

Alveolar Macrophages in Sarcoidosis Coexpress High Levels of CD86 (B7.2), CD40, and CD30L

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Alveolar macrophages (AM) from sarcoid patients have been shown to be good antigen presenting cells (APC) unlike normal AM which are usually ineffective. We demonstrate in ten consecutive sarcoid patients that most of their AM, unlike normal AM, do coexpress high levels of CD86, CD40, and CD30L, all known to be important for T-cell activation. CD80 is also slightly more expressed on sarcoid AM than on normal AM, but is detected on only $26 \pm 6\%$ (mean \pm SEM) of sarcoid AM. A good correlation is present between the percentage of sarcoid AM expressing CD86 and CD40 or CD86 and CD30L. However, no correlation is found between the percentage of CD80 and CD86 positive AM in these same patients. Blocking antibodies against CD86 were able to reduce by more than 80% allogeneic T-cell proliferation induced by the AM of sarcoid patients. This study provides evidence that AM can, in pathologic states such as sarcoidosis, express functional costimulatory molecules for T-cell activation such as CD86, thought to be rather specific for more professional APC such as dendritic cells. **Nicod, L. P., and P. Isler. 1997. Alveolar macrophages in sarcoidosis coexpress high levels of CD86 (B7.2), CD40, and CD30L. *Am. J. Respir. Cell Mol. Biol.* 17:91-96.**

Human alveolar macrophages (AM), resident phagocytic cells in the lung that derive from peripheral blood monocytes (1) are usually ineffective in presenting antigens to T cells (2). Among various mechanisms suggested to explain this poor function as antigen presenting cells (APC), it has been shown that AM have a defective expression of B7 costimulatory cell surface molecules (3). The same authors showed that even when AM were activated with interferon- γ (IFN- γ), they failed to express CD80 or CD86 antigens (3). CD80 or CD86 normally ligate CD28 on T cells to provide costimulatory signals required for the activation of T cells (4).

In contrast to AM from normal lungs, AM from sarcoid patients have been shown to have an enhanced capacity to mediate antigen-induced T-lymphocyte proliferation (5, 6). In addition, numerous data suggest that AM in sarcoidosis are in an immunologically activated state. Thus, while phenotypic studies have not shown an increased density of class II major histocompatibility molecules on sarcoid AM, they show an increased expression of the accessory molecules CD11 and CD18 known to play a role in T-cell activation (5, 7). Sarcoid AM also release proinflammatory cytokines such as tumor necrosis factor- α ,

interleukin (IL)-6 and IL-1 beta (8, 9), that may stimulate T-cell proliferation.

The mechanisms for the enhanced antigen presentation in sarcoid lung have, however, remained unclear and important ligands in the interaction between APC and T cells remain to be explored. B7 molecules, either CD80 or CD86, have been shown to be able to transmit essential signals for T lymphocytes through the ligand CD28 or CTLA-4. CD80 and CD86 are expressed by powerful APC such as human blood dendritic cells (10). We asked whether sarcoid AM express these molecules to explain why they are effective APC. Among other candidates to explain the APC function of sarcoid AM is CD40. CD40 present on APC is known to interact with its ligand (CD40L) expressed on activated T cells. This interaction is not only important to induce anti-tumoral immunity but also for cell-mediated immunity in general (11). Activated T cells expressing high levels of CD40L could also trigger CD40 present on dendritic cells to induce IL-12 production and enhanced CD80 and CD86 expression on the APC (12, 13). T cells expressing CD40L have also been shown to induce the release of key proinflammatory cytokines such as tumor necrosis factor- α , IL-1 β , IL-6 and IL-8 from APC (14). Thus the CD40 present on the APC can provide signals to T cells through their CD40L, and vice versa T cells can activate the APC in their functions, thus amplifying immune processes.

CD30L is another molecule present on APC which could be important for APC-T-cell interaction. The precise role of CD30L on the APC remains to be established. However, several reports have shown that CD30 ex-

(Received in original form September 17, 1996 and in revised form December 3, 1996)

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Abbreviations: alveolar macrophages, AM; antigen presenting cells, APC; bronchoalveolar lavage, BAL; standard error of the mean, SEM.

Am. J. Respir. Cell Mol. Biol. Vol. 17, pp. 91-96, 1997

pressed on T cells is a member of the tumor necrosis factor/nerve growth factor receptor family. CD30 could be preferentially expressed on CD4 and CD8 T-cell clones that produce T helper 2-type cytokines (15). Thus APC expressing CD30L could favor Th2 cytokines release.

We show that a high percentage of sarcoid AM express high levels of important accessory molecules, namely CD86, CD40, and CD30L. CD80 is present only on a subset of AM obtained from sarcoid patients and with a lower level of expression than CD86, although they are both from the B7 family. The expression of CD40 and CD30L, individually, correlates with the expression of CD86, whereas the expression of CD86 and CD80 shows little correlation.

Materials and Methods

Reagents

Control mouse IgG, anti-CD14 (clone IOM2) and anti-CD40 (clone MAB89) were from Immunotech (Marseille, France). Anti-B7.1 monoclonal antibody (mAb) (clone L 307.4) was purchased from Becton Dickinson (Mountain View, CA), anti-B7.2 mAb (clone IT2.2) was from Pharmingen (San Diego, CA). Anti-CD30L mAb was from Genzyme (Cambridge, MA). Anti-HLA-DR mAb was from DAKO (Glostrup, Denmark). Fluorescein (DTAF)-conjugated F(ab')₂ fragment goat anti-mouse IgG was provided by Immunotech.

Culture Medium

RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin was used (Gibco, Paisley, Scotland).

Study Populations

Ten consecutive patients with sarcoidosis were analyzed in our laboratory (three women and seven men, average age 35.8 ± 9.6 yr). In 8 of 10 cases, the diagnosis was made by biopsy obtained either from the lungs or from lymph nodes, and showed epithelioid cell granulomas with no evidence for pathogens known to cause granulomatous diseases. In 2 cases, the diagnosis was made by the presence of bilateral hilar adenopathies with bronchoalveolar lavage (BAL) lymphocytosis and a high CD4/CD8 ratio. Each patient underwent BAL analysis. The number of cells was $55 \pm 15 \times 10^4$ cells/ml (mean \pm SEM) with $51 \pm 5\%$ macrophages; $45 \pm 5\%$ lymphocytes; $2.1 \pm 0.4\%$ neutrophils, and $0.8 \pm 0.6\%$ eosinophils. The ratio of CD4/CD8 T lymphocytes in BAL was of 6 ± 1.7 (mean \pm SEM) as measured by flow cytometry using an EPICS V flow cytometer (Coulter Immunology, Hialeah, FL). Aside from the BAL analysis, the assessment of disease stage was done with chest radiographs. A stage I, II, or III disease on chest X-ray was found in 3, 4, and 3 cases, respectively. Systemic corticosteroids were administered at the time of BAL in 3 cases.

Preparation of Cell Suspension

After local anesthesia, BAL was performed by injecting a total of 150–200 ml of saline solution via fiberoptic

bronchoscopy in 50-ml aliquots, followed by immediate vacuum aspiration after each aliquot. Cells recovered from the BAL were washed three times with Hank's balanced salt solution without calcium and magnesium (HBSS; Gibco) and resuspended in RPMI 1640 complete medium at the concentration of 1×10^6 cells/ml.

Control Population

Human alveolar macrophages obtained by BAL of 7 normal surgical specimens next to pulmonary carcinomas were used as control (two women and five men, average age 65.1 ± 10.2 yr). As previously described, segments or lobes were lavaged at room temperature with 250–500 ml of sterile 0.9% NaCl using a 50-ml syringe introduced in the major bronchial airways (16). The cells obtained were $> 85\%$ macrophages and $< 2\%$ neutrophils as determined by differential counting of Wright-stained cytocentrifuge preparations. Alveolar macrophages were centrifuged, washed 3 times with HBSS (Gibco) and resuspended in RPMI 1640 complete medium at the concentration of 1×10^6 cells/ml.

Flow Cytometric Analysis

The expression of CD14, B7.1 (CD80), B7.2 (CD86), CD40, and CD30L on BAL macrophages from sarcoid patients was compared with alveolar macrophages obtained from control population. BAL cells were stained by incubating the cells with the different mAbs for 45 min at 4°C. To reveal the surface markers, fluorescein-conjugated (Fab')₂ fragments of goat anti-mouse IgG were used. BAL macrophages were gated in flow cytometry analysis using two different parameters: size (forward scatter) and complexity (side scatter) as shown in Figure 1. The purity of AM was assessed by May-Grünwald staining and by the

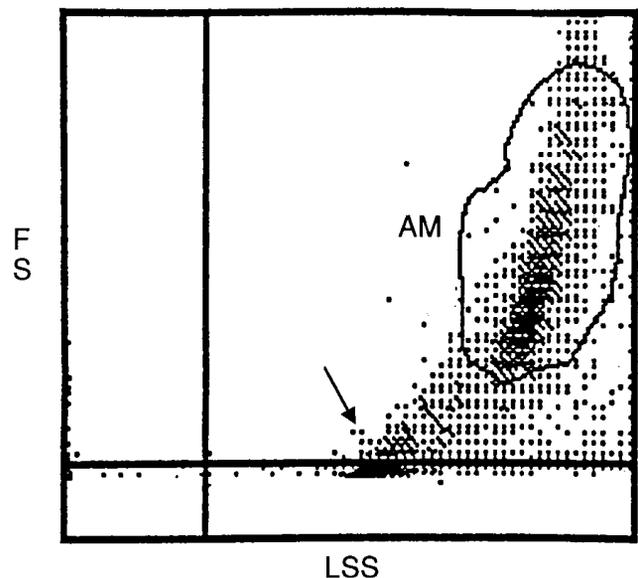
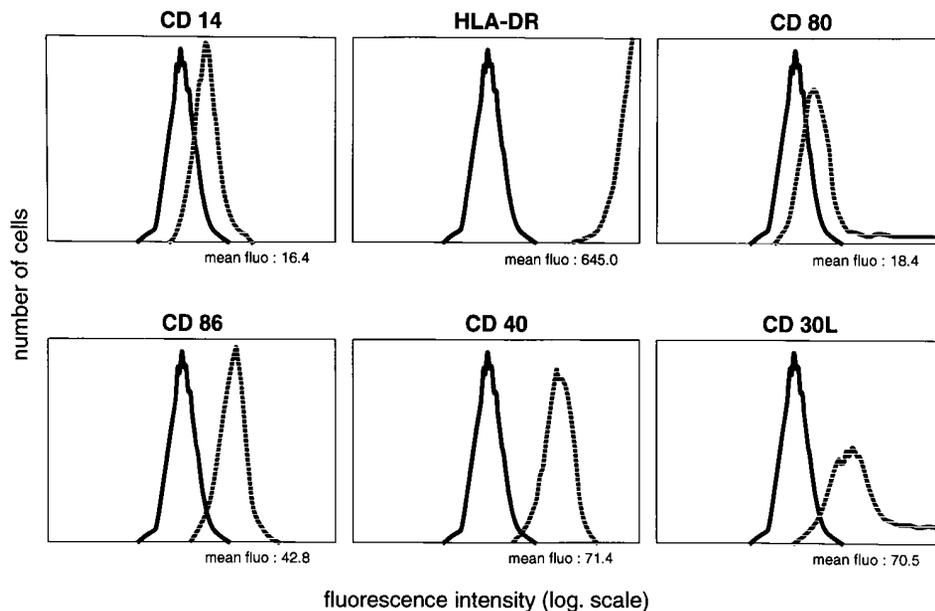


Figure 1. Flow cytometric analysis of bronchoalveolar cells. Cells are shown according to their size, forward scatter (F.S.), and their complexity, side scatter (S.S.). Lymphocytes are indicated with an arrow while alveolar macrophages (AM) are surrounded by the circle used to analyze them selectively.

Figure 2. Flow cytometric analysis of various markers in a patient with sarcoidosis. Five thousand cells having the typical macrophage scatter were analyzed. The expression of various markers is shown in *dashed lines* and compared with the fluorescence obtained with a control antibody all detected with a second fluorescent conjugated (Fab')₂ fragment goat anti-mouse IgG (*straight lines*). The mean fluorescence intensity of the control was of 7.8 arbitrary units in this case.



expression of HLA-DR antigens present in greater than 90% of this population. Five thousand cells bearing the typical size and complexity were scored. As BAL exhibited relatively homogeneous fluorescence intensity for the different markers, but diverged in their autofluorescent background, the data are presented mostly as percent of positive cells for CD14, CD80, CD86, CD40, and CD30L. The gates to define the number of positive cells were chosen in order to have < 1% positive cells with the control isotype matched mAb. Results are expressed in mean \pm SEM. When data are also given in mean fluorescence intensity, it is mentioned if it is done after subtraction of the nonspecific fluorescence obtained with the control irrelevant antibody and the naturally occurring autofluorescence of AM.

Mixed Leukocyte Reactions

BAL from sarcoid patients were irradiated with a cesium source (3,000 rads). BAL macrophages (25×10^3) were cocultured with purified T lymphocytes (15×10^4) in 200 μ l of RPMI 1640 complete medium in 96-well culture plates (Costar, Cambridge, MA) at 37°C. T lymphocytes were purified from buffy coats of healthy donors. Briefly, mononuclear cells were isolated from buffy coats by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Monocytes were removed by 1 h adherence on plastic (Costar, Cambridge, MA). To isolate T cells, non-adherent mononuclear cells were passed twice through a nylon wool column. To assess the function of CD80 and CD86 molecules on BAL cells from sarcoid patients, anti-CD80 mAb and anti-CD86 mAb (10 μ g/ml) were added to the MLR between BAL macrophages and T cells. After 5 days of cocultures, cells were pulsed with 0.5 μ Ci [³H]thymidine (Amersham, UK) 18 h before harvest. The cells were aspirated onto filter paper, and the radioactivity was analyzed in a liquid scintillation counter and data given in cpm.

Statistics

Statistics were performed using the program Statview 4.05 (Abacus Concepts, Berkeley, CA). The Mann-Whitney rank sum test was used to compare percent of cells expressing various markers among the sarcoid AM as compared with control subjects. Simple regression analysis was used to find out if a correlation was present between the percent of cells expressing the various markers in the sarcoid AM.

Results

Phenotypic Analysis of Sarcoid and Control AM

The percentage of AM obtained by BAL from sarcoid patients and expressing the costimulatory molecules for AM-T cells signaling was analyzed. A representative of the experiments of the various markers analyzed is shown in Figure 2. Besides HLA-DR, the marker expressed with highest intensity on more than 95% of the AM, it is apparent that CD86, CD40, and CD30L are also detected on a majority of sarcoid AM.

In contrast to HLA-DR or CD86, CD80 was present at rather low intensity (Figure 2). After subtraction of the fluorescence obtained with the control antibody, the fluorescence of CD80 was of 9.1 ± 1.6 arbitrary units (mean \pm SEM) on the sarcoid AM of all ten patients studied. In addition, CD80 was present on only $26.9 \pm 5.7\%$ of sarcoid AM (mean \pm SEM). This percentage was, however, higher than in the control AM as only $3.5 \pm 0.9\%$ of these cells expressed CD80 ($P < 0.01$ by Mann-Whitney rank sum test; Figure 3).

CD86 expression was strong on sarcoid AM (Figure 2). The CD86 expression in arbitrary units was of 34.9 ± 9.3 after subtraction of the fluorescence obtained with the control antibody in all ten patients, while its intensity was of 8.5 ± 3.1 arbitrary units in the control population. The percentage of CD86 positive sarcoid AM was $71.5 \pm 9.0\%$,

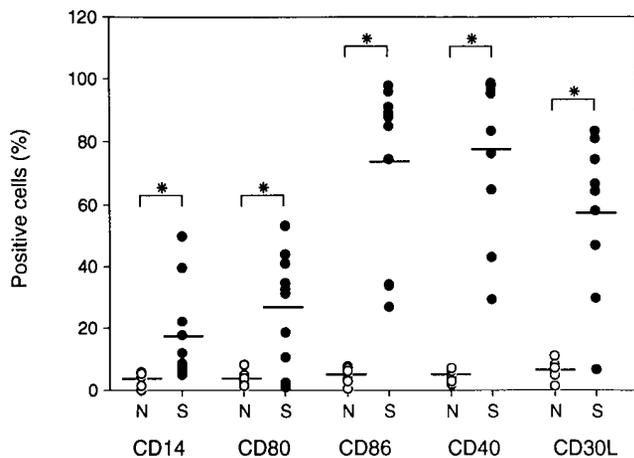


Figure 3. Percentage of AM from sarcoid patients expressing various surface markers. The data are presented as percentage of positive cells for CD14, CD80, and CD86. CD40 and CD30L analyzed with a gate were < one percent of control mouse mAb was detected. N = control subjects. S = sarcoid patients. * $P < 0.01$ by Mann-Whitney rank sum test.

by contrast only $4.5 \pm 1\%$ of control AM expressed CD86 ($P < 0.01$; Figure 3).

CD40 expression was also very high on sarcoid AM (Figure 2). The mean fluorescence intensity obtained with this marker, after subtraction of the fluorescence from the control antibody, was of 47.6 ± 7.8 arbitrary units. The mean percentage of CD40 positive sarcoid AM reached $78.3 \pm 7.9\%$ while it was present on only $3.7 \pm 0.9\%$ AM of control subjects (Figure 3). There was a close correlation between the number of AM expressing either CD86

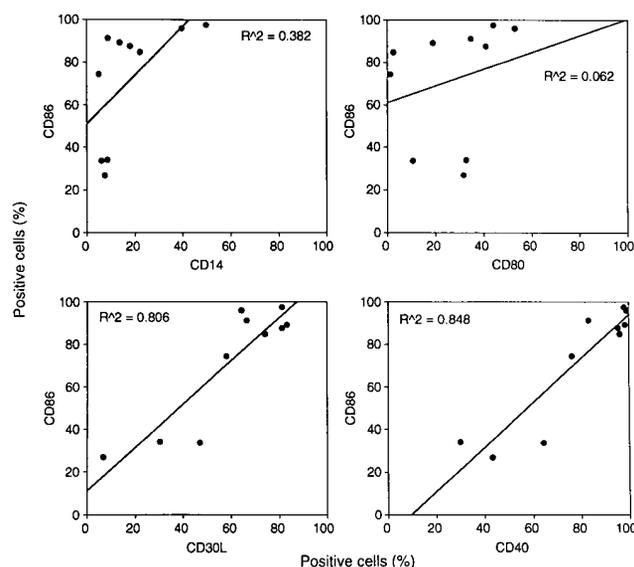


Figure 4. Correlation of percent sarcoid AM expressing various markers. The percentage of cells was established as described in Figure 2. The correlation between various markers was established by a simple regression analysis.

or CD40 in all patients with sarcoidosis ($r = 0.85$; $P < 0.001$ by simple regression analysis) (Figure 4). Meanwhile, no correlation was found between CD86 and CD80 ($r = 0.06$; $P = 0.4$) or between CD86 and CD14 ($r = 0.38$; $P = 0.06$).

CD30L was expressed by an average of $56 \pm 7.8\%$ of AM in sarcoid patients (Figure 3). CD30L was expressed on the same proportion of AM than CD86 in patients with sarcoidosis. The degree of correlation between CD30L and CD86 was close to the correlation obtained between CD86 and CD40 ($r = 0.81$; $P < 0.001$) (Figure 4). The co-expression of these molecules could imply that both are induced by a similar stimuli. Alternatively, when AM interact with T cells through CD40 and CD40L for instance, AM could be driven to express higher levels of other molecules such as CD86 or CD30L. Such a succession of events has been described for dendritic cells (13).

Functional Analysis of Sarcoid AM in Alloreactions

Addition of mAb directed against CD86 to allogeneic MLR induced with AM from sarcoid patients did markedly reduce T-cell proliferation (Figure 5). The addition of anti-CD80 mAb had low or no inhibitory effect as compared with irrelevant IgG. No effect was observed when alloreaction was conducted with the antibodies against CD40 and CD30L used in this study (data not shown). These results at least demonstrate that CD86 was functional on sarcoid AM.

Discussion

This study provides evidence that not only dendritic cells but also AM can in some pathologic states such as sarcoi-

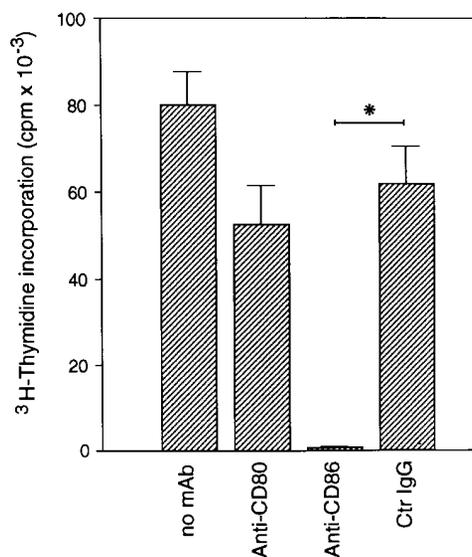


Figure 5. Effect of anti-CD80 and anti-CD86 mAb in a mixed leukocyte reaction induced by AM of sarcoid patients. BAL cells were inactivated with a cesium source (3,000 rads). BAL macrophages (25×10^3) were cocultured with purified allogeneic T cells (15×10^4) in 96-well culture plates in triplicate for 5 days. Cells were pulsed with $0.5 \mu\text{Ci}$ [^3H]thymidine 18 h before harvest and results are expressed in $\text{cpm} \pm \text{SD}$ of a representative experiment. * $P < 0.05$ by unpaired t test.

dosis express the accessory molecules CD80, CD86, CD40, and CD30L. In sarcoidosis, there is a good correlation between the expression of CD86, CD40, and CD30L, while no correlation between CD80 and CD86 is found. The expression of these molecules contribute to the good APC function of sarcoid AM described in the past (5, 6), as at least blocking antibodies against CD86 suppressed allogeneic T-cell proliferation induced by these macrophages.

CD80 and CD86 are expressed by human dendritic cells and are important costimulatory signals for the activation of CD4 and CD8 T lymphocytes (10). Monocytes have been shown to be able to express CD80 or CD86 only after prolonged stimulation by IL-4 and GM-CSF (17) or by IFN- γ (3). However, it has been impossible to induce CD80 on AM and CD86 only to a low extent in the same conditions (3). Our results provide evidence that AM in sarcoidosis can nevertheless express CD86 and efficiently activate T cells. Proliferation induced by allogeneic sarcoid AM is shown to be markedly inhibited by blocking antibodies against CD86, demonstrating its importance for the APC function of these cells. The conditions that lead to CD86 expression in sarcoidosis are unknown at present. Unidentified microbial constituents have been reported to induce B7 on murine macrophages (18). Unknown pathogen could similarly induce B7 on sarcoid AM. Alternatively, it is possible that freshly attracted monocytes could be induced to express these markers under unknown stimuli and then mature into AM, while the expression of these markers is preserved.

The fluorescence signal for CD86 was consistently higher on sarcoid AM than CD80. Along with this, we found no clear evidence for CD80 function on AM in sarcoidosis. The functional necessity for the two CD28 counterreceptors is presently unknown. In a murine model, CD80 and CD86 costimulate IL-2 and IFN- γ production equivalently, but CD86 has also been described to induce significantly more IL-4 than CD80 with the greatest difference seen in naive T cells (19). As the pattern of cytokines produced by activated T cells in this disease is not yet known (20), the role of the enhanced expression of CD86 on sarcoid AM remains to be established.

CD40 has been shown to be required for antigen-specific T-cell priming *in vivo* by the use of mice lacking CD40 ligand (20). The mice lacking CD40 ligand have a reduced inflammatory response and a reduced capacity of granuloma formation in a *Leishmania* model (11). The high expression of CD40 on sarcoid AM is therefore likely of importance. The good correlation between CD86 expression and CD40 could be partially explained by the observation that crosslinking of CD40 markedly increases CD80 and CD86 expression (21). This interaction of APC and T cells through CD40 and CD40L is thought to be one of the early events that could enhance the APC function of either dendritic cells or B cells (13). This would be followed by the increased expression of CD80 and CD86 and secondarily to a more effective triggering of T-cell proliferation and cytokine production. The absence of correlation between CD40 and CD80 contrarily to CD40 and CD86 could suggest however that CD80 and CD86 expression is not always linked.

The fact that antibodies against CD40 molecules did not decrease the MLR could be due to the fact that the sarcoid AM that we used had already been activated through this molecule to express high levels of CD86. We were perhaps too late in our assay *in vitro* to prevent then the alloreaction by blocking CD40 and CD40L interactions as CD86 was already expressed to a high level. Alternatively, it is possible that the antibodies used against CD40 in our assay were not blocking the appropriate epitope. Additional studies are currently being performed.

CD30 belongs to the tumor necrosis factor receptor super family and does activate T cells (22). Its preferential role in activation of Th2 cytokines remains controversial (23). CD30L on sarcoid AM may also be of importance in the physiopathology of sarcoidosis. This is supported by its coordinated expression with CD86 and CD40.

In conclusion, we have provided evidence that AM can, in pathological states such as sarcoidosis, express high levels of CD86, CD40, and CD30L. The mechanisms leading to the overexpression on AM of such markers and their role in sarcoidosis remain unclear and need to be clarified.

Acknowledgments: The authors thank Dr. K. Hauser for his lecture, Dr. P. Joliet for his assistance in the statistics analysis of the manuscript, and Mrs. F. Songeon for her technical assistance. This work was done with the support of the FNSRS fund No. 32-40844.94; the Lancardis Foundation and the "Association Vaud-Genève" project No. 19.

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