The Journal of Immunology

Active Wegener's Granulomatosis Is Associated with HLA-DR $^{+}$ CD4^{+} T Cells Exhibiting an Unbalanced Th1-Type T Cell Cytokine Pattern: Reversal with IL-10

Björn R. Lúdvíksson, Michael C. Sneller, Kevin S. Chua, Cheryl Talar-Williams, Carol A. Langford, Rolf O. Ehrhardt, Anthony S. Fauci and Warren Strober

J Immunol 1998; 160:3602-3609; ;
http://www.jimmunol.org/content/160/7/3602

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Active Wegener's Granulomatosis Is Associated with HLA-DR$^+$ CD4$^+$ T Cells Exhibiting an Unbalanced Th1-Type T Cell Cytokine Pattern: Reversal with IL-10

Björn R. Lúdvíksson,[1]* Michael C. Sneller,† Kevin S. Chua,* Cheryl Talar-Williams,† Carol A. Langford,† Rolf O. Ehrhardt,* Anthony S. Fauci,† and Warren Strober*

Wegener's granulomatosis (WG) is a granulomatous vasculitis that affects the upper respiratory tract, lung, and kidney. Since T cells make up a significant proportion of cells infiltrating granulomatous lesions in WG, we investigated the proliferative response and cytokine profile of T cells from these patients. PBMCs were isolated from 12 patients with active WG, 7 patients with inactive disease, and 12 healthy normal donors. PBMCs from clinically active WG patients exhibited increased proliferation following stimulation with either PMA/ionomycin or anti-CD2 and anti-CD28, when compared with normal donors. In addition, these PBMCs exhibited increased secretion of IFN-γ, but not of IL-4, IL-5, or IL-10. Furthermore, TNF-α production from PBMCs and CD4$^+$ T cells isolated from patients with WG was elevated, when compared with healthy donors. In further studies, we investigated the ability of WG patients' monocytes to produce IL-12 and showed that both inactive and active patients produced increased amounts of IL-12. Finally, the in vitro IFN-γ production by WG PBMC is inhibited in a dose-dependent manner by exogenous IL-10. These data suggest that T cells from WG patients overproduce IFN-γ and TNF-α, probably due to dysregulated IL-12 secretion, and that IL-10 may therefore have therapeutic implications for this disease. ***The Journal of Immunology*****, 1998, 160: 3602–3609.**

Wegener's granulomatosis (WG)[2] vasculitis is characterized by the presence of granulomatous inflammatory lesions consisting of focal accumulations of lymphocytes, macrophages, and epithelioid cells, and multinucleated giant cells in the parenchyma of tissue surrounding blood vessels and occasionally in the vessel walls (1, 2). These lesions appear to be initiated by CD4$^+$ T cells and macrophages, since immunohistochemical studies of early lung and kidney lesions reveal a predominance of these cell types (3, 4). The granulomas of WG are similar to those associated with a variety of intracellular infections; however, attempts to isolate an infectious agent from lesional tissue have failed (2, 5–7). It has thus been suggested that while lesions of WG patients are initiated by T cells responding to an exogenous agent, they are sustained by T cells that cross-react with autoantigens in the vessel walls or the immediately surrounding tissue. Evidence supporting this hypothesis comes from studies of giant cell arteritis, another granulomatous vasculitis in which the presence of a clonotypic expansion of CD4$^+$ T cells within vascular lesions suggests that the cellular infiltrate represents a localized Ag-driven immune response (8).

In recent years, studies of the pathogenesis of various autoimmune and infectious diseases have shown that the inflammatory lesions occurring in some of these diseases are driven by T cells that display either a Th1- or Th2-type pattern of cytokine secretion. For example, in infection due to *Mycobacterium tuberculosis* or in Crohn's disease (an inflammatory bowel disease of unknown etiology), the granulomatous lesions are dominated by T cells exhibiting a Th1-type pattern (9–12). In contrast, in the inflammatory lesions seen in schistosomiasis and extrinsic asthma, the associated infiltrates are dominated by T cells exhibiting a Th2-type pattern (13–16). This paradigm also holds for various animal models of infection and autoimmunity; in several of these models, treatment directed toward the blocking of the Th1-type or Th2-type T cell response leads to amelioration of disease (17). Thus, the cytokine pattern present in a given autoimmune disease is of more than theoretical interest.

In previous studies, it has been shown that the granulomatous lesions of giant cell arteritis contain cells that display a Th1-type cytokine pattern (18), and it is reasonable to ask whether WG falls into the same or a different category. In the present studies, we address the latter question by examining both the proliferative capacity and the profile of cytokine secretion of circulating T cell populations from patients with WG.

Materials and Methods

Study population characteristics

Seventeen patients with WG were studied. All 19 patients had biopsy-proven WG, characterized by necrotizing vasculitis, granulomatous inflammatory changes, or both, in a typical organ system. In addition, all patients met the American College of Rheumatology 1990 criteria for classification of WG. These patients were participating in clinical research protocols approved by the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board, and the Clinical Director (NIAID) of the Clinical Center of the National Institutes of Health. All patients provided written informed consent. The clinical features of the 19 patients are summarized in Table I. Healthy volunteers from the apheresis center of the

*Mucosal Immunity Section, Laboratory of Clinical Investigation, and †Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received for publication September 17, 1997. Accepted for publication December 5, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Björn Lúdvíksson, Mucosal Immunity Section, Laboratory of Clinical Investigation, NIAID, National Institutes of Health, Building 10, Room 11N-238, 10 Center Drive, Bethesda, MD 20892. E-mail address: bludviksson@atlas.niaid.nih.gov

[2] Abbreviations used in this paper: WG, Wegener's granulomatosis; NIAID, National Institute of Allergy and Infectious Diseases; PB, peripheral blood; SAC, *Staphylococcus aureus* Cowan I strain; PMN, polymorphonuclear; CD40L, CD40 ligand.

0022-1767/98/$02.00

Table I. *Clinical characteristics of 19 patients with Wegener's granulomatosis*

Patient No.	Age (Yr)/Sex	Active Disease Manifestations[a]	Prior Disease Manifestations	Immunosuppressive Treatment[b]	cANCA Titer
1	24/M	E, L, S, A	E, L, S, K, A	AZA, prednisone	1:40
2	31/M	E, K	E, L, S, K	CYC, prednisone	1:320
3	30/M	E, L	E, L, K	CYC	1:640
4	64/F	E, L, A	E, L	None	1:320
5	55/F	E, L, A, MNM	E, L	None	1:160
6	56/F	E, K, A	E, K, A	None	Positive[c]
7	44/F	E, L, A	E, L, K	None	Positive[c]
8	21/F	E, L, A	E, L, A	CYC, prednisone	1:80
9	57/M	L, A	E, L, A	MTX	1:320
10	35/M	EY, K, A	E, L, K	AZA	1:80
11	33/M	L, K	E, L, K	CYC, prednisone	1:640
12	41/M	E, K	E, L, K	None	1:160
13	24/M	Remission	L, K, EY	MTX, prednisone	Negative
14	54/M	Remission	L, K, EY, A	None	1:80
15	43/M	Remission	E, L, K, EY	MTX, prednisone	1:80
16	45/M	Remission	L, K, EY, A	MTX	Negative
17	19/M	Remission	E, L	MTX	Negative
18	28/F	Remission	E, L	MTX	1:20
19	41/M	Remission	E, L, K, EY	MTX	1:160

[a] Disease manifestations at time of *in vitro* studies: E, upper respiratory tract; L, lung; S, skin; K, kidney; A, rheumatic symptoms; MNM, mononeuritis multiplex; EY, inflammatory eye disease.
[b] Immunosuppressive treatment at time of *in vitro* studies: AZA, azathioprine; CYC, cyclophosphamide; MTX, methotrexate.
[c] cANCA, titer not done.

National Institutes of Health Department of Transfusion Medicine served as controls.

Preparation of purified PBMCs, T cells, and monocytes

PBMCs from WG patients and normal healthy human donor volunteers were isolated by Ficoll-Hypaque density gradient centrifugation of leukocyte concentrates obtained by the apheresis center of the National Institutes of Health by automated apheresis, as described previously (19). T cell-enriched populations from PBMCs were prepared by rosette formation with neuraminidase-treated sheep RBC, followed by recentrifugation over Ficoll-Hypaque gradient. Highly purified PB $CD4^+$ or PB $CD8^+$ T cells were prepared by negative selection using mAbs attached to immunomagnetic beads, as described previously (12). In brief, PB T cell populations were suspended at 2×10^7 cells/ml in calcium-free PBS with 1% FCS (coating medium) to which dilutions of ascites fluid containing the mAbs, anti-CD8, anti-MHC class II, anti-CD14, and anti-CD21 were added. The hybridoma cell lines OKT8 (anti-CD8), IVA12 (anti-MHC class II), 63D3 (anti-CD14), and THB5 (anti-CD21) were obtained from American Type Culture Collection (Rockville, MD), and the hybridoma cell lines 3G8 (anti-CD16) and 10F7 (anti-erythroglycoprotein) were obtained from Dr. Scott Fritz (NIAID, Frederick, MD). Each ascites sample was used at a dilution of 1:350 except IVA12, which was used at a dilution of 1:250. These Abs were prepared as murine ascites and filtered as described previously (20). The cells were incubated at 4°C for 30 min, washed twice, and resuspended in coating medium. The Ab-coated cell populations were then removed by an initial incubation with immunomagnetic beads coated with anti-murine IgG Ab (Advanced Magnetics, Cambridge, MA), followed by a subsequent incubation with immunomagnetic beads coated with anti-murine IgG Ab obtained from Dynal (Oslo, Norway). The resultant T cell population contained >95% $CD4^+$ T cells, as assessed by flow cytometric analysis. For use in flow cytometry studies, anti-CD3 (Leu 4), anti-CD4 FITC (Leu 3a + 3b), anti-CD8 phycoerythrin (Leu 2a), anti-CD14 (Leu M3), anti-CD20 (Leu 16), and control goat anti-mouse IgG FITC/phycoerythrin were purchased from Becton Dickinson (San Jose, CA). Purified monocytes (>95% pure) were isolated from normal human leukocyte concentrates (see above) using counterflow centrifugal elutriation. Purified monocytes were characterized by FACS analysis using fluoresceinated mAb (Becton Dickinson) with specificity for the following cell surface markers: Leu-M5 (monocytes), CD3 (T cells), CD57 (NK cells), and CD20 (B cells).

Reagents and mAbs

Cell cultures were performed in complete medium consisting of RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 3 mM L-glutamine, 10 mM HEPES buffer, 10 mg/ml gentamicin (BioWhittaker), 100 U/ml penicillin, and 10% heat-inactivated FCS. LPS isolated from *Escherichia coli* 01127:BS was obtained from Sigma Chemical Co. (St. Louis, MO) and used at 1 μg/ml. PMA was obtained from Calbiochem (San Diego, CA) and was used at 20 ng/ml; ionomycin was obtained from Sigma and was used at 100 ng/ml. Trimeric human CD40 ligand (CD40L)/leucine-zipper fusion protein was obtained from Immunex (Seattle, WA). Heat-killed, formalin-fixed *Staphylococcus aureus*, Cowan I strain (SAC) was obtained from Calbiochem. Recombinant IFN-γ (Genzyme, Cambridge, MA) was used at 1 μg/ml. Anti-CD28 Ab was the gift of Dr. Carl June (Bethesda Naval Research Institute, Bethesda, MD) and was used at a final concentration of 1 μg/ml. The anti-CD2 Ab pair $T11_2$ and $T11_3$, used to stimulate T cells (12, 21), was provided by Dr. Ellis Reinherz (Dana-Farber Cancer Institute, Boston, MA) and was used at a dilution of 1:1000 each.

Proliferation assays

To measure proliferation, 5×10^4 cells were cultured in 0.2 ml of complete medium with different stimuli in flat-bottom 96-well microtiter plates (Costar, Cambridge, MA). Plates were incubated at 37°C in a humidified incubator containing 6% CO_2 for 72 h. One mCi of [^{3}H]thymidine (DuPont NEN, Boston, MA) was added to each microwell 16 h before terminating the culture. Finally, cells were harvested onto glass filter paper with an automated multisample harvester (PHD cell harvester; Cambridge Technology, Cambridge, MA) and counted in a liquid scintillation counter.

Lymphokine production assays

To measure lymphokine production, PBMCs, T cells, or monocytes were cultured at 10^6 cells/well in 1 ml of complete medium with different stimuli in 24-well tissue culture plates (Costar). Plates were incubated at 37°C in a humidified incubator containing 6% CO_2 for 8 to 48 h as indicated, after which supernatants were collected and assayed for IL-4, IL-5, IL-10, IL-12, TNF-α, or IFN-γ using commercially obtained ELISA kits (BioSource International, Camarillo, CA). Methods used for these ELISAs were provided by the manufacturer.

Statistical analysis

Descriptive statistics and testing for significance of differences were assessed by Student's *t* test using the StatWorks and Microsoft Excel statistical analysis computer programs.

Results

Circulating T cells from patients with active WG exhibit markedly increased proliferation

In initial studies, we determined proliferative responses of PBMCs from WG patients with both active and inactive disease as well as

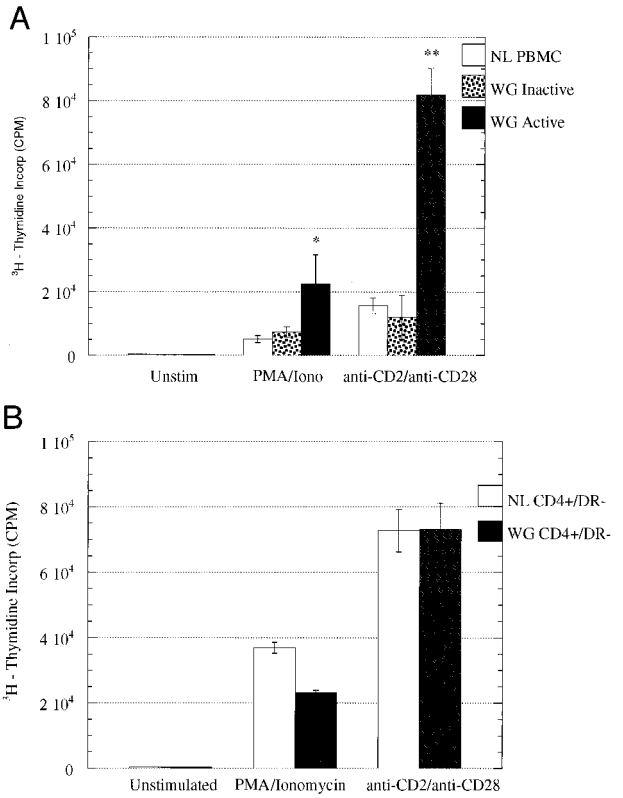

FIGURE 1. Proliferative responses of PBMCs and T cells isolated from patients with WG compared with healthy donors. *A*, PBMCs were isolated from WG patients with either active ($n = 10$; black bars) or inactive ($n = 7$; dotted bars) disease. Proliferative responses were evaluated after 72 h of stimulation and compared with those of healthy donors ($n = 12$; white bars). *$p < 0.01$; **$p < 0.001$. *B*, Purified CD4$^+$HLA-DR$^-$ T cells were similarly stimulated from patients with active WG ($n = 6$) and compared with those of healthy donors ($n = 6$).

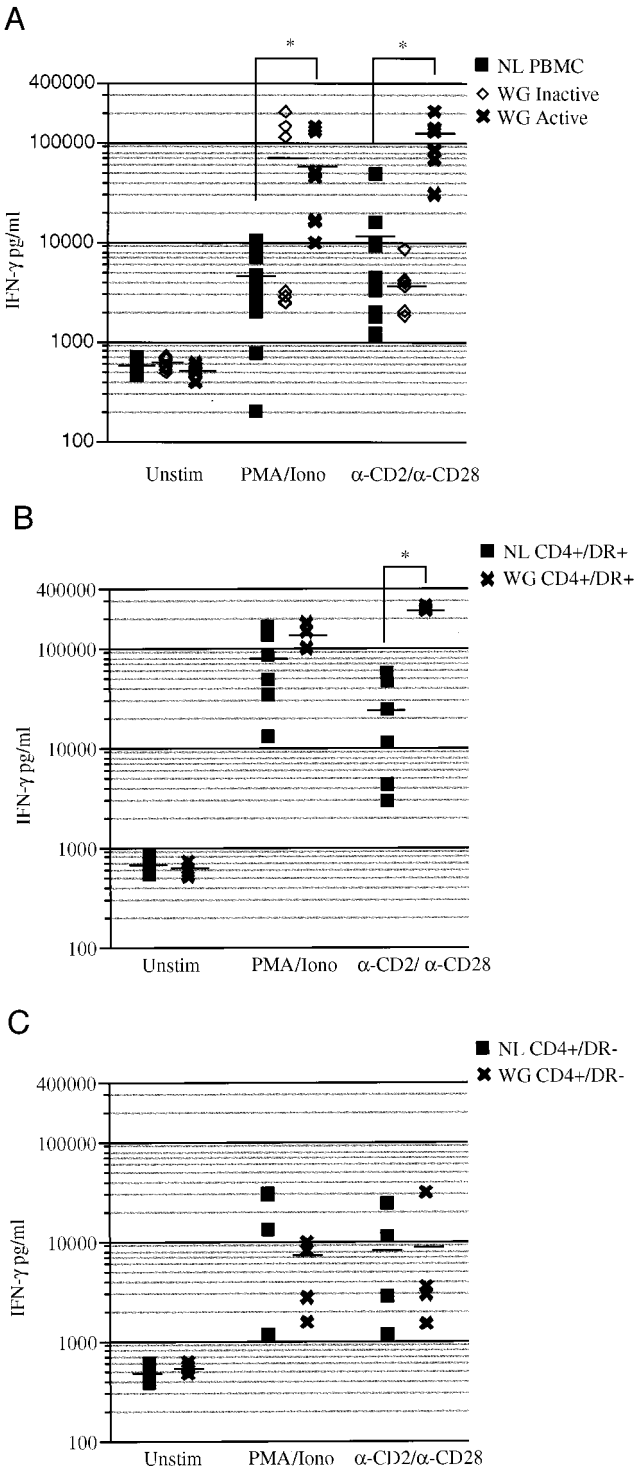

FIGURE 2. IFN-γ secretion in cell culture supernatants from PBMCs and CD4$^+$ T cells 48 h after stimulation, as analyzed by ELISA. *A*, IFN-γ secretion from PBMCs isolated from either WG patients with either active (×; $n = 10$) or inactive (◇; $n = 7$) disease compared with healthy donors (■; $n = 12$); *$p < 0.001$. Similarly, IFN-γ secretion was analyzed from highly purified CD4$^+$HLA-DR$^+$ (*B*) or CD4$^+$HLA-DR$^-$ (*C*) peripheral T cells from patients with active WG ($n = 4$) compared with those of healthy donors ($n = 6$). *$p < 0.001$.

from healthy donors. As shown in Figure 1*A*, PBMCs from WG patients with active disease stimulated with PMA/ionomycin exhibited a twofold higher proliferative response after 72 h of culture (mean [^{3}H]thymidine incorporation, 2.24×10^4) compared with that of patients with inactive disease or healthy donors (mean [^{3}H]thymidine incorporation, 0.73×10^4 and 0.52×10^4, respectively; $p < 0.01$). In addition, as also shown in Figure 1*A*, when stimulated via the CD2/CD28 signaling pathway, PB T cells from patients with active WG exhibited an approximately sevenfold higher proliferative response than did T cells from patients with inactive disease or normal controls. To determine whether this increased proliferative response was due to T cells previously activated in vivo, we isolated CD4$^+$HLA-DR$^-$ T cells by negative selection and stimulated them with PMA/ionomycin or anti-CD2/anti-CD28. As depicted in Figure 1*B*, the proliferative response of CD4$^+$HLA-DR$^-$ T cells did not differ between patients and controls. Taken together, these results indicate that patients with active WG have circulating, previously activated, CD4$^+$ T cells that exhibit a heightened proliferative response.

Circulating T cells from patients with active WG exhibit a Th1 cytokine profile

In the next set of studies, we determined the pattern of cytokine responses by stimulated PBMCs from patients with both active and inactive WG. PBMCs were stimulated with either PMA/ionomycin or anti-CD2/anti-CD28, and cytokine secretion into culture supernatants at 48 h was evaluated by ELISA (see *Materials and Methods*). As shown in Figure 2*A*, PBMCs from patients with active WG produced 10-fold higher amounts of IFN-γ than did those of healthy controls when stimulated in vitro with either PMA/ionomycin or anti-CD2/anti-CD28 ($p < 0.001$). In contrast, as shown

in Figure 2*A*, PBMCs from patients with inactive WG produced increased amounts of IFN-γ in only three of seven cases when stimulated by PMA/ionomycin and normal amounts of IFN-γ when stimulated by anti-CD2/anti-CD28.

This increased IFN-γ production was limited to previously activated T cells, since, as shown in Figure 2, *B* and *C*, anti-CD2/anti-CD28 stimulation of $CD4^+HLA\text{-}DR^+$ T cells from patients with active WG led to a 10-fold higher IFN-γ secretion than did stimulation of cells from normal controls ($p < 0.0001$; Fig. 2*B*). Similar stimulation of $CD4^+HLA\text{-}DR^-$ T cells did not lead to higher IFN-γ secretion. Parallel studies of T cells stimulated with PMA/ionomycin also tended to show higher mean secretion of IFN-γ in cultures from patients with active WG than in those of normal controls, but in this case, the difference was no longer statistically significant (Fig. 2*C*). The above abnormality in IFN-γ secretion was limited to $CD4^+$ T cells, since purified $CD8^+$ T cells from patients with active WG and normal controls produced similar amounts of IFN-γ when stimulated with anti-CD2/anti-CD28 or PMA/ionomycin (data not shown). While there was a strong correlation between proliferative responses and IFN-γ production in active WG patients (results not shown), there was no correlation between these parameters in inactive WG patients. In addition, we were not able to identify any clinical differences between the inactive WG patients who had relatively high IFN-γ production (when stimulated with PMA/ionomycin) and those with relatively low IFN-γ production.

In a final set of cytokine secretion studies, we also determined the ability of PBMCs and purified T cells from patients with active WG to secrete Th2 cytokines. As shown in Figure 3, no difference between WG patients and controls was found for IL-4 secretion from either PBMCs or purified $CD4^+$ T cells. Similarly, there was no significant difference in the amount of IL-5 secreted by the different groups (data not shown).

Taken together, these data demonstrate that circulating T cells from patients with active WG manifest a dysregulated cytokine secretion profile characterized both by a major increase in IFN-γ and by normal production of IL-4 and IL-5.

T cells, but not monocytes, from patients with active WG secrete increased amounts of TNF-α

TNF-α is another Th1-type cytokine that is potentially important in the pathogenesis of granulomatous inflammatory vascular lesions. Therefore, in the next set of studies, we evaluated TNF-α secretion by T cells and monocytes from patients with active WG. Purified $CD4^+$ T cells were stimulated either with PMA/ionomycin, with LPS + 3% human serum, or directly with anti-CD2/anti-CD28. As shown in Figure 4*A*, $CD4^+$ T cells from patients with active WG secreted significantly more TNF-α when stimulated with either LPS or anti-CD2/anti-CD28 than did $CD4^+$ T cells from normal controls ($p < 0.002$). In contrast, similar amounts of TNF-α were found in T cell supernatants from the two groups after PMA/ionomycin stimulation. Interestingly, as shown in Figure 4*B*, freshly isolated and purified monocytes (see *Materials and Methods*) from active WG patients did not secrete increased amounts of TNF-α compared with monocytes of normal controls when stimulated with either PMA/ionomycin or LPS. These results suggest that T cells, but not monocytes, isolated from the PB of patients with active WG secrete enhanced amounts of TNF-α.

Monocytes from WG patients secrete increased amounts of IL-12

In view of the above findings of increased secretion of Th1 cytokines in patients with active WG, we next determined whether monocytes from patients with WG exhibited increased production

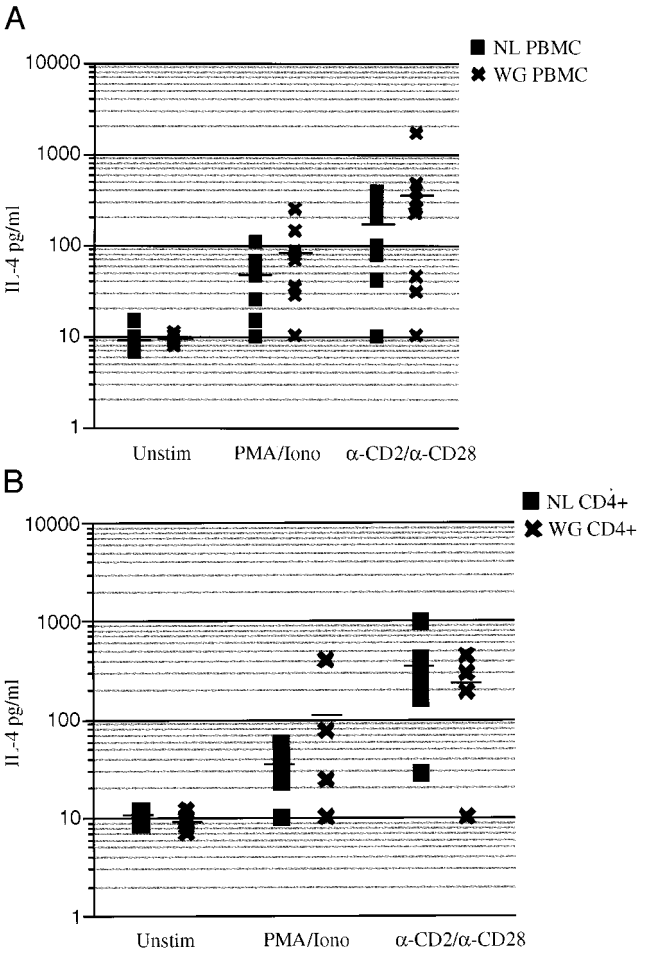

FIGURE 3. IL-4 secretion in cell culture supernatants from PBMCs and $CD4^+$ T cells 48 h after stimulation, as analyzed by ELISA. *A*, IL-4 secretion from PBMCs isolated from WG patients with active disease (×; $n = 10$) compared with healthy donors (■; $n = 12$). *B*, Secretion was analyzed from highly purified (>95%) $CD4^+HLA\text{-}DR^+$ peripheral T cells from patients with active WG ($n = 4$) compared with those of healthy donors ($n = 6$).

of IL-12, the major inducer of IFN-γ. For these studies, we first stimulated cryopreserved, purified monocytes from patients with inactive WG and normal controls either with LPS alone, LPS and IFN-γ, SAC and IFN-γ, or CD40L and IFN-γ. As shown in Figure 5*A*, monocytes that had been isolated from patients with inactive WG secreted increased amounts of IL-12 compared with normal donor monocytes in response to all of the stimulants tested. In further studies, we determined IL-12 production by stimulated monocytes isolated from two patients with active disease. As shown in Figure 5*B*, both of the patients studied secreted significantly increased amounts of IL-12 compared with normal controls ($p < 0.0001$). Thus, IL-12 production by circulating monocytes is enhanced in both clinically inactive and active patients with WG.

Addition of exogenous IL-10 to PBMC cultures of active WG patients inhibits the increased IFN-γ secretion

It was demonstrated in previous studies that IL-10 can prevent the development of a Th1 response and reduce IFN-γ production, most likely by inhibiting IL-12 secretion (22–24). To determine whether IL-10 can also inhibit increased IFN-γ secretion in patients with active WG, we cultured anti-CD2/anti-CD28 stimulated PBMCs from patients with active WG in the presence of increasing amounts of IL-10. As shown in Figure 6, IL-10 had a

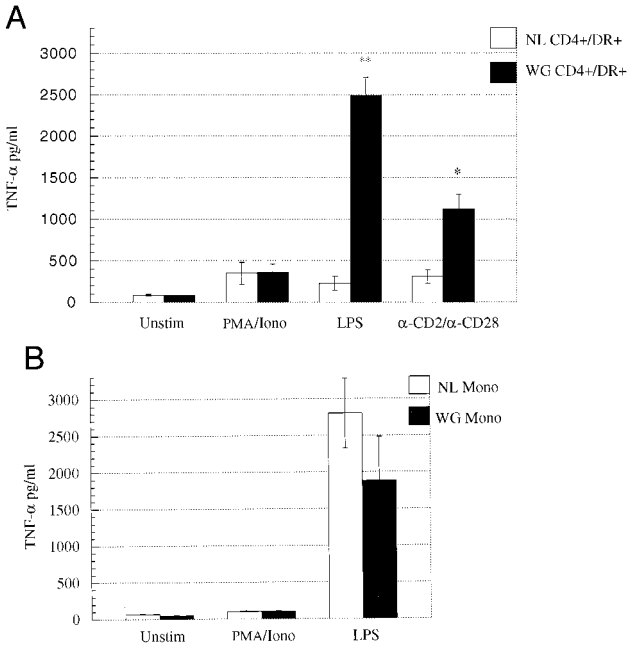

FIGURE 4. TNF-α secretion by CD4$^+$ T cells and monocytes of patients with WG compared with those of healthy donors. *A*, TNF-α secretion from highly purified CD4$^+$ T cells (>90%) isolated from patients with active WG ($n = 6$) compared with those of healthy donors ($n = 8$) 24 h after stimulation. *$p < 0.002$. *B*, TNF-α secretion from purified monocytes isolated from patients with active WG ($n = 8$; black bars) and from healthy donors ($n = 8$; white bars) 6 h after stimulation.

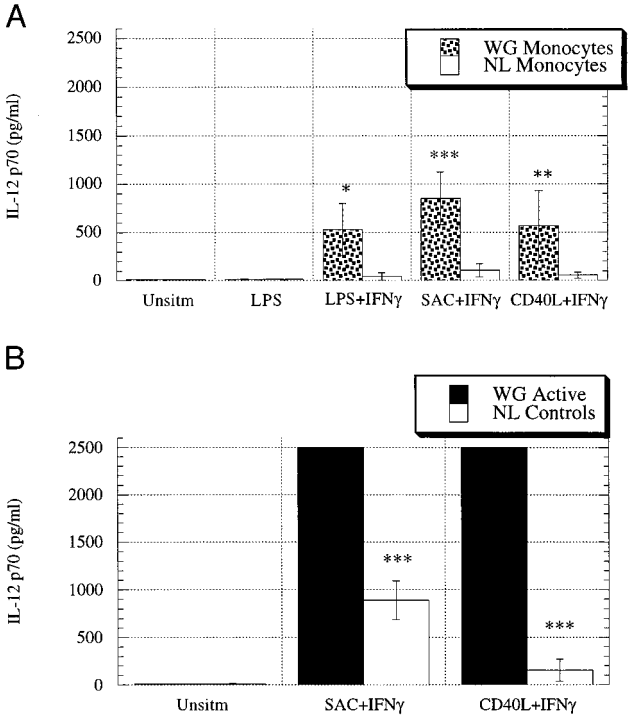

FIGURE 5. Secretory IL-12 (p40, part of the p70 heterodimer) secretion from purified cryopreserved monocytes isolated from patients with inactive WG (*A*; $n = 6$; dotted bars) or freshly isolated monocytes from active WG (*B*; $n = 2$; black bars) compared with cryopreserved (*A*; $n = 8$) and freshly isolated monocytes from healthy donors (*B*; $n = 6$; white bars). Monocytes were stimulated for 24 h with LPS (1 μg/ml), LPS (1 μg/ml) and 1 μg/ml IFN-γ, SAC (0.01%) and 1 μg/ml IFN-γ or CD40L (3 μg/ml), and 1 μg/ml IFN-γ. *$p = 0.032$; **$p = 0.014$; ***$p < 0.001$.

dose-dependent blocking effect on IFN-γ production by PBMCs isolated from patients with active WG. Finally, to determine whether an abnormality of IL-10 secretion accounts for the increased IFN-γ secretion in patients with active WG, we evaluated IL-10 production both by PBMCs and by purified monocytes from patients with active WG; as shown in Table II, these cell populations were found to secrete normal amounts of IL-10.

Discussion

WG was first described by Klinger in 1931 and was subsequently shown by Wegener in l939 to be a systemic disease characterized by necrotizing granulomas involving the upper and lower respiratory tracts, as well as other organs (25, 26). WG is now recognized as a distinctive, small-vessel granulomatous vasculitis that primarily involves the respiratory tract and is associated with a non-immune complex glomerulonephritis (27). That the basic pathophysiologic mechanism in WG is an immune dysfunction was initially suggested by the observations of Fauci and Wolff that immunosuppressive therapy with cyclophosphamide and glucocorticoids very effectively induced sustained remissions of this otherwise fatal disease (1, 28).

Immunohistochemical analyses have shown that the inflammatory lesions in WG contain significant numbers of CD4$^+$ T cells along with macrophages and neutrophils (29, 30). In the present study, we examined the function of circulating T cells on the assumption that disease-inducing T cells traffic through the peripheral circulation before localizing in sites of granulomatous inflammation. The feasibility of this approach is supported by our unpublished observations, as well as those of others, showing that patients with active WG have increased numbers of circulating HLA-DR$^+$ and CD25$^+$ T cells (31). In addition, we show in the present study that circulating T cells from patients with active WG (but not from patients with inactive disease) exhibit increased proliferative responses, and that the subpopulation of cells that accounts for this increased response is in the HLA-DR$^+$ subset.

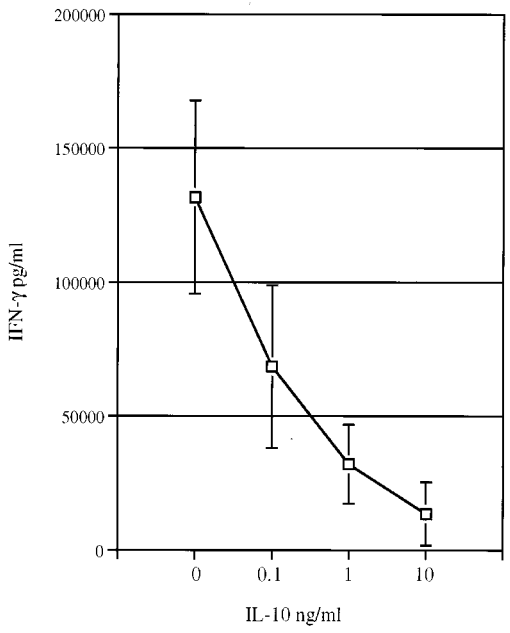

FIGURE 6. IL-10 down-regulates the secretion of IFN-γ. PBMCs were isolated from patients with active WG disease and stimulated with anti-CD2 and anti-CD28 for 48 h in titrating amounts of IL-10, after which cell culture supernatants were analyzed for IFN-γ secretion by ELISA ($n = 6$).

Table II. *IL-10 secretion by PBMCs and monocytes*[a]

	PBMC		Monocytes	
	NL ($n = 8$)	WG ($n = 8$)	NL ($n = 8$)	WG ($n = 8$)
Unstimulated	59 ± 2	60 ± 5	49 ± 3	45 ± 2
PMA/ionomycin	214 ± 87	262 ± 65	ND	ND
LPS[b]	1738 ± 599	1714 ± 474	ND	ND
TNF-α, 0.2 ng/ml	ND	ND	245 ± 130	385 ± 172
TNF-α, 1 ng/ml	ND	ND	579 ± 149	506 ± 220
TNF-α, 5 ng/ml	ND	ND	754 ± 144	925 ± 262

[a] PBMCs or monocytes (1×10^6 cells/ml) cultured as described in *Materials and Methods*; IL-10 pg/ml secretion was measured by ELISA in cell culture supernatants, after 48 h in the case of PBMCs and after 24 h in monocytes. Values are given as mean pg/ml ± SE.

[b] Stimulation with LPS was done in the presence of 3% human serum.

These results, taken together, suggest that these circulating HLA-DR^+ T cells are representative of the disease-inducing T cells present at sites of granulomatous inflammation.

The major finding of this study is that previously activated $CD4^+$HLA-DR^+ T cells in the circulation of patients with active WG produce vastly increased amounts of IFN-γ when stimulated by the T cell-specific anti-CD2/anti-CD28 stimulus. This finding, plus the observation that the same $CD4^+$ T cell population secretes normal amounts of IL-4 and IL-5 following stimulation, strongly suggests that the activated T cells associated with the granulomatous inflammation of WG are skewed toward a Th1 cytokine pattern. An additional potentially important finding is that circulating T cells, but not monocytes/macrophages, from patients with active WG secrete increased amounts of TNF-α. The latter cytokine is also produced by Th1 cells, and its overproduction by $CD4^+$ T cells provides further support for the hypothesis that an aberrant Th1 response may be associated with the granulomatous inflammatory lesion in WG. Findings leading to a similar conclusion have recently been reported with respect to giant cell arteritis and show that cells in lesional tissue were synthesizing mRNA for IFN-γ, but not for IL-4 and IL-5. It is noteworthy, in polymyalgia rheumatica, that temporal artery tissue samples were not found to contain mRNA for IFN-γ (18). Thus, assuming that the cell populations in these patient groups are compatible, a skewed Th1 cytokine secretion pattern appears to be a phenomenon limited to the granulomatous vasculitides.

Increased production of IFN-γ and TNF-α by circulating, previously activated T cells (and, presumably, by lesional T cells in active WG) may help to further define some of the clinical and pathologic features of the disease. In particular, such increased production provides a potential explanation for the generation of the granulomatous lesions in WG via their effects on cellular recruitment and cellular activation in developing lesions. With regard to cellular recruitment, Th1 cytokines such as IFN-γ and TNF-α up-regulate not only the expression of adhesion molecules that are critical to the entry of cells into inflammatory sites but also to the costimulation of the cells that have entered these sites (32). For example, in the rabbit model of granulomatous vasculitis, increased expression of the adhesion molecules ICAM-1 and VCAM-1 has been found to be an early and persistent feature of the inflammatory lesion (33). In addition, IFN-γ is the first cytokine appearing at lesional sites, followed by TNF-α and IL-1. In WG, increased ICAM-1 and VCAM-1 expression can be demonstrated in renal biopsy specimens, and soluble forms of these molecules are elevated in patients with active disease (34–38). Similarly, in T cells isolated from the lesions of giant cell arteritis, the expression of LFA-1 and VLA-2 (the ligands of ICAM-1 and VCAM-1, respectively) is increased. Finally, in TNF-α-induced experimental hemorrhagic vasculitis, soluble TNF-α receptor treatment inhibits both granuloma formation and ICAM-1 expression (39).

With regard to cellular activation in lesional tissue, it is now well established that IFN-γ and TNF-α act synergistically in vitro to induce activation of monocytes/macrophages and their production of proinflammatory cytokines. In addition, in various animal models of granulomatous disease such as that caused by *Leishmania* infection, mice with a high capacity for IFN-γ production manifest granuloma formation and monocyte activation, which clears the infection. In contrast, mice with a low capacity for IFN-γ production lack monocyte activation and develop progressive and often fatal disease (40–44).

Polymorphonuclear (PMN) cells are also significant contributors to the inflammatory lesion in WG, either directly, via the release of toxic materials, or because of an autoimmune response to PMN cell granular proteins. With regard to the latter, Abs against the neutrophil cytoplasmic Ag proteinase-3 (cANCA) are uniquely associated with WG and are frequently elevated during periods of increased disease activity (45–51). While it is unlikely that these Abs play a primary role in the generation of granulomatous lesions, they can conceivably contribute to tissue injury in a secondary fashion. With regard to the present discussion, aberrant production of Th1 cytokines such as IFN-γ and TNF-α may enhance autoantibody production in WG by several mechanisms. First, these cytokines have been shown to induce surface expression of proteinase-3 on endothelial cells and neutrophils (52, 53). Second, these cytokines may facilitate macrophage uptake and presentation of proteinase-3 that has been released from neutrophils and endothelial cells or that has been generated by cells in the lesions undergoing apoptosis. This, in turn, could lead to T cell-dependent B cell production of autoantibodies reactive with proteinase-3. Furthermore, it has recently been shown that neutrophils can be a potential source of IL-12 and, therefore, it is conceivable that during the initial influx of neutrophils they not only are attracting mononuclear cells to the inflammatory site but also promoting a Th1 cytokine response (54).

The finding that WG lesions are associated with T cells markedly skewed toward Th1 cell differentiation implies an abnormality in the regulation of IL-12, the APC-derived cytokine that is the primary inducer of T cells producing IFN-γ. The most important data bearing on this point came from studies of WG monocytes/macrophages showing that monocytes isolated from patients with WG produced greatly increased levels of IL-12 when stimulated with any of a variety of stimulants. Interestingly, while the highest levels of IL-12 production were obtained with monocytes from patients with active disease, monocytes from patients with inactive disease also produced increase amounts of IL-12. This finding implies that the increase in IL-12 production is not a secondary effect to the inflammatory process itself, but rather a primary feature of WG. Also bearing on this issue of the relation of IL-12 to the increased IFN-γ secretion was the demonstration that IL-10 exerts a dose-dependent inhibition of IFN-γ production in culture of WG T cells. Since IL-10 down-regulates IFN-γ production via a more primary inhibitory effect on IL-12 production (55, 56), this finding provides further substantiation to the concept that the increased Th1 response in active WG is mediated by excess IL-12 production. The observation that WG is associated with an IL-12-driven Th1 T cell response is parallel to the finding in sarcoidosis, another granulomatous inflammatory disease in which it has been demonstrated that cells in bronchoalveolar lavage fluids exhibit increased IL-12 production associated with a Th1-like cytokine profile (57).

The cytokine studies described here allow us to postulate a series of pathologic events in WG responsible for the granulomatous

vasculitis that characterizes the disease. The initial event is an exposure to an environmental Ag(s) (perhaps an infectious agent) that induces an excessive macrophage IL-12 response and leads to unbalanced T cell response characterized by overproduction of IFN-γ and TNF-α. This is followed by the establishment of a granulomatous inflammation via changes in cellular adhesion and activation of monocytes and T cells, as described above. The final event is inflammation-induced tissue breakdown with the release of intracellular materials from infiltrating cells, such as proteinase-3, which causes further autoimmune responses that amplify the primary lesion. The environmental Ag(s) capable of initiating this cascade is presently unknown. The earlier suggestion that it was proteinase-3 seems unlikely (at least in relation to the granuloma formation), since proteinase-3 expression is not strictly related to disease activity, and we have found that purified proteinase-3 does not stimulate T cells from WG patients to undergo either proliferation or cytokine secretion (our unpublished observations). Another candidate is a superantigen associated with a respiratory pathogen; this would explain both the initiation of disease in the respiratory area and the exacerbation of disease related to the occurrence of infection.

The findings reported here have important implications for the treatment of WG. In particular, they suggest that any of a variety of approaches to the down-regulation of the Th1 T cell pathway and IL-12 production at the time of disease initiation or exacerbation may be effective in aborting the inflammation. One obvious possibility along these lines is the administration of IL-10 to patients; we are currently exploring this possibility.

Acknowledgments

We thank Charles Carter for providing elutriated monocytes and Nancy Shulman for excellent editorial assistance.

References

1. Fauci, A. S., and S. Wolff. 1973. Wegener's granulomatosis: studies in eighteen patients and a review of the literature. *Medicine 52:535.*
2. Hoffman, G. S., G. S. Kerr, R. Y. Leavitt, C. W. Hallahan, R. S. Lebovics, W. D. Travis, M. Rottem, and A. S. Fauci. 1992. Wegener granulomatosis: an analysis of 158 patients. *Ann. Intern. Med. 116:488.*
3. ten Berge, I., J. M. Wilmink, C. J. Meyer, J. Surachno, K. ten Veen, T. G. Balk, and P. T. Schellekens. 1985. Clinical and immunological follow-up of patients with severe renal disease in Wegener's granulomatosis. *Am. J. Nephrol. 5:21.*
4. Rasmussen, N., and J. Petersen. 1993. Cellular immune responses and pathogenesis in c-ANCA positive vasculitides. *J. Autoimmun. 6:227.*
5. Baltaro, R. J., G. S. Hoffman, J. M. Sechler, A. F. Suffredini, J. H. Shelhamer, A. S. Fauci, and T. A. Fleisher. 1991. Immunoglobulin G antineutrophil cytoplasmic antibodies are produced in the respiratory tract of patients with Wegener's granulomatosis. *Am. Rev. Respir. Dis 143:275.*
6. Hoffman, G. S., J. M. G. Sechler, J. I. Gallin, J. H. Shelhamer, A. Suffredini, F. P. Ognibene, R. J. Baltaro, T. A. Fleisher, R. Y. Leavitt, W. D. Travis, M. F. Barile, M. Tsokos, R. P. Holman, S. E. Straus, and A. S. Fauci. 1991. Bronchoalveolar lavage analysis in Wegener's granulomatosis: a method to study disease pathogenesis. *Am. Rev. Respir. Dis. 143:401.*
7. Savage, C. 1991. Pathogenesis of systemic vasculitis. In *Systemic Vasculitides*. A. Churg and J. Churg, eds. Igaku-Shoin, New York, p. 7.
8. Weyand, C. M., J. Schonberger, U. Oppitz, N. N. Hunder, K. C. Hicok, and J. J. Goronzy. 1994. Distinct vascular lesions in giant cell arteritis share identical T cell clonotypes. *J. Exp. Med. 179:951.*
9. Barnes, P. F., S. Lu, J. S. Abrams, E. Wang, M. Yamamura, and R. L. Modlin. 1993. Cytokine production at the site of disease in human Tuberculosis. *Infect. Immun. 61:3482.*
10. Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams. 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to tuberculosis infection. *J. Immunol. 151:518.*
11. Kindler, V., A. P. Sappino, G. E. Grau, P. F. Piguet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell 56:731.*
12. Fuss, I. J., M. Neurath, M. Boirivant, J. S. Klein, C. de la Motte, S. A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate $CD4^+$ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease: Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J. Immunol. 157:1261.*
13. Center, D. M., H. Kornfeld, M. J. Wu, M. Falvo, A. C. Theodore, J. Bernardo, J. S. Berman, W. W. Cruikshank, R. Djukanovic, L. Teran, and S. Holgate. 1994. Cytokine binding to $CD4^+$ inflammatory cells: implications for asthma. *Am. J. Respir. Crit. Care Med. 150:S59.*
14. Djukanovic, R. 1996. Bronchoscopy as a research tool for the study of asthma pathogenesis and effects of antiasthma drugs. *J. Allergy Clin. Immunol. 98:S64.*
15. Virchow, J., Jr., C. Kroegel, C. Walker, and H. Matthys. 1996. Inflammatory determinants of asthma severity: mediator and cellular changes in bronchoalveolar lavage fluid of patients with severe asthma. *J. Allergy Clin. Immunol. 98:S33.*
16. Secor, W. E. 1994. Sizing up granulomas. *Exp. Parasitol. 78:336.*
17. Ehrhardt, R. O., B. R. Ludviksson, B. Gray, M. Neurath, and W. Strober. 1997. Induction and prevention of colonic inflammation in IL-2-deficient mice. *J. Immunol. 158:566.*
18. Weyand, C. M., K. C. Hicok, G. G. Hunder, and J. J. Goronzy. 1994. Tissue cytokine patterns in patients with polymyalgia rheumatica and giant cell arteritis. *Ann. Intern. Med. 121:484.*
19. Abrahamsen, T. G., C. S. Carter, E. J. Read, M. Rubin, H. G. Goetzman, E. F. Lizzio, Y. L. Lee, M. Hanson, P. A. Pizzo, and T. Hoffman. 1991. Stimulatory effect of counterflow centrifugal elutriation in large-scale separation of PB monocytes can be reversed by storing the cells at 37°C. *J. Clin. Apheresis 6:48.*
20. Yokoyama, W. M. 1991. Monoclonal antibody supernatant and ascites fluid production. In *Current Protocols in Immunology*. J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober, eds. Greene Publishing and Wiley Interscience, New York.
21. Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. *Cell 36:897.*
22. Windhagen, A., D. E. Anderson, A. Carrizosa, R. E. Williams, and D. A. Hafler. 1996. IL-12 induces human T cells secreting IL-10 with IFN-γ. *J. Immunol. 157:1127.*
23. Gerosa, F., C. Paganin, D. Peritt, F. Paiola, M. T. Scupoli, M. Aste-Amezaga, I. Frank, and G. Trinchieri. 1996. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-γ and interleukin-10. *J. Exp. Med. 183:2559.*
24. Gong, J. H., M. Zhang, R. L. Modlin, P. S. Linsley, D. Iyer, Y. Lin, and P. F. Barnes. 1996. Interleukin-10 down-regulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression. *Infect. Immun. 64:913*
25. Wegener, F. 1939. Uber eine eigenartige rhinogene granulomatose mit besonderer beteiligung des arterien systems und der nieren. *Beitr. Pathol. Anat. Allg. Pathol. 36:202.*
26. Klinger, H. 1931. Grenzformen der periarteritis nodosa. *Frankf. Z. Pathol. 42: 455.*
27. Jennette, J. C., R. J. Falk, K. Andrassy, P. A. Bacon, C. J., W. L. Gross, E. C. Hagen, G. S. Hoffman, G. Hunde, C. G. M. Kallenberg, R. T. McCluskey, R. A. Sinico, A. Rees, L. A. Vanes, R. Waldherr, and A. Wiik. 1994. Nomenclature of systemic vasculitides: proposal of an International Consensus Conference. *Arthritis Rheum. 37:187.*
28. Fauci, A. S., S. M. Wolff, and J. S. Johnson. 1971. Effect of cyclophosphamide upon the immune response in Wegener's granulomatosis. *N. Engl. J. Med. 285: 1493.*
29. Noronha, I. L., C. Kruger, K. Andrassy, E. Ritz, and R. Waldherr. 1993. In situ production of TNF-α, IL-1β and IL-2R in ANCA-positive glomerulonephritis. *Kidney Int. 43:682.*
30. Ronco, P., P. Verroust, F. Mignon, O. Kourilsky, P. Vanhille, A. Meyrier, J. P. Mery, and M. L. Morel. 1983. Immunopathological studies of polyarteritis nodosa and Wegener's granulomatosis: a report of 43 patients with 51 renal biopsies. *Q. J. Med. 52:212.*
31. Schlesier, M., T. Kaspar, J. Gutfleisch, G. Wolff-Vorbeck, and H. H. Peter. 1995. Activated $CD4^+$ and $CD8^+$ T-cell subsets in Wegener's granulomatosis. *Rheumatol. Int. 14:213.*
32. Baudeau, C., F. Delarue, C. J. He, G. Nguyen, C. Adida, M. N. Peraldi, J. D. Sraer, and E. Rondeau. 1994. Induction of MHC class II molecules HLA-DR, -DP and -DQ and ICAM-1 in human podocytes by γ-interferon. *Exp. Nephrol. 2:306.*
33. Coers, W., E. Brouwer, J. T. Vos, A. Chand, S. Huitema, P. Heeringa, C. G. Kallenberg, and J. J. Weening. 1994. Podocyte expression of MHC class I and II and intercellular adhesion molecule-1 (ICAM-1) in experimental paucimmune crescentic glomerulonephritis. *Clin. Exp. Immunol. 98:279.*
34. Pall, A. A., A. J. Howie, D. Adu, G. M. Richards, C. D. Inward, D. V. Milford, N. T. Richards, J. Michael, and C. M. Taylor. 1996. Glomerular vascular cell adhesion molecule-1 expression in renal vasculitis. *J. Clin. Pathol. 49:238.*
35. Boehme, M. W., W. H. Schmitt, P. Youinou, W. R. Stremmel, and W. L. Gross. 1996. Clinical relevance of elevated serum thrombomodulin and soluble E-selectin in patients with Wegener's granulomatosis and other systemic vasculitides. *Am. J. Med. 101:387.*
36. Mrowka, C., and H. G. Sieberth. 1995. Detection of circulating adhesion molecules ICAM-1, VCAM-1 and E-selectin in Wegener's granulomatosis, systemic lupus erythematosus and chronic renal failure. *Clin. Nephrol. 43:288.*
37. Mrowka, C., and H. G. Sieberth. 1994. Circulating adhesion molecules ICAM-1, VCAM-1 and E-selectin in systemic vasculitis: marked differences between Wegener's granulomatosis and systemic lupus erythematosus. *Clin. Invest. 72:762.*
38. Stegeman, C. A., J. W. Tervaert, M. G. Huitema, P. E. de Jong, and C. G. Kallenberg. 1994. Serum levels of soluble adhesion molecules intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin in patients with Wegener's granulomatosis. Relationship to disease activity and relevance during followup. *Arthritis Rheum. 37:1228.*

39. Ythier, A., M. P. Gascon, P. Juillard, C. Vesin, D. Wallach, and G. E. Grau. 1993. Protective effect of natural TNF-binding protein on human TNF-induced toxicity in mice. *Cytokine 5:459.*
40. Kopf, M., F. Brombacher, G. Kohler, G. Kienzle, K. H. Widmann, K. Lefrang, C. Humborg, B. Ledermann, and W. Solbach. 1996. IL-4-deficient BALB/c mice resist infection with *Leishmania major*. *J. Exp. Med. 184:1127.*
41. Brown, D. R., K. Swier, N. H. Moskowitz, M. F. Naujokas, R. M. Locksley, and S. L. Reiner. 1997. T helper subset differentiation in the absence of invariant chain. *J. Exp. Med. 185:31.*
42. Brown, J. A., R. G. Titus, N. Nabavi, and L. H. Glimcher. 1996. Blockade of CD86 ameliorates *Leishmania major* infection by down-regulating the Th2 response. *J. Infect. Dis. 174:1303.*
43. Guler, M. L., J. D. Gorham, C. S. Hsieh, A. J. Mackey, R. G. Steen, W. F. Dietrich, and K. M. Murphy. 1996. Genetic susceptibility to *Leishmania*: IL-12 responsiveness in TH1 cell development. *Science 271:984.*
44. Afonso, L. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott. 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. *Science 263:235.*
45. Cohen, T. J., F. J. van der Woude, A. S. Fauci, J. L. Ambrus, J. Velosa, W. F. Keane, S. Meijer, M. van der Giessen, G. van der Hem, and T. H. The. 1989. Association between active Wegener's granulomatosis and anticytoplasmic antibodies. *Arch. Intern. Med. 149:2461.*
46. Davis, D., J. E. Moran, J. F. Niall, and G. B. Ryan. 1982. Segmental necrotising glomerulonephritis with antineutrophil antibody. *Br. Med. J. 285:606.*
47. Goldschmeding, R., C. E. vanderSchoot, D. tenBokkelHuinink, C. E. Hack, M. E. vandenEnde, C. G. M. Kallenberg, and A. E. G. vondemBorne. 1989. Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. *J. Clin. Invest. 84:1577.*
48. Niles, J. L., R. T. McCluskey, M. F. Ahmad, and M. Amin Arnaout. 1989. Wegener's granulomatosis autoantigen is a novel neutrophil serine proteinase. *Blood 74:1888.*
49. Nolle, B., U. Specks, J. Ludemann, M. S. Rohrbach, D. A. DeRemee, and W. L. Gross. 1989. Anticytoplasmic autoantibodies: their immunodiagnostic value in Wegener's granulomatosis. *Ann. Intern. Med 111:28.*
50. Specks, U., C. L. Wheatley, T. J. McDonald, M. S. Rohrbach, and D. A. DeRemee. 1989. Anticytoplasmic autoantibodies: their immunodiagnostic value in Wegener's granulomatosis. *Mayo Clin. Proc. 64:28.*
51. Van der Woude, F. J., S. Lobatto, H. Permin, M. van der Giessen, N. Rasmussen, and A. Wiik. 1985. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet 1:425.*
52. Mayet, W. J., E. Csernok, C. Szymkowiak, W. L. Gross, and K-H. Meyer zum Buschenfelde. 1993. Human endothelial cells express proteinase-3, the target antigen of anticytoplasmic antibodies in Wegener's granulomatosis. *Blood 82:1221.*
53. Csernok, E., M. Ernst, W. Schmitt, D. F. Bainton, and W. L. Gross. 1994. Activated neutrophils express proteinase-3 on their plasma membrane in vitro and in vivo. *Clin. Exp. Immunol. 95:244.*
54. Romani, L., A. Mencacci, E. Cencei, R. Spaccapelo, G. Del Sero, I. Nicoletti, G. Trinchieri, F. Bistoni, and P. Puccetti. 1997. Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. *J. Immunol. 158:5349.*
55. Dai, W. J., G. Kohler, and F. Brombacher. 1997. Both innate and acquired immunity to *Listeria monocytogenes* infection are increased in IL-10-deficient mice. *J. Immunol. 158:2259.*
56. Gong, J. H., M. Zhang, R. L. Modlin, P. S. Linsley, D. Iyer, Y. Lin, and P. F. Barnes. 1996. Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression. *Infect. Immun. 64:913.*
57. Moller, D. R., J. D. Forman, M. C. Liu, P. W. Noble, B. M. Greenlee, P. Vyas, D. A. Holden, J. M. Forrester, A. Lazarus, M. Wysocka, G. Trinchieri, and C. Karp. 1996. Enhanced expression of IL-12 associated with Th1 cytokine profiles in active pulmonary sarcoidosis. *J. Immunol. 156:4952.*