Insights into Gene Modulation by Therapeutic TNF and IFNγ Antibodies: TNF Regulates IFNγ Production by T Cells and TNF-Regulated Genes Linked to Psoriasis Transcriptome

Asifa S. Haider[1], Jules Cohen[1], Ji Fei[2], Lisa C. Zaba[1], Irma Cardinale[1], Kikuchi Toyoko[1], Jurg Ott[2] and James G. Krueger[1]

Therapeutic antibodies against tumor necrosis factor (TNF) (infliximab) and IFNγ (fontolizumab) have been developed to treat autoimmune diseases. While the primary targets of these antibodies are clearly defined, the set of inflammatory molecules, which is altered by use of these inhibitors, is poorly understood. We elucidate the target genes of these antibodies in activated human peripheral blood mononuclear cells from healthy volunteers. While genes suppressed by fontolizumab overlap with known IFNγ-induced genes, majority of genes suppressed by infliximab have previously not been traced to TNF signaling. With this approach we were able to extrapolate new TNF-associated genes to be upregulated in psoriasis vulgaris, an "autoimmune" disease effectively treated with TNF antagonists. These genes represent potential therapeutic targets of TNF antagonists in psoriasis. Furthermore, these data establish an unexpected effect of TNF blockade on IFNγ synthesis by T cells. Synthesis of IFNγ, a cytokine of Th1-polarized T cells, is suppressed by 8.1-fold ($P<0.01$) at the mRNA level, while synthesis of IFNγ is eliminated in >60% of individual T cells. These data suggest that TNF blockade with infliximab can suppress a major pathway of the adaptive immune response and this observation provides a key rationale for targeting TNF in "Type-1" T-cell-mediated autoimmune diseases.

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INTRODUCTION

Cytokines like tumor necrosis factor (TNF) and IFNγ are key regulators of cellular immune reactions in both normal immune responses and autoimmune diseases. Some diseases, for example, psoriasis, rheumatoid arthritis, or inflammatory bowl disease, are effectively treated with cytokine inhibitors like infliximab (Remicade®), an anti-TNF antibody, or etanercept (Enbrel®), a fusion protein that binds to TNF and lymphotoxin alpha (LTA) (Jacobi *et al.*, 2003; Gottlieb *et al.*, 2005). The anti-IFNγ antibody, fontolizumab (HuZAF™), is currently being tested in a phase 2 clinical trial for rheumatoid arthritis. Psoriasis also shows a strong genomic signature of IFNγ-regulated genes (Lew *et al.*, 2004), suggesting that IFNγ blockage might also produce therapeutic benefits in this disease.

Cytokines like TNF and IFNγ largely regulate immune responses by acting as inducers of inflammatory gene products. Through receptors on target cells, secreted TNF activates NF-κB, whereas secreted IFNγ activates STAT1, which then increases transcription of a large group of immunity-related genes (Boehm *et al.*, 1997; Watts, 2005).

While TNF inhibitors are in widespread clinical use, very little is known about the molecular and cellular effects of cytokine blockade in human tissues.

At least one problem with detailed study of anti-inflammatory mechanisms of TNF inhibitors is that the array of genes regulated by TNF in leukocytes is largely unknown. In contrast, IFNs have been much better studied with respect to their ability to induce transcription of inflammation related genes, and a set of ~150 IFNγ-regulated genes have been codified and established from many independent research studies (Boehm *et al.*, 1997).

In most previous experiments, gene sets regulated by proinflammatory cytokines have been identified by exposing resting cells to single cytokines or to defined cytokine mixtures (Banno *et al.*, 2003). However, this does not model therapeutic use of cytokine inhibitors in inflammatory diseases, since activation of cells within mixed cell populations is ongoing at the time inhibitors are administered.

[1]*Department of Investigative Dermatology, Laboratory of Investigative Dermatology, The Rockefeller University, New York, New York, USA and* [2]*Department of Statistical Genetics, Laboratory of Statistical Genetics, The Rockefeller University, New York, New York, USA*

Correspondence: Dr James G. Krueger, The Rockefeller University, Dermatology, 1230 York Ave, Manhatten, New York 10021, USA. E-mail: kruegej@mail.rockefeller.edu

Abbreviations: IL-12R, IL-12 receptor; LTA, lymphotoxin alpha; NK, natural killer; PBMC, peripheral blood mononuclear cells; PSORS, psoriasis susceptibility; TNF, tumor necrosis factor

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Furthermore, immune reactions are known to be regulated by varying ratios of cytokines made in complex mixtures during cellular immune responses.

In this report, we present an approach to elucidate how genes expressed in mixtures of activated mononuclear leukocytes (T cells, B cells, and natural killer (NK) cells) may be regulated by blocking TNF or IFNγ with neutralizing antibodies specific for these cytokines. These TNF- and IFNγ-regulated genes further served to re-evaluate the genes known to be expressed in psoriasis vulgaris. IFN blockade suppresses (by $>$1.5-fold) expression of 19 genes (most previously identified as IFN-regulated in other cell types), whereas TNF blockade suppresses (by $>$1.5-fold) expression of 50 genes and most of these genes have not previously been identified as being TNF regulated. The new insight into TNF-regulated gene products not only helps to identify a larger TNF signature in psoriasis (a common ''autoimmune'' disease in humans), but also a strong interaction between TNF and function of Th1 T cells in the adaptive immune system.

RESULTS

Analysis of genes induced by T-cell activation in peripheral blood mononuclear cells *versus* purified T cells

T lymphocytes were activated by co-ligation of CD3 and CD28 with bead-bound antibodies, using either purified T cells (prepared by negative selection) or T cells contained in a mixture of peripheral blood mononuclear cells (PBMCs). From past work with analysis of cytokines produced by T cells using intracellular staining and flow cytometry detection, both methods yielded activated T cells (confirmed by increased CD69 expression) that produce high levels of TNF and IFNγ (Figure S1). Since secreted cytokines may regulate gene transcription in cells other than T cells, we reasoned that genes induced in PBMC preparations might be more representative of cytokine-regulated genes in inflammatory diseases, where complex mixtures of leukocytes are present at sites of inflammation.

T-cell activation stimulated by ligation of CD3 and CD28 is shown in Figure 1, along with experimental conditions where IFNγ is blocked by incubation with fontolizumab, or TNF is blocked by incubation with infliximab. A strong T-cell activation response to CD3/CD28 ligation is shown by upregulation of CD69 in $>$80% of $CD3^+$ T cells (Figure 1a), by an increase in annexinV+ and annexinV+/propidium iodide+ T cells (activation-induced apoptosis/cell death; Figure 1b), and by induction of IFNγ synthesis in a subset of T cells (Figure 1c). As discussed in more detail later, the conditions of IFNγ or TNF blockade with antibodies do not grossly alter T-cell activation response (CD69 induction; Figure 1a) or overall levels of apoptosis/cell death (annexinV and propidium iodide staining; Figure 1b). In fact, we did not detect any significant effects on total $CD3^+$ T cells, B cells, or NK cells (Figure 1d).

Figure 2 shows representative data of gene expression in PBMCs that were cultured for 24 hours with or without T cell stimulated by CD3/CD28 antibodies. We detected a large set of genes ($>$1,200) whose expression increased by 1.2-fold in a statistically significant manner in activated PBMCs (see Table 1 for a short list and Table S1 for a complete list of these genes, while a subset is illustrated in the heat maps in Figure 2). The number of activation-induced genes in PBMCs was larger (571 genes) than genes induced in purified T-cell cultures (487 genes), which could reflect regulation of T-cell genes by accessory cells (Table S2). However, by comparing how ''pure'' T-cell genes related to genes induced in PBMC preparations (Table S2), it is clear that activated PBMCs contain a mixture of genes detected in activated T cells (overlap of 559 genes between activated T cells and PBMCs) and genes which are not detected in purified T-cell preparations (571 genes), many of which are associated with myeloid cells. Several of the highly upregulated genes unique to PBMC cultures, for example, matrix metalloproteinase-12 (MMP-12) and CCL18 (Table S2), are gene products, which are detected in myeloid dendritic cells and that are not typically associated with T cells (Schutyser *et al.*, 2001; Rust *et al.*, 2006). We interpret these data as being consistent with paracrine effects of T-cell-derived cytokines on blood monocytes that are present in PBMC preparations.

As gene products of myeloid cells are highly upregulated in psoriasis skin lesions (Zhou *et al.*, 2003a, b), we believe that the PBMC system better reflects T-cell/myeloid cell interactions that may occur in autoimmune inflammation in peripheral tissues.

Identification of genes regulated by cytokine-neutralizing antibodies in activated PBMC cultures

To define the gene that are regulated by cytokines, we used the approach of measuring changes in gene expression in PBMCs in the presence of available antibodies that block cytokines like TNF or IFNγ. These antibodies bind to TNF or IFNγ produced after activation of T cells in PBMCs and thus inhibit the expression of genes that are regulated by these cytokines. These antibodies consist of two domains, one that neutralizes the cytokines and another nonspecific IgG1 domain. To ascertain the contribution of the cytokine-binding domain to broad gene activation that occurs in activated PBMCs, we first compared the gene expression in stimulated PBMCs with control humanized IgG1 that lacked relevant antigen specificity. As shown in the heat map in Figure 2a and Table S3, there were seven genes that were modestly ($>$1.2-fold) downregulated by treatment with the control antibody, but none by $>$1.5-fold. We therefore, used the cut-off of 1.5-fold difference to define the effects of therapeutic antibodies in stimulated PBMCs.

Identification of genes suppressed by blocking IFNγ in activated PBMC cultures

We then proceeded to study the suppression of inflammatory genes with humanized or chimeric cytokine antibodies that have been used in clinical trials of patients with a variety of inflammatory diseases to block IFNγ or TNF. Consistent with cell activation and viability measures, addition of cytokine-blocking antibodies did not significantly affect expression of most activation-associated genes ($\sim$80% were unchanged). Stimulated genes selected as being cytokine-regulated had expression reduced by at least 1.5-fold, with a *P*-value $<$0.01. The IFNγ-regulated genes identified by this approach

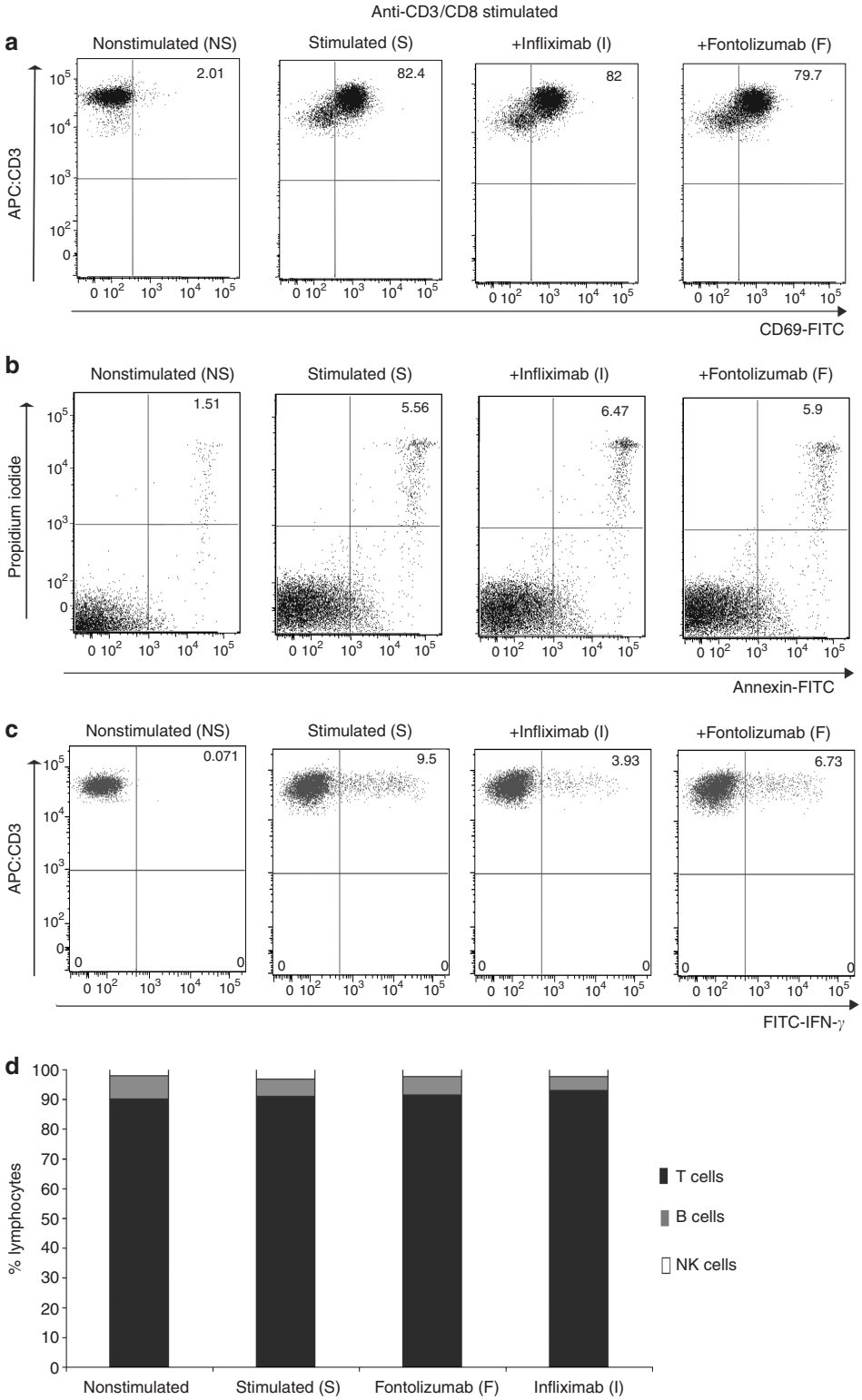

Figure 1. T-cell activation, apoptosis, detection of IFNγ on T cells, and effects on T cells, B cells, and NK cells. PBMC stimulation with anti-CD3/CD28 antibody (S) was compared with non-stimulated PBMCs (NS), activation in the presence of anti-IFNγ antibody (fontolizumab) and anti-TNF antibody (infliximab). Representative plots out of four individual experiments are presented. (**a**) T-cell activation: percentage of expression of CD3/CD69-positive T cells is indicated in the upper right-hand corner of dot plots. (**b**) Apoptosis: percentage of propidium iodide/annexinV-FITC-positive cells is indicated in the upper right-hand corner of the dot plots. (**c**) Intracellular IFNγ positive T-cell measurements: percentage of expression of CD3/IFNγ-positive T cells indicated in the upper right-hand corner of dot plots. (**d**) Effects on T cells, B cells, and NK cells: percentages of lymphocytes ($CD3^+$ T cells, $CD3^-CD19^+$ B cells, and $CD3^-CD56^+CD16^+$ NK cells) are presented as column bars.

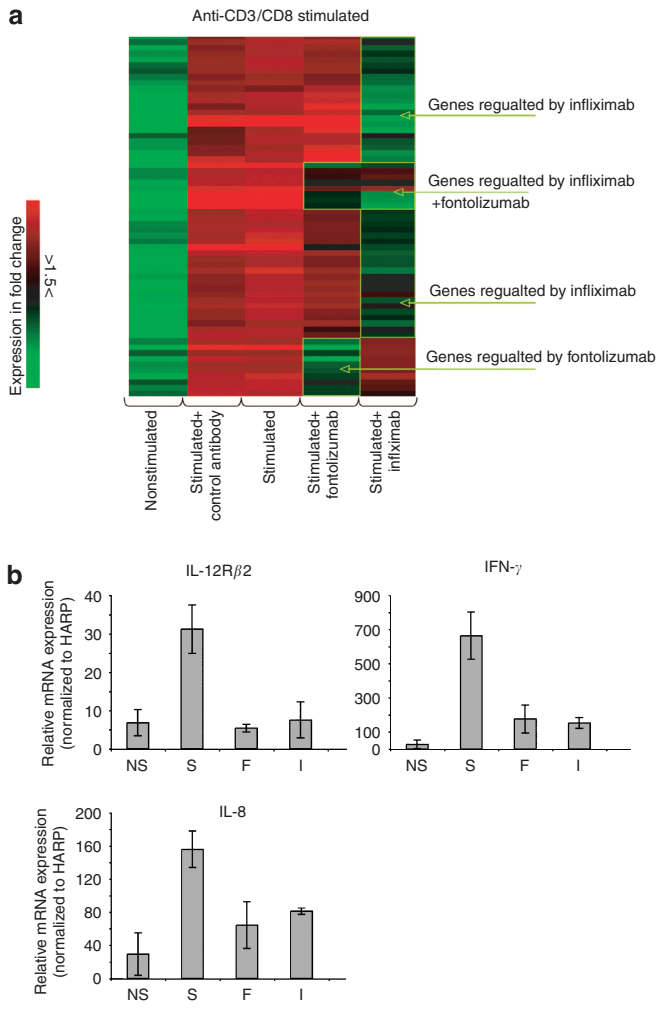

Figure 2. mRNA analysis. (**a**) Heat maps of activation genes regulated by IFNγ, TNF. mRNA from cultured PBMCs was hybridized to individual oligonucleotide arrays containing ~12,000 human genes (Affymetrix HG-U95Av2 chips). Heat maps show unsupervised hierarchical clusters of genes regulated by TNF or IFNγ in PBMCs by >1.5-fold, using similarity measure, Pearson correlation. Gene lists were created after significance analysis as described in Materials and Methods. The mean expression levels of genes regulated by >1.5-fold are represented by red lines (upregulated) and green lines (downregulated). Genes in PBMCs activated with anti-CD3/CD28 (stimulated) are compared with non-stimulated, stimulated in the presence of control antibody (IgG1), TNF inhibitor infliximab and IFNγ inhibitor fontolizumab. Genes exclusively regulated by >1.5-fold by either infliximab or fontolizumab are listed in Table 2 and complete lists of genes, with fold changes and *P*-values, and confidence intervals in Table S4 and Table S5. (**b**) Real-time reverse transcription-PCR confirmation of IL-12Rβ2, IFNγ, and IL-8. mRNA from PBMCs stimulated with anti-CD3/CD28 antibody (S) was compared with non-stimulated PBMCs (NS), activation in the presence of anti-IFNγ antibody (fontolizumab, F), anti-TNF antibody (infliximab, I). Mean of relative mRNA expression normalized to human acidic ribosomal protein is plotted as column bars ($n=3$).

correspond to genes previously identified in other cell types as being IFNγ regulated, through exposing those cells directly to IFNγ *in vitro* (Boehm *et al.*, 1997; Banno *et al.*, 2003). We identified several genes that have not been previously associated with regulation by TNF (gene symbols are in bold inTables 2 and 3) when compared with studies defining TNF-regulated genes (Schwamborn *et al.*, 2003; Zhou *et al.*, 2003a) in cell lines.

Figure 2a shows clear blocks of genes that are suppressed by IFNγ or TNF blockade after these selection criteria. There are genes exclusively suppressed by blocking IFNγ or TNF, but also a few genes that are blocked by fontolizumab or infliximab. Nine genes were exclusively suppressed by fontolizumab (anti-IFNγ) (Table 2a), while another seven genes were blocked by both fontolizumab and infliximab (Table 2b). Interestingly, IFNγ mRNA was among the highest regulated genes, with its mRNA suppressed by seven-fold (detected by microarray analysis or four-fold (detected by real-time reverse transcription-PCR analysis) in fontolizumab-treated cultures (Table 2b; Figure 2b). In addition, very low levels of IFNγ protein were detected in fontolizumab-treated cultures (Figure 3), but this effect is due to neutralization of secreted cytokine by fontolizumab. Furthermore, IL-12 receptor (IL-12Rβ2) mRNA was suppressed by 6.4-fold in these cultures (Figure 2b). Strong suppression of IL-1α and IL-1β proteins is shown in Figure 3. We also detected a ~30% reduction in the number of T cells synthesizing IFNγ protein, as detected by intracellular cytokine staining (Figure 1c). This approach seems to faithfully identify IFNγ-regulated genes, as there is a good overlap between identified genes with those previously associated with IFNγ regulation.

Identification of genes suppressed by blocking TNF in activated PBMC cultures

Infliximab, which is a chimeric (mouse/human) antibody-binding TNFα, was used to block TNF in anti-CD3/CD28-stimulated PBMC cultures. This antibody was chosen, as it has been the most commonly used TNF inhibitor in treatment of inflammatory diseases in humans. However, some previous reports have suggested that infliximab induces apoptosis in activated leukocytes (van den Brande *et al.*, 2005). Since our gene expression measures assume functional blockade of cytokine signaling, rather than killing of lymphocyte populations, we assessed cell viability in infliximab-treated cultures by annexinV/propidium iodide staining (Figure 1b). As shown in this figure, T-cell activation occurred in a normal manner in PBMCs cultured with infliximab and no increase in apoptosis was measured by annexinV/PI staining. Other experiments gating on B cells or NK cells showed no induction of apoptosis in these cell populations (Figure 1d). Overall cell recovery and total RNA amounts obtained from infliximab-treated cultures were not significantly reduced from other cultures that were included as controls.

As shown by the heat maps in Figure 2a, TNF blockade with infliximab reduces expression of a much larger set of stimulated genes compared with IFNγ blockade. Overall, 50 genes (all genes are listed in Table S4) were significantly suppressed by >1.5-fold ($P< 0.01$) by infliximab. Genes that were exclusively suppressed by infliximab (Table 2c) included MMP-12 and IL-1β, which are known to be regulated by TNF/NF-κB. However, IFNγ mRNA was also strongly suppressed by infliximab (Table 2b, ~9.5-fold

Table 1. Genes upregulated in activated PBMCs

			Stimulated *versus* non-stimulated *P*-value < 0.01)		
Gene ID	**Symbol**	**Gene product**	**Fold increase**	**LowF**	**HighF**
1611_s_at	IFNγ	*Homo sapiens* interferon-gamma (IFNG) gene	12.5	3.3	47.6
1534_at	IL-12RB2	Interleukin 12 receptor, beta 2	7.3	3.5	15.4
1481_at	MMP-12	Matrix metalloproteinase 12 (macrophage elastase)	6.8	1.2	38.7
39802_at	MCP-3	*Homo sapiens* mRNA for monocyte chemotactic protein-3	4.6	0.8	25.2
37823_at	CCL8	Chemokine (C–C motif) ligand 8	4.3	2.0	9.4
408_at	CXCL1	Human gene for melanoma growth stimulatory activity (MGSA)	4.2	2.0	8.6
38299_at	IL-6	Interleukin 6 (interferon, beta 2)	3.2	1.2	8.4
1702_at	IL-2RA	Interleukin 2 receptor, alpha	3.1	2.1	4.8
40385_at	CCL20	Chemokine (C–C motif) ligand 20	3.1	1.3	7.3
36296_at	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	2.6	2.0	3.4
39402_at	IL-1B	Interleukin 1, beta	2.5	1.5	4.2
1369_s_at	IL-8	Human interleukin 8 (IL-8) gene, complete cds	2.4	1.9	3.1
38326_at	G0S2	Human G0S2 protein gene, complete cds	2.4	1.8	3.3
34037_at	IL-9	Interleukin 9	2.4	1.8	3.1
1852_at	TNF	Human gene for tumor necrosis factor (TNF-alpha)	2.2	1.5	3.1
32128_at	CCL18	Chemokine (C–C motif) ligand 18	2.2	1.5	3.1
34916_s_at	TNFRSF4	Tumor necrosis factor receptor superfamily, member 4	2.1	1.7	2.7
41592_at	SOCS1	Suppressor of cytokine signaling 1	2.1	1.6	2.9
34022_at	CXCL3	Chemokine (C–X–C motif) ligand 3	2.1	1.2	3.9
37137_at	GZMB	Serine protease-like protein precursor	2.1	1.9	2.4
668_s_at	MMP-7	Human matrilysin gene, exon 6 and complete cds	2.1	1.2	3.6
436_at	IL-5	Interleukin 5 (colony-stimulating factor, eosinophil)	2.0	1.5	2.7
41677_at	IL-15RA	Interleukin 15 receptor, alpha	1.9	1.6	2.4
37014_at	MX1	Interferon-inducible protein p78 (mouse)	1.9	1.4	2.5
36377_at	IL-18R1	Interleukin 18 receptor 1	1.7	1.5	2.0
34021_at	IL-2	Interleukin 2	1.6	1.0	2.6

PBMC, peripheral blood mononuclear cell.
Selected list of genes (top 25) upregulated after stimulation of PBMCs with anti-CD3/28 (stimulated) for 24 hours, as compared with non-stimulated PBMCs. Fold change are mean expression values of stimulated *versus* non-stimulated, with corresponding confidence intervals (LowF and HighF); $P < 0.01$. Complete lists with *P*-values, fold changes, and confidence intervals are online in Table S1. Gene symbols and products are described in Gene Ontology annotations.

reduction), and suppression to this extent was not expected. As shown in Figure 2b, reduced mRNA for IFNγ led to a large reduction in IFNγ protein synthesis in infliximab-treated PMBC cultures (Figure 3). As an independent measure of IFNγ synthesis by T cells, cytokine levels were determined by intracellular cytokine staining in infliximab-treated cultures. Infliximab treatment reduced the number of IFNγ-positive T cells by 59% and, in addition, reduced the amount of IFNγ protein produced (as assessed by mean fluorescence intensity measures; Figure 1c). However, many genes, which are not regulated by IFNγ, are also suppressed by infliximab (Table 2c). It thus appears that gene sets regulated by infliximab partly overlap with genes regulated by IFNγ (Table 2b), but the majority of TNF-regulated genes are distinct from IFNγ-regulated genes. Suppression of genes such as MMP-12, CCL17, CCL18, and CCL20 suggest that TNF formed by activated T cells is acting in a paracrine manner to stimulate genes in monocytes or myeloid dendritic cells that are contained in PBMC cultures.

New insights into pathogenesis of psoriasis

Psoriasis is an inflammatory skin disease in which mRNAs for many inflammatory gene products are upregulated, as detected via array-based approaches or by real-time reverse transcription-PCR . For example, a large set of genes, which are characteristic of cellular responses to IFNs are present in skin lesions, and these products can potentially explain activation of several cell types involved in this disease process (Zhou *et al.*, 2003b). Although TNF antagonists have emerged as effective therapeutics for patients with psoriasis, the role of TNF in disease pathogenesis is much less clear with respect to inflammatory pathways. In this study using

Table 2. Genes regulated by IFNγ inhibitor (fontolizumab) and/or TNF inhibitor (infliximab)

a. Genes suppressed by fontolizumab

			Stimulated *versus* infliximab				Stimulated *versus* fontolizumab ($P<0.01$)			
Gene ID	Symbol	Gene product	*P*-value	Fold change	LowF	HighF	*P*-Value	Fold decrease	LowF	HighF
39802_at	MCP-3	*Homo sapiens* mRNA for monocyte chemotactic protein-3	0.2500	2.6	0.6	11.8	0.0156	2.9	0.8	10.6
37823_at	CCL8	Chemokine (C-C motif) ligand 8	0.0625	2.0	1.0	3.9	0.0156	4.5	2.3	8.7
41677_at	IL-15RA	Interleukin 15 receptor, alpha	0.0156	1.5	1.4	1.6	0.0156	1.7	1.6	1.9
1440_s_at	TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	0.0156	1.5	1.2	1.7	0.0156	1.6	1.3	2.0
38299_at	IL-6	Interleukin 6 (interferon, beta 2)	0.0625	1.4	1.0	2.0	0.0156	2.2	1.0	4.9
38549_at	cig5	Viperin	0.1406	1.2	0.9	1.7	0.0156	2.5	2.0	3.1
37014_at	MX1	Myxovirus (influenza virus) resistance 1	0.1094	1.2	0.9	1.5	0.0156	1.6	1.4	1.9
1107_s_at	G1P2	Interferon, alpha-inducible protein (clone IFI-15K)	0.2031	1.1	0.9	1.4	0.0156	1.6	1.4	1.8
626_s_at	IFI35	Interferon-induced protein 35	0.2344	1.1	0.9	1.3	0.0156	1.6	1.5	1.7
35095_r_at	LILRA3	Leukocyte immunoglobulin-like receptor	0.2969	1.1	0.9	1.3	0.0156	1.5	1.1	2.0

b. Genes commonly suppressed by fontolizumab and infliximab

			Stimulated *versus* infliximab ($P<0.01$)				Stimulated *versus* fontolizumab ($P<0.01$)			
Gene ID	Symbol	Gene product	*P*-Value	Fold decrease	LowF	HighF	*P*-value	Fold decrease	LowF	HighF
1611 _s_at	**IFNγ**	*Homo sapiens* interferon-gamma (IFNG) gene, complete cds	0.0156	9.5	3.1	29.2	0.0156	7.0	2.0	24.6
1534_at	***IL-12RB2***	Interleukin 12 receptor, beta 2	0.0156	5.5	3.0	10.0	0.0156	4.9	2.6	9.4
408_at	*CXCL1*	Human gene for melanoma growth stimulatory activity (MGS	0.0156	2.8	1.6	4.8	0.0156	3.2	1.9	5.4
36776_at	**LAG3**	Lymphocyte-activation gene 3	0.0156	2.3	1.2	4.4	0.0156	1.8	1.2	2.8
33849_at	***PBEF1***	Pre-B-cell colony enhancing factor 1	0.0156	1.6	1.3	2.0	0.0156	1.6	1.4	1.9
32868_at	***TGM3***	Transglutaminase 3	0.0156	1.6	1.2	2.0	0.0156	1.7	1.2	2.4
1852_at	TNF	Human gene for tumor necrosis factor (TNF-alpha).	0.0156	1.5	1.3	1.9	0.0156	1.5	1.2	1.9

c. Genes suppressed by infliximab

			Stimulated *versus* infliximab ($P<0.01$)				Stimulated *versus* fontolizumab			
Gene ID	Symbol	Gene product	*P*-Value	Fold decrease	LowF	HighF	*P*-Value	Fold change	LowF	HighF
1482_g_at	**MMP12**	Matrix metalloproteinase 12 (macrophage elastase)	0.0156	5.3	1.0	29.2	0.0156	0.3	0.1	0.7
1702_at	IL-2RA	Interleukin 2 receptor, alpha	0.0156	2.3	1.7	3.1	0.0469	1.5	1.0	2.1
39402_at	IL-1B	Interleukin 1, beta	0.0156	2.2	1.5	3.2	0.3906	0.9	0.8	1.1
1520_s_at	EDN1	*Homo sapiens* endothelin-1 (EDN1) gene, complete cds.	0.0156	2.1	1.5	3.0	0.4844	1.0	0.8	1.1

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Table 2. cotinued

c. Genes suppressed by infliximab

			Stimulated *versus* infliximab ($P<0.01$)				Stimulated *versus* fontolizumab			
Gene ID	Symbol	Gene product	*P*-Value	Fold decrease	LowF	HighF	*P*-Value	Fold change	LowF	HighF
38326_at	*G0S2*	Human G0S2 protein gene, complete cds.	0.0156	2.0	1.6	2.6	0.0938	0.7	0.4	1.1
40385_at	**CCL20**	Chemokine (C-C motif) ligand 20	0.0156	2.0	1.2	3.3	0.2188	1.3	0.8	2.1
32128_at	***CCL18***	Chemokine (C-C motif) ligand 18	0.0156	1.9	1.5	2.5	0.0313	0.4	0.2	0.8
1183_at	***CCL17***	Chemokine (C-C motif) ligand 17	0.0156	1.9	1.3	2.9	0.0156	0.0	0.0	0.3
34037_at	***IL-9***	Interleukin 9	0.0156	1.9	1.5	2.3	0.0156	0.6	0.6	0.7
37485_at	**SLC27A2**	Solute carrier family 27 (fatty acid transporter), member 2	0.0156	1.9	1.6	2.1	0.0156	1.5	1.3	1.7
544_at	NFKB2	Nuclear factor of kappa 2	0.0156	1.8	1.3	2.5	0.0156	1.2	1.1	1.5
1983_at	CCND2	Cyclin D2	0.0156	1.8	1.5	2.0	0.0156	1.4	1.3	1.6
38909_at	***CYP27B1***	Cytochrome P450, family 27, subfamily B, polypeptide 1	0.0156	1.7	1.4	2.1	0.0313	1.2	1.1	1.3
31853_at	***EED***	Embryonic ectoderm development	0.0156	1.7	1.5	1.9	0.0156	1.2	1.1	1.4
36296_at	***LTA***	Lymphotoxin alpha (TNF superfamily, member 1)	0.0156	1.7	1.5	2.0	0.0156	1.2	1.1	1.3
436_at	***IL-5***	Interleukin 5 (colony-stimulating factor, eosinophil)	0.0156	1.7	1.4	2.2	0.7500	1.0	0.9	1.1
33513_at	***SLAMF1***	Signaling lymphocytic activation molecule family member 1	0.0156	1.7	1.4	2.1	0.0156	1.4	1.2	1.6
39827_at	***DDIT4***	DNA-damage-inducible transcript 4	0.0156	1.7	1.4	2.1	0.3281	1.1	0.9	1.3
39120_at	***MT1X***	Metallothionein 1X	0.0156	1.7	1.2	2.3	0.0156	1.4	1.1	1.7
1788_s_at	*DUSP4*	Dual specificity phosphatase 4	0.0156	1.7	1.3	2.2	0.0625	1.2	1.0	1.5
1536_at	**CDC6**	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	0.0156	1.7	1.4	1.9	0.0625	1.2	1.0	1.5
37282_at	***MAD2L1***	MAD2 mitotic arrest deficient-like 1 (yeast)	0.0156	1.6	1.2	2.1	0.2031	1.2	0.9	1.5
32186_at	***SLC7A5***	Solute carrier family 7, member 5	0.0156	1.6	1.3	2.0	0.0156	1.3	1.1	1.5
34916_s_at	***TNFRSF4***	Tumor necrosis factor receptor superfamily, member 4	0.0156	1.6	1.4	1.8	0.5625	1.0	0.9	1.2
37137_at	***GZMB***	Serine protease-like protein precursor	0.0156	1.6	1.4	1.8	0.0156	1.2	1.1	1.3
31540_at	***TNFRSF9***	Tumor necrosis factor receptor superfamily, member 9	0.0156	1.6	1.4	1.8	0.1406	0.8	0.5	1.1
1369_s_at	IL-8	Human interleukin 8 (IL8) gene, complete cds.	0.0156	1.6	1.3	1.8	0.0156	1.4	1.3	1.4
494_at	**IL-13**	Human interleukin-13 (IL-13) precursor gene, complete cds.	0.0156	1.6	1.1	2.3	0.5156	1.1	0.9	1.4
849_g_at	***TRAF1***	TNF receptor-associated factor 1	0.0156	1.5	1.4	1.7	0.0313	1.1	1.0	1.3
39942_at	***BATF***	*Homo sapiens* B-ATF gene, complete cds.	0.0156	1.5	1.3	1.8	0.0156	1.2	1.1	1.4
33812_at	***ADAM19***	A disintegrin and metalloproteinase domain 19 (meltrin beta)	0.0156	1.5	1.3	1.8	0.8281	1.0	0.8	1.4

Table 2 continued on the following page

Table 2. cotinued

c. Genes suppressed by infliximab

			Stimulated *versus* infliximab ($P<0.01$)				Stimulated *versus* fontolizumab			
Gene ID	Symbol	Gene product	*P*-Value	Fold decrease	LowF	HighF	*P*-Value	Fold change	LowF	HighF
41439_at	***MYO1B***	Myosin IB	0.0156	1.5	1.3	1.8	0.0156	0.8	0.7	1.0
572_at	***TTK***	Human kinase (TTK) mRNA, complete cds.	0.0156	1.5	1.3	1.8	0.0313	1.2	1.0	1.3
40357_at	***INHBA***	Inhibin, beta A (activin A, activin AB alpha polypeptide)	0.0156	1.5	1.3	1.9	0.1719	1.2	0.9	1.6
1461_at	NFKBIA	Nuclear factor of kappa inhibitor, alpha	0.0156	1.5	1.4	1.7	0.0625	1.1	1.0	1.2
35992_at	*MSC*	Musculin (activated B-cell factor-1)	0.0156	1.5	1.2	1.9	0.0469	0.8	0.6	0.9
36909_at	**WEE1**	WEE1 homolog (*S. pombe*)	0.0156	1.5	1.3	1.8	0.0938	1.1	1.0	1.4

TNF, tumor necrosis factor.
List of genes, which are downregulated by >1.5-fold after treatment of activated PBMCs with a. IFNγ inhibitor, fontolizumab exclusively; b. IFNγ inhibitor fontolizumab and TNF inhibitor, infliximab; and c. TNF inhibitor, infliximab, exclusively corresponding to the heat map in Figure 2a. Fold decreases are mean expression values of stimulated *versus* treated with fontolizumab, or infliximab with corresponding confidence intervals (LowF and HighF) and *P*-values. Gene symbols and products are described in Gene Ontology annotations. Full lists of all genes regulated by infliximab and fontolizumab are available in Table 4S and Table 5S, respectively. Genes in bold have not been previously associated with regulation by IFNγ or TNF.

very stringent criteria, we have identified a set of six genes that are exclusively IFNγ regulated as compared with 15 TNF-regulated inflammatory genes in leukocytes that are also upregulated in psoriasis lesions (Table 3). Hence, a strong genomic signal for TNF can be associated with psoriasis lesions. In considering pathogenic roles for TNF in inflammatory diseases, including psoriasis, much emphasis has been placed on its role in inducing ''immediate'' genes (IL-1, IL-6, and IL-8) that regulate innate immune responses to potential pathogens (Dinarello *et al.*, 1986). Although these are important inflammatory cytokines, the way in which TNF could serve to regulate a complex cellular infiltrate that contains several types of dendritic cells and T-cell subsets is not readily explained by this trio of cytokines. Our data show that infliximab strongly downregulates key chemokines (eg, CCL20/MIP-3α; Table 2c) that control dendritic cell and T-cell infiltration into peripheral tissues, and key effector molecules (IL-1β, IL-8, granzyme B) of T cells and/or NK cells. In addition, infliximab suppresses several cell surface receptors (Table 3, for example, IL-12R that lead to activation and differentiation of T-cell subsets implicated in psoriasis). To the extent that TNF appears to serve as a strong inducer of IFNγ production by T-cells, and that infliximab also regulates many classical IFN response genes, much of the therapeutic activity of infliximab in psoriasis might be through suppression of the IL-23/γ-IFN-regulated ''Type-1'' pathway (Kim *et al.*, 2004). Hence, in a chronic inflammatory disease, the pathogenic activities of TNF may be much more associated with cellular infiltrates and inflammatory products of the adaptive arm of immunity, rather than those of an ''immediate'' innate response.

DISCUSSION

We have pursued identification of inflammatory (leukocyte derived) genes that are regulated when activated cells are exposed to antibodies that neutralize IFNγ or TNF. In part, the impetus for this work has been to derive a better understanding of how specific cytokines may contribute to inflammation in an array of cell-mediated ''autoimmune diseases'' like psoriasis vulgaris, inflammatory bowel disease, and rheumatoid, or psoriatic arthritis. Infliximab was chosen as the model TNF-neutralizing antibody, since it has shown effectiveness in reversing inflammation in each of these conditions (Chaudhari *et al.*, 2001; Reich *et al.*, 2005). However, very little is known about the therapeutic mechanism of infliximab in humans, even though it has been administered to >200,000 people with an array of autoimmune inflammatory diseases. One of the potential paradoxes in the treatment of psoriasis with infliximab is that psoriasis has a very strong signature of upregulated genes that can be assigned to IFN-induced signaling (Zhou *et al.*, 2003b; Lew *et al.*, 2004), but the number of induced genes that can be assigned to TNF/NF-κB signaling is quite small. Furthermore, psoriasis is strongly associated with cutaneous infiltrates of Type-1 T cells (defined by secretion of IFNγ), and the array of activated genes strongly implies T cells are acting as pathogenic activators in this disease. Thus, there is little direct evidence that TNF serves as a pathogenic cytokine in psoriasis. In trying to explain therapeutic activity of TNF inhibitors in human inflammatory diseases, numerous investigators have promoted the view that beneficial effects of TNF inhibitors are likely to be mediated by modulating pathways of innate immunity, which in turn, are strongly

Table 3. Overlap between genes upregulated in psoriasis lesions and cytokine-regulated genes in leukocytes

Systematic	LS/NL	Regulated by	Symbol	Gene product	Lesional (LS) *versus* non-lesional (NL) fold change (LS/NL)
38549_at	5.443	IFNγ	MX1	Myxovirus (influenza virus) resistance 1,	7.0
626_s_at	1.493	IFNγ	G1P2	Interferon, alpha-inducible protein (clone IFI-15K)	5.7
41677_at	1.257	IFNγ	Cig5	Viperin	5.4
1520_s_at	2.138	IFNγ	IFI35	Interferon-induced protein 35	1.5
33513_at	1.377	IFNγ	IL-15RA	Interleukin 15 receptor, alpha	1.3
34916_s_at	1.275	IFNγ	CCL8	Chemokine (C–C motif) ligand 8	1.2
1107_s_at	5.667	TNF	CCL20	Chemokine (C–C motif) ligand 20	7.1
37823_at	1.242	TNF	IL-8	Human interleukin 8 (IL-8) gene, complete cds.	5.1
32868_at	3.926	TNF	MMP-12	Matrix metalloproteinase 12 (macrophage elastase)	3.2
1534_at	1.361	TNF	*GZMB*	Serine protease-like protein precursor	2.5
40385_at	7.093	TNF	IL-1B	Interleukin 1, beta	2.2
1369_s_at	5.139	TNF	EDN1	*Homo sapiens* endothelin-1 (EDN1) gene, complete cds.	2.1
1481_at	3.24	TNF	SLC7A5	Solute carrier family 7	2.0
37137_at	2.487	TNF	TTK	Human kinase (TTK) mRNA, complete cds.	1.9
39402_at	2.247	TNF	CDC6	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	1.9
32186_at	1.957	TNF	MAD2L1	MAD2 mitotic arrest-deficient-like 1 (yeast)	1.5
572_at	1.877	TNF	*MYO1B*	Myosin IB	1.5
1536_at	1.86	TNF	*BATF*	*Homo sapiens* B-ATF gene, complete cds.	1.4
1721_g_at	1.487	TNF	*SLAMF1*	Signaling lymphocytic activation molecule family member 1	1.4
39942_at	1.387	TNF	*TNFRSF4*	Tumor necrosis factor receptor superfamily, member 4	1.3
33812_at	1.224	TNF	*ADAM19*	A disintegrin and metalloproteinase domain 19 (meltrin beta)	1.2
37014_at	6.977	TNF+IFNγ	CXCL1	Human gene for melanoma growth stimulatory activity (MGSA).	14.0
408_at	14.02	TNF+IFNγ	PBEF1	Pre-B-cell colony enhancing factor 1	4.2
33849_at	4.154	TNF+IFNγ	*TGM3*	Transglutaminase 3	3.9
41439_at	1.459	TNF+IFNγ	*IL-12RB2*	Interleukin 12 receptor, beta 2	1.4

Psoriasis genes, which are regulated by IFNγ or TNF.
Genes that are downregulated (>1.5-fold) after inhibition of IFNγ with fontolizumab or TNF with infliximab in stimulated PBMCs were compared with genes upregulated in psoriasis lesions, as compared with non-lesional skin biopsies (Zhou *et al.*, 2003a,b). Fold change are mean expression values in lesional (LS) *versus* non-lesional (NL) skin biopsies. Gene symbols and products are described in Gene Ontology annotations.
TMF, tumor necrosis factor.

linked to tissue infiltration by neutrophils and other leukocytes of the innate immune system (De Rycke *et al.*, 2005; Pay *et al.*, 2005). In contrast, our data suggest that TNF blockade could have major regulatory effects on adaptive immune responses by suppressing synthesis of IFNγ and the β2 subunit of the IL-12R, as well as other T-cell-derived gene products. Note that the IL-12R functions to promote Th1 polarization of T cells and also the level of IFNγ synthesis by T cells. Hence, suppression of the IL-12Rβ2 subunit might control the measured decrease in IFNγ synthesis by infliximab.

The data presented in this study significantly expand the roster of genes, which are regulated (directly or indirectly) by TNF. We acknowledge that these data are derived from samples of only eight subjects, and could limit findings some relevant associations. Study of larger populations might demonstrate statistical significance to some genes now excluded or variations in individual responses might be appreciated, accounting for variability in clinical responses to cytokine antagonists.

In contrast to human keratinocytes, where IFNγ regulates many more genes than TNF (Banno *et al.*, 2003, 2004), our results in activated PBMCs suggest that TNF regulates many more genes than IFNγ in leukocytes. The set of genes, which are suppressed by infliximab strongly argues that TNF may play a key role in stimulating T cells, and thus effector pathways, which are generally assigned to adaptive immune responses. The strong suppression of IFNγ, arguably the key

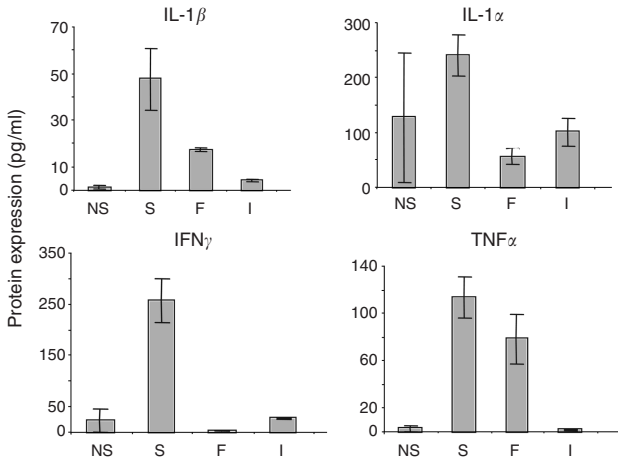

Figure 3. Protein expression of IL-1α and IL-1β, IFNγ and TNF. Cytokine levels in the supernatant from PBMCs activated with anti-CD3/CD28 antibody (S) were compared with non-stimulated PBMCs (NS), activation in the presence of anti-IFNγ antibody (fontolizumab, F) or anti-TNF antibody (infliximab, I). Mean of cytokine expression is plotted as column bars ($n = 3$).

cytokine produced by activated Th1 T cells, in infliximab-treated PBMCs suggests that important regulatory/effector cytokines of T cells are modulated by TNF within an inflammatory environment. While prior work has established that TNF can serve as co-stimulus of T-cell activation (Banerjee *et al.*, 2005), and also that NF-κB-binding sites are present in the promoter of the IFNγ gene (Sica *et al.*, 1997), the extent to which infliximab suppresses IFNγ levels in PBMC cultures and particularly in $CD3^+$ T cells was unexpected and is a surprising result of this study. The consequences of reducing IFNγ synthesis in T cells are many. Relevant outcomes could be significant impairment of macrophage activation for killing of intracellular organisms (Marodi *et al.*, 1993).

The relevance of our findings to clinical administration of TNF inhibitors is suggested by two observations. First, treatment of psoriasis with etanercept, a fusion protein that neutralizes TNF and lymphotoxin, does lead to gradual reductions in levels of IFNγ mRNA in skin lesions, as well as reductions in IFNγ-induced gene products such as the chemokines MIG and IP-10 (Gottlieb *et al.*, 2005). The gene Mx-1 has been identified to be regulated by type I IFNs as well as type 2 IFNγ (Banno *et al.*, 2003; Pletneva *et al.*, 2006). Mx-1 is suppressed in psoriasis lesions by etanercept, in parallel with reductions in IFNγ mRNA (Gottlieb *et al.*, 2005). We acknowledge that the effects may also be derived from regulation of type 1 IFNs that are also present in psoriasis. Hence, there is *in vivo* evidence that TNF inhibition can suppress or cross-regulate cytokine response pathways that are considered to be hallmarks of the adaptive immune system. Secondly, direct suppression of IFNγ synthesis in T cells by infliximab could explain cases of severe mycobacterial infection and/or reactivation of tuberculosis in patients undergoing treatment with infliximab (Keane *et al.*, 2001). Our results thus suggest that pathways, which regulate innate responses (suppression of IL-8, TNF, or IL-1), as well as effector responses of T cells associated with adaptive immunity, are likely to be suppressed by infliximab. In contrast, inhibition of IFNγ with fontolizumab leads to more restricted suppression of genes and most suppressed genes have been previously assigned to IFNγ responses in an array of individual cell types (Boehm *et al.*, 1997). Although there is some overlap in gene sets derived by inhibiting IFNγ *versus* TNF, clear sets of genes that are uniquely regulated by each inhibitor can be identified. In turn, these gene sets can be applied to help interpret cytokine-associated genomic signals in complex inflammatory diseases like psoriasis. Please note that our cytokine response gene sets are not specific to psoriatic leukocytes, as we sought to derive generalizable expression profiles that could be used to study many different inflammatory diseases. However, since response of psoriatic leukocytes to TNF or IFNγ might differ, for example, as an effect of varying psoriasis susceptibility (PSORS) genes in different individuals, and since psoriatic T cells may be more activated (Cooper *et al.*, 2003), it would be interesting to eventually compare genomic responses of normal *versus* psoriatic leukoctyes with therapeutic cytokine inhibitors. Furthermore, there could be key differences in induced gene sets between responding and non-responding psoriasis patients to a given inhibitor, as we have recently demonstrated for alefacept (Haider *et al.*, 2007).

In the previous work, we have identified ~1,300 genes upregulated in psoriasis skin lesions (Zhou *et al.*, 2003b), but very few genes could be assigned to TNF or NF-κB-regulated pathways. This study identifies 18 genes that are over-expressed in psoriasis, which are suppressed by infliximab in PBMCs (Table 3). Accordingly, a significant ''signal'' for TNF-regulated genes can now be assigned to psoriasis vulgaris.

MATERIALS AND METHODS

Samples used in the study

Institutional review board approval was obtained before inviting healthy volunteers to participate in the study. Informed consent was obtained from volunteers before their participation, and the study was performed with strict adherence to the Declaration of Helsinki Principles. PBMCs from seven donors and T cells from three donors were isolated from freshly drawn human blood (in heparin tube) using Ficoll-Paque Plus, according to standard protocol. T cells were negatively isolated using Dynal® T-cell negative isolation kit (Dynal Biotech, Invitrogen Corp, Carlsbad, CA, cat. no. 113-11D) according to the manufacturer's protocol. PBMCs and T cells were resuspended at 1×10^6 cells/ml in Roswell Park Memorial Institute (Invitrogen Corp, Carlsbad, CA, cat. no 21870) + 5% heat-inactivated human serum (Hyclone, Logan, UT, SH30071.03,) and PenStrep (penicillin 10 KU/ml, streptomycin 10 mg/ml; Invitrogen, cat. no. 15140-148). Cultures were set up in 20 ml per dish (35 × 20 mm; Corning, NY, cat. no. 25020). In cultures with PBMCs, monocytes were allowed to adhere for 1 hour at 37°C before activation and addition of inhibitors.

Non-stimulated PBMCs or T cells (NS) served as control to stimulation (S) with anti-CD3/CD28 using Dynabeads® CD3/CD28 T-Expander (Dynal Biotech, 111.31). PBMCs were also stimulated in the presence of IFNγ inhibitor fontolizumab (F) (100 g/ml, HuZAF™, anti-IFNγ, PDL Biopharma), TNF inhibitor, infliximab (I) (100 g/ml,

Remicade®, anti-TNF, Centocor), and the control IgG1 antibody MSL109 (200 g/ml, PDL Biopharma). All reagents were added at time 0 and cells (adherent and floating) and supernatant were collected at 24 hours post-stimulation. Cytokines/chemokine levels were measured in the supernatant and the cells were lysed and stored at at −80°C for later RNA preparation, as described previously (Haider *et al.*, 2006).

The description for the following measurements are available online in the Supplementary material for Materials and Methods:

1. Measurement of IFNγ, IL-1α, IL-1β and TNF level by Luminex.
2. PBMC phenotype and apoptosis.
3. Real-time reverse transcription-PCR and microarray analysis of tissue mRNA gene expression.
4. Statistical comparisons of microarray analysis.

Complete lists of genes with *P*-values, fold changes in mean expression values, upper and lower confidant intervals (F-upper, F-lower), and description of relevant functions of genes are provided in Tables S1–S6 (see http://www.rockefeller.edu/labheads/krueger/supplemental)

CONFLICT OF INTEREST

Dr JG Krueger has been a consultant to PDL BioPharma and Centocor.
The opinions, findings, conclusions, or recommendations put forth in this position paper are those of the authors and do not necessarily reflect the views of the organizations with which they are employed or engaged.

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SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Figure S1. IFNγ and TNF staining in activated T cells.

Table S1. List of genes activated after stimulation of peripheral blood mononuclear cells.

Table S2. List of genes expressed after stimulation of PBMCs as compared to isolated T-cells.

Table S3. List of genes regulated by control antibody.

Table S4. List of genes regulated by infliximab.

Table S5. List of genes regulated by fontolizumab.

Table S6. List of all genes in PBMCs treated with antiCD3/CD28, control antibody, fontolizumab or infliximab. The *P*-values, gene symbols, gene product and confidence intervals are shown.

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