFNγ	356	312	316	604	1238	1103	1867	2103	828	374	972	393
IL-17 +TNFα	118	149	NT	NT	786	734	1155	1646	588	309	767	444