Sarcoidosis is characterized by extensive local inflammation (granuloma, cytokine secretion) associated with anergy (poor response to antigens in vitro and in vivo). We postulated that this paradoxical situation would correspond to a disequilibrium between effector and regulatory T lymphocytes (T reg cells). We show that CD4+CD25brightFoxP3+ cells accumulate at the periphery of sarcoid granulomas, in bronchoalveolar lavage fluid, and in peripheral blood of patients with active disease. These cells exhibited powerful antiproliferative activity, yet did not completely inhibit TNF-α production. Sarcoidosis is therefore associated with a global T reg cell subset amplification whose activity would be insufficient to control local inflammation. At the same time, peripheral T reg cells exert powerful antiproliferative activity that may account for the state of anergy. Altogether, these findings advance our conceptual understanding of immune regulation in a way that resolves the immune paradox of sarcoidosis and permit us to envisage a profound clinical impact of T reg cell manipulation on immunity.
suppress naive T cell proliferation, but are unable to totally suppress TNF-α secretion.

RESULTS

Definition of T reg cells
The percentage of CD4+ lymphocytes expressing very high levels of CD25 was initially determined using cytofluorometric analysis. The forkhead/winged helix transcription factor FoxP3 is crucial to the development and function of T reg cells (21–23) and so far considered the best marker available to identify naturally occurring T reg cells. Using real-time PCR, we confirmed that only CD4+CD25bright cells, as defined according to our gating strategy (Fig. 1 a), express high levels of FoxP3 among CD4+ T cells (Fig. 1 b). We also used a single cell PCR approach to calculate precisely the fraction of FoxP3+ cells present among sorted cells. Although very few CD4+CD25− cells expressed FoxP3 (0.7%), we determined by this approach that, on average, 86.5% of sorted CD4+CD25bright cells were Foxp3+ (Fig. 1 d). Furthermore, CD4+CD25bright T cells present other typical features of naturally occurring T reg cells such as intracellular expression of CTLA-4 (24, 25) and cell surface CCR4 expression (19) (Fig. 2 a). More importantly, these cells are the only CD4+ population that exerts strong dose-dependent inhibition on the CD4+CD25− T cell proliferation (16) (Fig. 1 c). These combined phenotypic and functional findings allow us to define the CD4+CD25bright cells enumerated herein as T reg cells.

High levels of blood and BALF CD4+CD25bright T cells in active sarcoidosis
We compared the percentage of CD25bright cells among circulating CD4+ T cells in patients and controls (Fig. 2 a, representative staining patterns). As shown in Fig. 3 a, significant increase in the mean value for patients with active sarcoidosis is evidenced when this group is compared with healthy controls (5.05 ± 5.33%, n = 29 vs. 1.32 ± 0.39%, n = 55, P < 0.0001; and 1.18 ± 0.81%, n = 18, P = 0.001, respectively). No significant difference was observed between inactive sarcoidosis and healthy controls (P = 0.25). We noted a modest but nevertheless significant decrease in the mean percentage of circulating CD4+CD25bright T cells in patients presenting with active tuberculosis compared with healthy controls (0.89 ± 0.41%, n = 13, P = 0.0016). Finally, CD4+CD25bright T cells are also abundant in sarcoidosis-involved organs. A significant increase in the mean percentage of BALF CD4+CD25bright T cells is noted in active sarcoidosis as compared with diseases that mimic sarcoidosis (7.01 ± 3.96%, n = 19, vs. 1.18 ± 0.85%, n = 24, P < 0.0001). On one occasion, we were able to extract lymphocytes from a sarcoid skin biopsy and determine that 7.8% of the skin CD4+ T cells were CD25bright (Fig. 2 a).

BALF CD4+CD25bright T cells overexpress CXCR3
In an attempt to identify a potential mechanism that would explain why many CD4+CD25bright T cells are found in the lung, whereas others recirculate to the periphery, we studied their chemokine receptor expression. All CD4+CD25bright T cells express CCR4 (Fig. 2 a), but only a minority of them express CCR3 in control blood (Fig. 2 b). In contrast, we found that in sarcoidosis patients, all BALF CD4+CD25bright T cells express high levels of CXCR3, whereas only a low level display of this receptor is found on the corresponding blood subset. The CD4+CD25bright T cell expression pattern of other chemokine receptors (CCR1 to CCR9, CXCR4, CXCR6, and CX3CR1) was also studied (26), but no clear difference between patients and control was observed (Fig. 2 b).

CD4+CD25bright T cells from a sarcoidosis patient express a polyclonal TCR repertoire
To address the question of whether CD4+CD25bright T cell numbers are increased during active sarcoidosis as a result of oligoclonal expansion, we investigated the T cell repertoire usage of sorted CD4+CD25bright T cells. As described previously (27, 28), we chose to study nine BV families that span >50% of the TCRBV repertoire expressed in healthy individuals (29, 30). We used the Immunoscope technique (31), which is based on the analysis of TCR CDR3 length polymorphism to assess the clonal composition of the CD4+CD25bright T cell subset. 5 × 10⁶ of these cells, as well as a corresponding number of CD4+CD25− T cells, were purified from a lymph node obtained from one patient with active sarcoidosis. It was verified that the sorted CD4+CD25bright T cells used for repertoire analysis were indeed immunosuppressive (Fig. 2 a). Immunoscope analysis of cells isolated from one patient shows (Fig. 4) that both subsets are similarly diverse and also display similar TCRBV size distributions.

Contraction of the CD4+CD25bright subset upon resolution of sarcoidosis
Because we observed that active sarcoidosis is characterized by the expansion of CD4+CD25bright T cells, we next questioned whether this subset size would vary within the same individual in relation to disease activity. In the 11 individuals tested longitudinally, there was a marked contraction of the initially expanded CD4+CD25bright circulating subset that returned to normal levels upon resolution of disease activity (5.2 ± 4.7% vs. 1.2 ± 0.4%, P = 0.02 using the Wilcoxon Signed-Rank test; Fig. 2). Amplification of the CD4+CD25bright T cell subset is therefore clearly related to the disease process.

CD4+ FoxP3+ cells accumulate in the periphery of noncaseating granulomas
We subsequently sought to visualize where T reg cells were localized in affected organs using bicolor microscopic analysis. As shown in Fig. 5, T reg cells identified as CD4+FoxP3+ cells accumulate in the granuloma periphery. These cells are also clearly more abundant in sarcoidosis-involved lymph nodes than in normal lymphoid tissue from control subjects (6.48 ± 2.12% of CD4+ cells, n = 10 vs. 1.67 ± 0.67%, n = 2).
CD4+CD25bright T cells from active patients suppress T cell proliferation

Phenotypic characterization of CD4+CD25bright cells as being also CCR4+CTLA-4+ is not sufficient to define them as immunosuppressive T reg cells. We therefore tested whether the CD4+CD25bright subset found expanded during active sarcoidosis is functionally active by purifying and testing it ex vivo. As expected, CD4+CD25− T cells from sarcoidosis patients have a reduced proliferation capacity as compared with healthy controls (29,700 ± 24,130 cpm, n = 18 vs. 62,300 ± 20,200 cpm, n = 19, P = 0.0005). However, irrespective of their tissue of origin (blood, BALF, or lymph node), CD4+CD25bright T cells from sarcoidosis patients inhibit T cell proliferation as efficiently as control CD4+CD25bright T cells (Fig. 6 a).

Sarcoidosis T reg cells abolish IL-2 but not IFN-γ or TNF-α secretion by autologous and allogeneic cells

It is believed that TNF-α could play a central role in sarcoidosis physiopathology (3, 4). The effect of patients isolated T reg cells on cytokine production was therefore studied. Cell cultures of each of nine patients and nine controls were analyzed three times over a period of 5 d (Fig. 6 b). In the control branch of the study, T reg cells completely inhibited TNF-α and IFN-γ secretion as measured on day 5 after stimulation (partial inhibition on day 3), whereas in the experimental arm, a clear residual cytokine secretion was apparent on day 5. These data are summarized in Fig. 6 c. Intriguingly, patients’ T reg cells completely inhibit IL-2 production (mean residual secretion 4.21 ± 2.57%, n = 18 vs. 4.23 ± 2.90%, n = 19, in controls), but not that of IFN-γ (19.02 ± 12.37% vs. 4.07 ± 2.91%) or TNF-α (27.23 ± 15.29% vs. 2.45 ± 2.7%). In comparison, control T reg cells inhibit the secretion of all three cytokines.

We then sought to determine if the expanded CD4+CD25bright subset could itself represent a notable source of TNF-α in patients suffering from sarcoidosis. In five patients and four controls, we obtained enough CD4+CD25bright cells to study cytokine secretion in an isolated manner. As shown, CD4+CD25bright cells contribute only slightly to the residual level of TNF-α secretion observed in the patient according to morphologic parameters. (b) Only CD25bright cells express high levels of FoxP3 among CD4+ T cells. Quantification of relative FoxP3 mRNA levels in CD4+ T cell subsets were sorted as indicated. cDNA samples were subjected to real-time quantitative PCR analyses using primers and an internal fluorescent probe specific for FoxP3 or HPRT. (c) CD25bright cells strongly inhibit the proliferation of autologous CD4+CD25− cells. 1 representative healthy control out of 10 was analyzed for natural T reg cell activity. (d) Detection of FoxP3 transcripts in single cells sorted according to the aforementioned defined parameters. One lane corresponds to one cell. Multiplex PCR was performed on 190 CD4+CD25bright cells and 282 CD4+CD25− cells to detect FoxP3 and CD3δ simultaneously (chosen as a lymphocyte house-keeping gene). As shown, six out of seven representative CD4+CD25bright cells are also FoxP3+ (164 cells out of 190, 86.3%). Only two CD4+CD25− cells were FoxP3+ (0.7%).

Figure 1. Isolation and characterization of human natural T reg cells. (a) Red background fluorescence upper level (x axis) is used to define the CD4+CD25− sorting gate (top left). The CD4+CD25++ (CD25bright) gate was adjusted to contain CD4+ T cells that express CD25 more brightly than CD4+CD25− cells (top right). CD4+CD25− cells are mainly CD4high, but a tiny fraction is also CD4low. CD4 fluorescence levels were therefore used to define CD4+CD25+ and CD4+CD25++ subsets as shown (bottom). Analysis was restricted to lymphocytes defined according to morphologic parameters. (b) Only CD25bright cells express high levels of FoxP3 among CD4+ T cells. Quantification of relative FoxP3 mRNA levels in CD4+ T cell subsets were sorted as indicated. cDNA samples were subjected to real-time quantitative PCR analyses using primers and an internal fluorescent probe specific for FoxP3 or HPRT. (c) CD25bright cells strongly inhibit the proliferation of autologous CD4+CD25− cells. 1 representative healthy control out of 10 was analyzed for natural T reg cell activity. (d) Detection of FoxP3 transcripts in single cells sorted according to the aforementioned defined parameters. One lane corresponds to one cell. Multiplex PCR was performed on 190 CD4+CD25bright cells and 282 CD4+CD25− cells to detect FoxP3 and CD3δ simultaneously (chosen as a lymphocyte house-keeping gene). As shown, six out of seven representative CD4+CD25bright cells are also FoxP3+ (164 cells out of 190, 86.3%). Only two CD4+CD25− cells were FoxP3+ (0.7%).
Figure 2. Phenotype and proportion of CD4+CD25bright T cells in patients and controls. (a) CD4+CD25bright T cells are abundant in blood, BALF, lymph nodes, and skin of patients with active sarcoidosis. Representative cytofluorometric analysis of (from left to right) healthy donor PBMCs, active sarcoidosis (AS) PBMCs, AS BALF lymphocytes, AS lymphocytes isolated from a lymph node (LN), and AS skin lymphocytes is shown. The percentage of CD4+ T cells expressing very high levels of CD25 (CD-25bright) is indicated in each case. Gates used to count and sort CD4+CD25bright cells are shown. CD4+CD25− (unshaded histograms) and CD4+CD25bright cells (shaded histograms) were simultaneously analyzed for their expression of membrane CCR4 and intracellular CTLA-4 (iCTLA-4). (b) Comparative expression of CXCR3 on BALF and blood CD4+CD25bright cells. CD4+CD25bright cells were analyzed by flow cytometry for their expression of membrane CXCR3 in one patient with active sarcoidosis and in one healthy control (left). Results of one representative experiment out of five are presented. Expression of other indicated chemokine receptors in a representative control subject and in one AS patient is shown. Flow cytometry analysis of CD4+CD25bright and CD4+CD25− gated lymphocytes is presented. Results of one representative experiment out of two are presented. (c) Contraction of the CD4+CD25bright circulating subset upon resolution of sarcoidosis. One representative longitudinal cytofluorometric analysis (left and middle). Longitudinal monitoring of CD4+CD25bright T cells in 11 patients with resolutive sarcoidosis (right). Percentage of peripheral blood CD4+CD25bright cells was initially measured when sarcoidosis was active and after resolution (mean time interval between two measures: 5 mo ± 2.5). The horizontal dashed line represent the average percentage of CD4+CD25bright T cells in healthy controls (n = 55). Comparisons were made using the Wilcoxon Signed Rank test.
group after 5 d of stimulation (Fig. 6 b). Further control experiments confirmed that early production (day 1) of TNF-α is of accessory cell (AC) origin, whereas the bulk of TNF-α released during the course of activation is produced by CD25− T cells (Fig. 6 d). As shown, only the latter source of TNF-α is sensitive to T reg–mediated suppression.

We then examined whether this confirmed partial loss of regulatory function on cytokine secretion was the result of a decrease in T reg cell activity or to a resistance of the patients’ CD4+CD25− cells to inhibition. To address this issue, “criss-cross” experiments were performed. T reg cells from patients were tested on healthy donors’ responder cells, and vice versa (Fig. 6 c). T reg cells from patients do not completely block cytokine secretion from either patients or healthy controls. However, residual amounts of TNF-α are also detected in the supernatants of sarcoidosis CD4+CD25− cells stimulated in the presence of control T reg cells.

The aforementioned results were obtained using low-dose anti-CD3 (16), and allogeneic ACs as stimulus according to previous defined protocols (12–14, 32, 33). To verify that the persistence of TNF-α and IFN-γ would not in fact be secondary to strong allogeneic responses, we repeated the functional analyses of T reg cells using only autologous ACs and anti-CD3 (0.5 μg/ml). Under these conditions, TNF-α and IFN-γ production are not totally suppressed by sarcoidosis T reg cells (n = 4) compared with controls (n = 5, P < 0.02, Fig. 6 d). Therefore, the incomplete control of TNF-α and IFN-γ production is not only observed in the context of strong allogeneic responses.

Most IFN-γ/TNF-α–secreting cells do not produce IL-2
It follows from our results that (a) TNF-α and IFN-γ production and (b) IL-2 production would be differentially affected by the presence of T reg cells. We postulated that this could be partially explained by the fact that TNF-α and IFN-γ are usually produced by cells that no longer (or never) made IL-2. To address that issue, we used four-color cytometry to monitor IL-2, TNF-α, and IFN-γ at a single cell level in three patients and five controls after a short-term in vitro stimulation (Fig. 7). We show that IFN-γ and IL-2 production are almost mutually exclusive. Although some TNF-α–producing CD4+ cells can secrete IL-2, a subset of CD4+IL2− TNF-α+IFN-γ+ cells can be clearly defined in patients’ and controls’ blood (Fig. 7). Altogether, we conclude on the existence of a defect in regulatory function exerted by sarcoidosis T reg cells, as well as a concurrent relative resistance on the part of highly differentiated (TNF-α+, IFN-γ+) autologous effector T cells.

**DISCUSSION**

It is generally accepted that sarcoidosis is a disorder mediated by excessive local helper T lymphocyte activity (1). Until now however, it has been difficult to explain why the intense inflammatory activity observed in sarcoidosis patients is commonly accompanied by a state of anergy. It was originally proposed that suppressive CD8 T cells could be involved in the down-modulation of cellular responses (1). More recently, a high percentage of CD4+CD25bright cells was reported in the blood and BALF of HLA-DR17+ patients with...
active disease (34), but the regulatory activity of these cells was not demonstrated. We show here that the T reg population is indeed globally amplified in circulating blood and BALF of patients presenting with active sarcoidosis. In vitro, the capacity of these cells to strongly inhibit the proliferation and IL-2 secretion of CD4+CD25− T lymphocytes contrasts with their inability to completely inhibit the secretion of TNF-α (and to a lesser extent IFN-γ). The latter observation could be particularly relevant as TNF-α plays a key role in granuloma formation (35) and because anti-TNF-α drugs have proved useful in the treatment of refractory sarcoidosis (36). Because T reg cells do not completely block TNF-α secretion, they might only partially control granuloma evolution in vivo. A limited effect of T reg cells on TNF-α secretion has previously been reported in patients suffering from rheumatoid arthritis (37). T reg function was also found impaired in multiple sclerosis (38). Given our data, we cannot conclude that T reg cells found in sarcoidosis patients have an intrinsic defect. It is equally probable that these cells are simply incapable of controlling an abnormally abundant secretion of TNF-α. Our analysis of cytokine production at a single cell level (Fig. 7) delineates nonoverlapping subsets of CD4+ cells as defined according to their cytokine secretion profile. In particular, a CD4+IL2−IFN-γ+TNF-α+ subset can be clearly defined. More work will be necessary to determine whether the later subset would be less sensitive to T reg suppression functionality in sarcoidosis patients. In any case, it is possible that phenotypically and functionally distinct subsets of CD4+ cells would be differentially regulated by T reg cells.

Although unable to totally suppress TNF-α and IFN-γ production in sarcoidosis patients, T reg cells exert a profound antiproliferative activity and are able to abolish IL-2 secretion as expected (mean suppression 95.8 ± 2.6%, Fig. 6 c). T cell expansion represents a crucial aspect of memory responses. Memory T lymphocytes specific for a given antigen are scarce. The latter could have their proliferation effectively suppressed when they encounter their recall antigen in lymphoid organs harboring an abnormal proportion of T reg cells. We show that T reg cells are indeed abundant in affected lymph nodes, but are infrequently found in the lymph nodes of healthy controls, lending support to a T reg cell/T memory disequilibrium as a possible mechanism of anergy.

We therefore postulate that the aforementioned paradox can be solved if one considers that T reg cells tightly control T cell homeostasis mainly through the control of T cell expansion (39), and that this mechanism is overly efficient in sarcoidosis patients. Alternatively, the same mechanism would not efficiently control the inflammation of injured tissues.

In active sarcoidosis patients, the chemokine receptor CCR4 is preferentially expressed on CD4+CD25bright T reg cells irrespective of their tissue of origin. In contrast, CXCR3 is overexpressed on BALF CD4+CD25bright cells. It was recently demonstrated that the specific recruitment of T reg cells in human ovarian tumors is mediated by the secretion of the CCR4 ligand CCL22 (macrophage-derived chemokine) by tumor cells and microenvironmental macrophages (19). However, lung macrophages from sarcoidosis patients do not secrete CCL22 (40), whereas they are known to produce high levels of the CXCR3 ligand CXCL10 (IFN-γ-inducible 10-kd protein [IP-10]) (41). It is therefore possible that it is in response to IP-10 secretion by activated tissue macrophages that CXCR3+ T reg cells are recruited to affected organs. Only cells that down-modulate CXCR3 would recirculate to the periphery. To explore another potential mechanism that would induce T reg migration and expansion,
we also studied the clonal diversity of this subset (Fig. 4). Using CDR3-length polymorphism analysis (27), we found that CD4+CD25bright T reg cells are polyclonal in one sarcoidosis patient. It is therefore unlikely that in this case CD4+CD25bright expansion would result from direct antigenic stimulation. Unfortunately, it was not possible until now to purify enough CD4+CD25bright cells from sarcoidosis BALF to perform additional reliable repertoire studies. It is known that oligoclonal population of αβ+ CD4+ T cells collect at granulomatous sites (42–44) and at Kveim reaction sites (45). Mycobacterial catalase-peroxydase was recently identified as one target of the adaptive immune response in sarcoidosis (46), but it remains unknown whether granuloma-associated T cells actually include mycobacterial catalase-peroxydase–specific T cells. In light of the data presented here, it could be interesting to study the reactivity of sarcoidosis T reg cells against mycobacterial antigens. In a first step, it will be necessary to solve the issue of the T reg repertoire diversity at granulomatous sites of inflammation.

To our knowledge, there is no clinical situation in which a T reg amplification of such magnitude (up to 21% of CD4+ T cells) has been found in the blood of patients. It is possible that the observed spontaneous release of IL-2 by lung T lymphocytes in active pulmonary sarcoidosis could be one of the factors that participate to their amplification (47). Circulating CD4+CD25bright cells were reported elevated during chronic graft-versus-host disease (48), but never exceeded 11% of CD4+ T cells. Accumulation of T reg cells has also been observed in the joints of rheumatoid arthritis patients (49) and in Hodgkin lymphoma–infiltrated lymph nodes (50). More studies are underway to confirm whether the expansion of circulating T reg cells is specific enough to distinguish sarcoidosis from any other granulomatosis. There currently exists no gold standard to confirm the diagnosis of sarcoidosis. We will therefore work to determine the peripheral blood or BALF T reg cell concentration that supports this diagnosis.

Finally, it is proposed that modulation of either the number or function of T reg cells could represent a means of controlling autoimmunity or other immunopathologic conditions (7, 18). Our data clearly support this assertion, but also indicate that such potential therapies might be, in the long term, detrimental to immune surveillance. Indeed, sarcoidosis patients are predisposed to opportunistic infections (51) and cancer (52). It was previously proposed that the increased cancer risk would result from chronic inflammation and subsequent tissue damage in affected organs (52). In light of the data presented here, we propose that a T reg cell/Th1 imbalance could also be evoked to explain why sarcoidosis patients have such a predisposition.

**MATERIALS AND METHODS**

**Patients.** 47 consecutive adult patients (25 women and 22 men; mean age: 43 yr; range: 23–65 yr) who had a clinical picture consistent with sarcoidosis (3) were recruited for this study. Patients were divided into two groups: patients with active, newly diagnosed sarcoidosis (n = 29) and patients with inactive disease (n = 18). Patients with active disease were studied before any treatment was initiated. Patients with inactive disease were either on low dose steroid therapy (<10 mg/day; n = 13) or not treated at all (n = 5). Blood samples from 55 age and sex matched healthy donors were obtained from Etablissement Français du Sang (Hôpital Pitié-Salpêtrière). A granulomatous
Figure 6. Sarcoidosis T reg cells suppress T cell proliferation but not IFN-γ or TNF-α secretion. (a) Control and sarcoidosis CD4+CD25bright cells strongly inhibit proliferation of autologous CD4+CD25− cells. Results are shown of add-back experiments in a representative healthy volunteer (out of 19 tested, see panel b) and in a representative sarcoidosis patient. CD4+CD25− T cells were flow sorted...
From blood \((n = 18)\), BAL \((n = 3)\) or lymph node \((n = 2)\) and stimulated with anti-CD3 and irradiated autologous AC in the presence of different numbers of flow sorted CD4<sup>+</sup>CD25<sup>+</sup> bright T cells CD25<sup>+</sup> ratios are indicated. (b) Time course analysis of cytokine secretion in patients and controls. IL-2, TNF-α, and IFN-γ were measured in supernatants from individual wells that were prepared for the in vitro suppression assays. In indicated patients and controls, cytokine secretion of CD4<sup>+</sup>CD25<sup>+</sup> alone was also studied. Individual analyses are presented. Mean values on day 5 and at a 1:1 ratio of CD25<sup>+</sup>/CD25<sup>−</sup> autologous coculture are as follows: IL-2, 1.47 ± 1.80 pg/ml vs. 1.82 ± 1.53 pg/ml, \(P = 0.49\); TNF-α, 2113 ± 152.7 pg/ml vs. 183.9 ± 237.4 pg/ml, \(P < 0.0001\); and IFN-γ 1,530.6 ± 1,782 pg/ml vs. 868.5 ± 1,232 pg/ml, \(P = 0.0003\) (patients, \(n = 18\) vs. controls, \(n = 19\)). (c) Sarcoidosis T reg cells strongly inhibit autologous CD4<sup>+</sup>CD25<sup>+</sup> proliferation and IL-2 secretion, but not IFN-γ or TNF-α. In comparison, control T reg cells strongly inhibit all three cytokines. Sarcoidosis T reg cells do not completely block allogeneic secretion of IFN-γ and TNF-α. Note that control T reg cells do not either completely block patient's TNF-α secretion. Mean percentage of residual proliferation and corresponding mean percentage of residual cytokine secretion of CD4<sup>+</sup>CD25<sup>+</sup> cells cocultured with autologous or allogeneic flow sorted T reg cells are presented. (d) Lack of complete TNF-α and IFN-γ suppression is not related to strong allogeneic responses. Add-back experiments presented in a–c have been repeated in the absence of allogeneic AC. Blood CD4<sup>+</sup>CD25<sup>−</sup> T cells were instead stimulated with anti-CD3 (0.5 pg/ml) and irradiated autologous AC in the presence or in the absence of CD4<sup>+</sup>CD25<sup>+</sup> T cells CD25<sup>+</sup>/CD25<sup>−</sup> ratios are indicated. Proliferation of AC alone was also tested (AC). Indicated cytokines were measured in the culture supernatant at indicated times (days). Note that early TNF-α secretion is contributed by AC. One representative experiment in a representative control and a representative sarcoidosis patient (top and middle) are shown. Results from four independent patients and five independent controls are presented (bottom). Comparisons were made using the nonparametric Mann–Whitney U test.
Figure 7. Most cells that coproduce TNF-α and IFN-γ do not secrete IL-2. Cytokine production profile of CD4⁺ T cells after stimulation with PMA. Fresh PBMCs from healthy controls or patients were cultured for 24 h in the presence of PMA. Cells were permeabilized, stained with a cocktail of anti-CD4-FITC and anti–IFN-γ-PE, anti-TNF-α-allophycocyanin, and anti–IFN-γ-FITC and analyzed on a FACScalibur flow cytometer. Analysis on gated CD4 cells is presented (left). Proportions of IFN-γ- and/or TNF-α-secreting cells are indicated. IL-2 detection in the indicated gated subsets is presented (right). Representative results of independent experiments in three sarcoidosis patients and five healthy controls are shown.

Real-time FoxP3 PCR. Real-time PCR was performed with a TaqMan assay on an ABI 7700 system (Applied Biosystems). Total RNA extracted from FACS sorted cells was immediately reverse transcribed in a 50 μL reaction volume (ProSTAR, First Strand; Stratagene) according to the manufacturer’s instructions. FoxP3 and HPR T-1 Assays-on-Demand gene expression probes (Hs00239589 and Hs99999909, respectively; Applied Biosystems) were used. In each reaction, HPR T-1 was amplified as a housekeeping gene to standardize the standard curve and to correct for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to HPR T-1 by the instrument software.

Single cell RT-PCR. Peripheral blood lymphocytes were stained with anti-human CD4-FITC and anti-human CD25-PE (BD Biosciences). Single cells were sorted using a FACScalibur flow cytometer. Fresh PBMCs from healthy controls or patients were cultured with PMA. Cells were permeabilized, stained with a cocktail of anti-CD4-FITC and anti–IFN-γ-PE, anti-TNF-α-allophycocyanin, and anti–IFN-γ-FITC and analyzed on a FACScalibur flow cytometer. Analysis on gated CD4 cells is presented (left). Proportions of IFN-γ- and/or TNF-α-secreting cells are indicated. IL-2 detection in the indicated gated subsets is presented (right). Representative results of independent experiments in three sarcoidosis patients and five healthy controls are shown.

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