

Review

A human *in vitro* granuloma model for the investigation of multinucleated giant cell and granuloma formation

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Summary. A method for the *in vitro* generation of granulomas and its use in the analysis of the human granulomatous response is summarized. As a target for the cellular response L3 larvae of *Nippostrongylus brasiliensis* are coincubated with human mononuclear blood cells, and within seven to fourteen days the development of blood monocytes to mature macrophages and to epithelioid cells and multinucleated giant cells (MGC) as typical constituents of granulomas clustered around the nematode is observed. The following review describes the uses and applications of this model for phenotyping, functional, formation and modulating studies of granulomas and MGCs, taking into account its unique features compared to other *in vitro* models.

With respect to MGC formation, procedures are described and examples are given which allow the phenotyping of these cells using immunofluorescence and immunohistological techniques. In addition, the potential of this model for illuminating functional aspects of MGC is described applying an isolation protocol for MGC and a subsequent reverse-transcriptase polymerase chain reaction method for the analysis of single cells. Moreover, the significance and relevance of using this granuloma model is discussed in the follow up analysis of *in vivo* findings of interleukin-6 expression in MGC of granulomas of patients with sarcoidosis. These *in vivo* results implicated a role for interleukin-6 in granuloma and MGC development. The *in vitro* granuloma model was used to investigate potential modulatory effects of this cytokine by analysing the cell numbers and the number of MGC per *in vitro* granuloma, the size of the MGC formed, the fusion index and the morphology of the *in vitro* granuloma. The results demonstrated significant modulatory effects of interleukin-6 on the cell number per *in vitro* granuloma and on the morphology of the cells involved. Conceivably, elevated interleukin-6 levels may modulate granuloma formation with respect to the number of cells involved and in influencing distinct cell populations

involved in granuloma formation.

Key words: Granuloma, Interleukin-6, Multinucleated giant cell, Sarcoidosis

Introduction

The term granuloma is applied to any small nodular delimited aggregation of mononuclear inflammatory cells or a collection of modified macrophages, usually surrounded by a rim of lymphocytes and often containing multinucleated cells. Some granulomas contain eosinophils and plasma cells, and fibrosis is commonly seen around the lesion. Granuloma formation represents a chronic inflammatory response initiated by various infectious and noninfectious agents. The rather imprecise terminology reflects the complexity of the granulomatous responses found with respect to different granulomatous diseases, the cellular constituents involved, and with respect to the factors and mechanisms leading to granuloma formation and resolution. Since the precise mechanism involved in the recruitment, activation, and maintenance of immune cells at granulomatous foci is not clearly understood, animal *in vivo* and human *in vitro* models mimicking granuloma formation are useful tools for the investigation of the mechanisms underlying granuloma formation. A large variety of *in vivo* granuloma models have been developed in the murine system (Warren, 1982; Kunkel et al., 1998) and with the advent of gene knock-out technology these models have become understandably attractive and effective for dissecting the mechanisms of granuloma formation and for elucidating the individual role of the mediators involved. They have thus contributed greatly to the understanding of the evolution of the granulomatous response from initiation to resolution or progression of disease. Nonetheless, even though these animal models involve mediator systems that are similar in both the human and the experimental model system, unique differences do exist that preclude them from exactly duplicating the human corollary disease (Kunkel et al.,

1998). As a consequence, human *in vitro* models have been developed and used at the level of antigen-coated beads (Bentley et al., 1985), *Schistosoma mansoni* eggs (Doughty and Phillips, 1982), artificial microparticles (Sato et al., 1991) and heat killed *Candida albicans* (Heinemann et al., 1997) as a supplementation to animal models. However, none of these models exactly imitates human granuloma formation with respect to the cellular components involved, the differentiation of monocytes/macrophages to epithelioid and multinucleated giant cells and with respect to phagocytosis and elimination of the granuloma-inducing agent.

The macrophage and its derivatives, epithelioid and multinucleated giant cells (MGC), are considered a histological hallmark of granulomas. Many *in vitro* models using defined conditions for studying MGC formation have been described, and these have been and are invaluable in defining stimulatory factors and in uncovering mechanisms of homotypic cell fusion leading to the formation of MGC (see Fais et al., 1997; Di Virgilio et al., 1999; Anderson, 2000, for recent reviews). Thus, it is now well documented that the T-cell cytokines interleukin-4 (McNally and Anderson, 1995; Dugast et al., 1997; McInnes et al., 1988), interleukin-13 (DeFife et al., 1997), interferon-gamma (Möst et al., 1990; Takashima et al., 1993; Fais et al., 1994) and interleukin-3 (Enelow et al., 1992a) as well as the monocyte/macrophage-derived cytokines tumor necrosis factor alpha (Sorimachi et al., 1995), macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (Lemaire et al., 1996a) play a role in MGC formation from monocytes/macrophages. Additional efforts have been put into uncovering the molecules involved in the actual homotypic aggregation and fusion of monocytes/macrophages to MGC and have revealed the involvement of HLA-DR Class II molecules (Orentas et al., 1992; Matsuyama et al., 1994), CD44 (Sterling et al., 1998), a new putative receptor termed macrophage fusion receptor (MFR) (Saginario et al., 1998), macrophage mannose receptor (MMR) (McNally et al., 1996), the purinergic P2Z/P2X7 receptor (Chiozzi et al., 1997), and CD98 (Ohgimoto et al., 1995). With respect to downstream events taking place after the engagement of fusion proteins, cytoskeletal elements have been implicated (Suga et al., 1995; DeFife et al., 1999).

Although it is undisputed that MGC form by fusion of monocytes/macrophages rather than by abnormal cell division (Murch et al., 1982) it is unclear which cells of the monocytic lineage at what stage of differentiation fuse to form MGC. Contradictory results and the description of distinct MGC types being formed *in vitro* may be attributed to this fact, besides the involvement of cytokines and other stimulatory factors. It is believed that MGC represent a specialized form of macrophage differentiation, and apparently the fusion event depends on the differentiation state of the cells involved (Gerberding and Yoder, 1993; Möst et al., 1997). In addition, it appears that freshly recruited cells from the

periphery rather than resident tissue macrophages are responsible for MGC formation in granulomas (Fais and Pallone, 1995).

Despite the advancements in the understanding of MGC formation, the function of these cells remains evasive. It has been postulated that MGC are highly stimulated cells of the monocyte-macrophage lineage at a terminal stage of maturation (Kreipe et al., 1988), but they have also been thought to be merely a disposal device for metabolically exhausted macrophages (Mariano and Spector, 1974). To substantiate the former hypothesis, these cells have been attributed with an enhanced candidacidal activity (Enelow et al., 1992b), elevated enzyme equipment and increase in oxygen free radicals in response to zymosan (Kreipe et al., 1988), have been proposed to represent a confinement mechanism at sites of infection (Hill, 1992) and may be involved in granuloma formation and sustenance and destruction of the irritant as demonstrated by the synthesis of relevant cytokines and inducible nitric oxide synthase by MGC (Seitzer et al., 1997; Hernandez-Pando et al., 2000).

As delineated above, granuloma and MGC formation is a highly complicated process, the outcome depending on the concerted action of many known and unknown factors. No single *in vivo* or *in vitro* model for granuloma or MGC formation is likely to be able to deliver all answers pertinent to the elucidation of these processes. The following will describe an *in vitro* model for human granuloma formation exhibiting some unique aspects compared to the models mentioned above. It is an experimental system which allows both the study of granuloma and MGC formation *in vitro* using human cells and thus combines these two important features, usually studied separately from each other. The model may thus provide the potential to answer questions pertinent to both granuloma and MGC formation as it is typically found in the case of granulomatous disease.

Generation of *in vitro* granulomas and MGC

For the *in vitro* generation of granulomas and multinucleated giant cells the coculture of human peripheral blood mononuclear cells (PBMC) with the cellular target *Nippostrongylus brasiliensis* is performed essentially as described (Seitzer et al., 1997) and is presented in more detail here.

For the preparation of *N. brasiliensis* larvae Wistar rats are infected by subcutaneous injection with 500 third-stage larvae (the larvae were a kind gift from Prof. K. Ishizaka, La Jolla, CA) of *N. brasiliensis* in 0.5 ml phosphate buffered saline (PBS; 137 mM NaCl, 3.9 mM KH_2PO_4 , 16 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$). Faeces from the infected rats are collected at days 5-8 after infection and mixed with humidified small styrofoam beads to which a small amount of charcoal has been added (1:20, w/w) (Warren, 1982). After 10-14 days, when the eggs have hatched, the mixture is covered with lukewarm liquid agar (1% in PBS). The third-stage larvae appearing on

the surface of the solidified agar are washed off with PBS, washed 5 times in PBS, and then 3 times in PBS containing 1000 U/ml penicillin G sodium salt, 1000 µg/ml streptomycin and 500 µg/ml gentamycin (Gibco). After one night in PBS containing 100 U/ml penicillin G sodium salt, 100 µg/ml streptomycin and 50 µg/ml gentamycin, they are transferred into complete culture medium and kept in the CO₂-incubator at 37 °C, where they are viable for at least six weeks. To extend the supply of worms, a part of the larvae may be frozen and kept in liquid nitrogen. Freezing is performed in 100% fetal calf serum (FCS). After thawing, about 70% of the larvae survive for 10-20 days.

The preparation of human PBMC is performed using heparinized blood and Ficoll-Hypaque density gradient centrifugation. Heparinized blood is diluted 1:2 with physiological saline, layered on Ficoll-Hypaque (Pharmacia) in a 1:4 ratio, and centrifuged at 22 °C for 30 min at x325g. PBMC are removed from the interphase, washed once with saline containing 20% FCS (10 min at x450g), then washed three more times with saline containing 20% FCS (10 min at x220g). All washes are performed at 4 °C. For cell culture, Iscove's modified Dulbeccos's medium (IMDM) (Gibco) is supplemented to final concentrations of 100 U/ml penicillin G sodium salt, 100 µg/ml streptomycin (Gibco), and 5% heat-inactivated autologous human serum.

The coculture of 0.75x10⁶/ml PBMC and 20 worms/well is performed in a volume of 2 ml in 24-well tissue culture plates (Nunc A/S, Roskilde, Denmark).

Using this protocol, cell clusters adhering only to living nematodes after one day of cocultivation predominantly consist of mononuclear cells comprising T-cells, B-cells, monocytes and macrophages. MGC and epithelioid cells are typically observed between the fourth and the seventh day of cocultivation. Both Langhans MGC (LHMGC) and foreign body MGC (FBMGC) develop in the culture. After 14 days, MGC represent the predominant constituent of the *in vitro* granuloma around *N. brasiliensis* reaching sizes of up to 1 mm in diameter and containing over 100 nuclei. In some cases, MGC appear to engulf *N. brasiliensis* entirely, leading to the destruction of the nematode. These features distinguish this model from the *in vitro* granuloma models presented in the introduction, since the cellular constituents and cellular development in the model display a strong relation to human granuloma formation *in vivo*. This makes it advantageous over previously described *in vitro* granuloma models, which predominantly focused on the parameters by which the size of the cell clusters could be influenced, since differentiation of macrophages to epithelioid cells and MGC could not be observed. In addition, prolonged coculture results in the phagocytosis and elimination of the nematode by degradatory processes, as demonstrated by the absence of nematodes and the presence of rather huge MGC containing fragments of the worm. Such a degradation process has not been observed or described

for other *in vitro* models. Figure 1 in the figure panel shows a hematoxylin eosin staining of such a specimen, with one enormous MGC depicted showing remnants of the nematode in the center of the cell.

Analysis of *in vitro* granulomas and MGC

The fact that single, separable granulomatous foci are generated in the coculture allows the determination of the cells per cluster and MGC per cluster in addition to the usually determined fusion index (fusion index (%) = (number of nuclei within MGC) / (total number of nuclei counted) x 100) as parameters of granuloma formation. MGC may additionally be evaluated with respect to size and number of nuclei. A major advantage of this *in vitro* model lies in the fact that not many of the cell clusters adhere to the tissue culture surface making the cell clusters, including MGC, amenable to selection procedures for further analysis. This accessibility may be further optimized by performing the culture under conditions where adherence to the culture surface is prevented, for example in teflon bags (Andreesen et al., 1983). Cytospin preparation slides may be prepared from worms with adherent cell clusters aspirated from the primary cultures under microscopic control by a micropipet and used for histological (e.g. May-Grünwald-Giemsa technique) and immunohistological procedures. For APAAP staining (Cordell et al., 1984) using New Fuchsin development, specimens are fixed for 10 minutes in acetone, incubated with the primary mouse monoclonal antibody for 15 minutes followed by a short wash in Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.5). The slides are then incubated with rabbit anti-mouse Ig antiserum (Dako) diluted 1:10 in TBS with 1:8 prediluted inactivated human serum for 15 minutes, washed in TBS and incubated with the APAAP complex diluted 1:20 in TBS for 15 minutes. The incubation with the secondary reagents is repeated once. After the enzyme development the slides are counterstained with hematoxylin and mounted.

Figures 2a-d, 3a,b show examples of phenotyping analysis performed on specimens prepared in this way. Figure 2 shows a series of stainings for CD54 (ICAM-1), a molecule implicated as playing a role in MGC fusion (Möst et al., 1990; Fais et al., 1994). Interestingly, single macrophages express CD54 on the entire cell surface, but as homoaggregation takes place, expression is polarized (Fig. 2a) and is reduced to the outer surface of the cells on the periphery of a cluster of cells apparently aggregating to fuse to an MGC (Fig. 2b). MGC themselves exhibit varying degrees of CD54 expression. Figure 2c shows an MGC with CD54 expression on the entire cell surface, reflecting perhaps a freshly fused MGC. An MGC with slight CD54 expression possibly implicates a reduced CD54 expression with time from the fusion event (Fig. 2d).

Figure 3 shows examples for staining of a macrophage marker, CD163, detected by the antibody

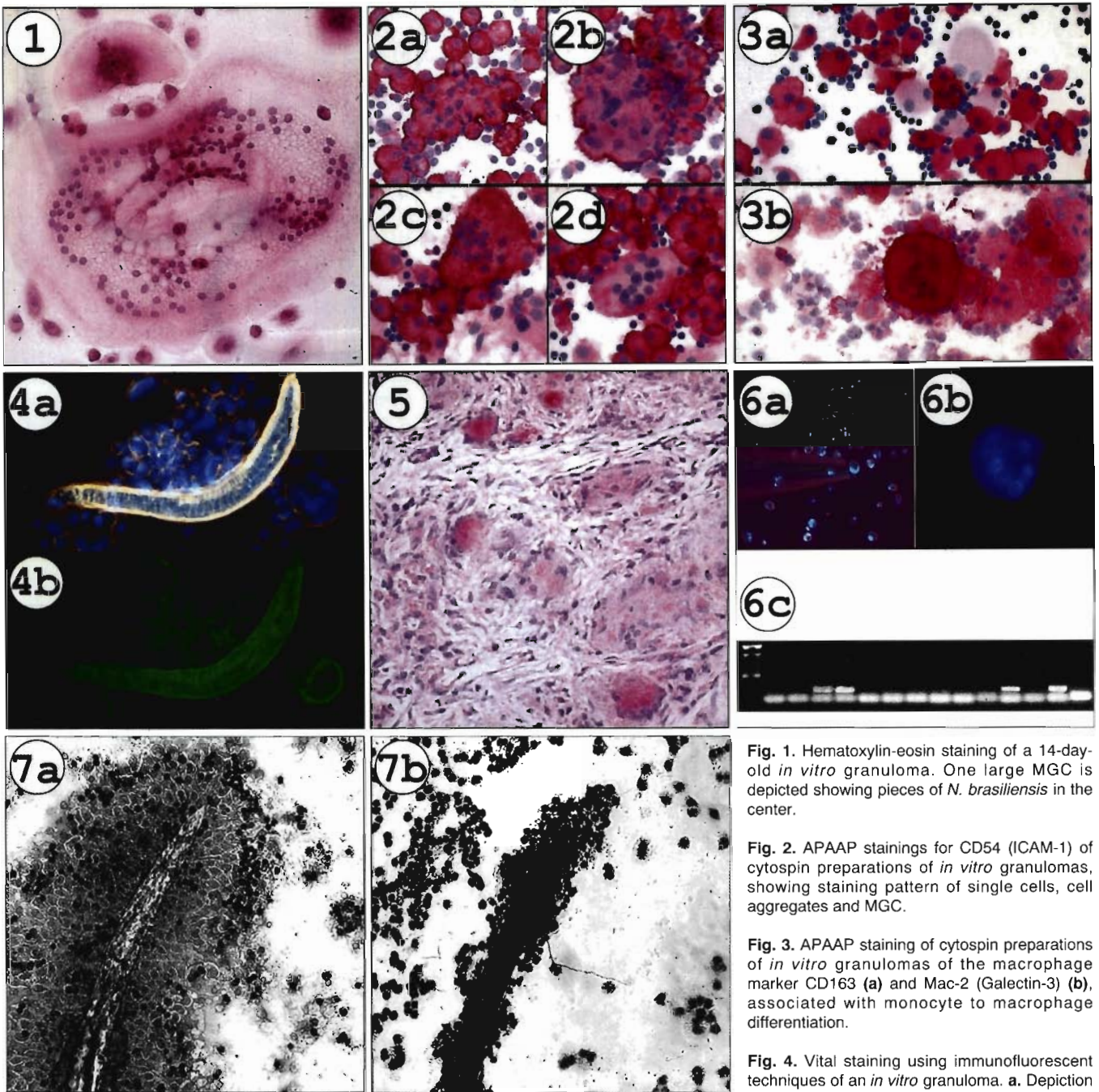


Fig. 1. Hematoxylin-eosin staining of a 14-day-old *in vitro* granuloma. One large MGC is depicted showing pieces of *N. brasiliensis* in the center.

Fig. 2. APAAP stainings for CD54 (ICAM-1) of cytospin preparations of *in vitro* granulomas, showing staining pattern of single cells, cell aggregates and MGC.

Fig. 3. APAAP staining of cytospin preparations of *in vitro* granulomas of the macrophage marker CD163 (a) and Mac-2 (Galectin-3) (b), associated with monocyte to macrophage differentiation.

Fig. 4. Vital staining using immunofluorescent techniques of an *in vitro* granuloma. a. Depiction using double exposure of phase contrast and marker.

nuclei staining with Hoechst 33342. b. Depiction of the same specimen also stained with a FITC-conjugated anti-CD29 antibody as a membrane marker.

Fig. 5. APAAP staining for IL-6 on a frozen section from a lymphnode of a sarcoidosis patient, demonstrating IL-6-positive MGC.

Fig. 6. Micropreparation (a) of single cell MGC (b) for RT-PCR analysis of IL-6 transcripts (c).

Fig. 7. Pappenheim staining of *in vitro* granulomas treated with IL-6 (a) and untreated (b) after 4 days of coculture. More cells also exhibiting a different phenotype than in the control culture are found in the IL-6-treated culture.

BerMac-3 (Fig. 3a) and of an antigen associated with monocyte maturation to macrophages, Mac-2 (Galectin-3), detected with the M3/38 antibody (Fig. 3b). Since no positive selection marker for MGC is known (Mechtersheimer and Möller, 1989), an interesting finding with respect to the immunophenotype of MGC is that macrophages are positive for CD163, whereas epithelioid cells show a weaker expression and MGC entirely lack this marker (Fig. 3a). This *in vitro* finding parallels the results obtained *in situ* (Backé et al., 1991), where Ber-Mac3 reacted strongly with macrophages in normal and diseased tissue but not with epithelioid type macrophages or multinucleated giant cells. The negativity of MGC for this macrophage marker thus makes BerMac-3 a valuable exclusion marker for these cells.

Galectin-3, also known as the Mac-2 antigen, is described as a differentiation marker for monocyte to macrophage maturation, showing an increased surface expression in the process (Leenen et al., 1986; Liu et al., 1995). As immunohistological analysis revealed, the differentiation of monocytes to macrophages to MGC presumably also correlates with an increase in galectin-3 expression. Figure 3b shows some highly positive macrophages as well as a deeply positive MGC. This was true for the majority of MGC. However, this staining pattern was not consistently found. A slightly positive MGC beside the deeply positive one and also the lesser positive macrophages alongside the positive ones demonstrate this in Figure 3a. Possibly macrophages at different maturation stages may fuse to form distinct MGC, as is implicated by the investigation of Möst et al. (1997). As a result, this observation raises the question as to whether heterogeneous macrophage populations are possibly present in the cultures which as a consequence give rise to a heterogeneous MGC population.

Besides the analysis of *in vitro* granulomas, single MGC may also be prepared and analysed. For the micropreparation of single MGC *in vitro* granulomas may be aspirated under microscopic control with a micropipette and then subjected to an appropriate protocol staining for desired selection criteria of MGC. After incubating in PBS/10% FCS at 4 °C for 30 min, the cells constituting the *in vitro* granulomas dissociate into a single cell suspension. Since MGC are too large to be used in conventional flow cytometric analysis, this approach gives the unique opportunity for multiparameter analysis of these cells using immunofluorescence techniques. In addition, MGC may be manually sorted applying desired selection criteria and subjected to further analyses.

As an example, samples may be stained with Hoechst 33342 (determination of the number and arrangement of nuclei per MGC), propidium iodide (viability of MGC), CD163 (negative selection marker) and FITC-conjugated anti-CD29 (as a membrane marker to distinguish cell clusters with many nuclei from MGC with many nuclei). Figure 4 depicts a selected vital *in*

vitro granuloma stained according to this procedure. Figure 4a represents a double exposure showing phase contrast and the Hoechst 33342 staining of the nuclei. Figure 4b shows the detection of CD29 as a membrane marker of the same specimen, allowing the discrimination of MGC. Upon leaving stained vital specimens in cold PBS/10% FCS the cells dissociate, permitting the micropreparation of single MGC using a micromanipulation and cell transfer device such as the micromanipulator 5171 and CellTram Oil (Eppendorf) under phase contrast microscopic control. A single aspirated cell may be transferred to a well of a Terasaki plate for immunofluorescence analysis of the selection criteria. Selection and evaluation criteria which can be applied using this protocol are the verification of only one cell being present in the well (phase contrast and CD29 as a membrane marker), documentation of the number and arrangement of nuclei present per MGC (Hoechst 33342) and cell viability (negativity for propidium iodide) and negativity for the macrophage marker CD163. This procedure allowed the establishment of the cytokine profile of MGC (Seitzer et al., 1997) using an RT-PCR method for single cells (Toellner et al., 1996), contributing to the elucidation of the function of MGC in granulomas. In addition, a functional discrimination with respect to the presence of interleukin-6 (IL-6) transcripts was observed between the morphologically distinct FBMGC and LHMGC, prompting questions as to the role of IL-6 in granuloma and MGC formation.

Modulation of *in vitro* granuloma and MGC formation by interleukin-6

To investigate the *ex vivo* relevance of the *in vitro* demonstration of IL-6 transcripts in MGC, we performed immunoenzymatic stainings for the presence of interleukin-6 protein (Biosource International, clone B-E8) in stimulated human monocytes, MGC of the *in vitro* granuloma, and on frozen sections of lymph node biopsies of sarcoidosis patients (kindly provided by Dr. Hans-Heinrich Wacker, Institute of Pathology, Christian-Albrechts-University Kiel, Germany). Immunoenzymatic staining for IL-6 was found to be specific for IL-6 protein by neutralization of the antibody staining pattern with recombinant IL-6 in tonsil tissue. Staining for IL-6 was only found to be positive in cells known to produce IL-6 (LPS-stimulated PBMC) and in cells known to contain IL-6 transcripts (*in vitro*-generated MGC).

A total of seven different lymphnode biopsies were stained for the presence of IL-6 protein. The results of these stainings are outlined in Table 1. Staining of sarcoid lymph nodes showed positive IL-6 staining in varying degrees of intensity in the granulomas, which was mainly attributable to macrophages and epithelioid cells. MGC, predominantly of the Langhans type present in sarcoid granulomas were also positive for IL-6 in varying degrees of staining intensity (Fig. 5). To

determine whether *ex vivo* MGC not only display the presence of immunoreactive IL-6 but are also producers of the cytokine, RT-PCR analysis of single MGC isolated from the bronchoalveolar lavage (BAL) of a sarcoidosis patient (kindly provided by Prof. Joachim Müller-Quernheim, Division of Clinical Immunopathophysiology, Dept. of Clinical Medicine, Research Center Borstel, Germany) was performed. Figure 6 shows the accumulation (Fig. 6a) and the isolation of single MGC (Fig. 6b) from a cell suspension of BAL using micropreparation techniques. In the RT-PCR performed according to the single cell protocol (Toellner et al., 1996) interleukin-6 transcripts could be demonstrated in some but not all BAL-MGC (Fig. 6c), perhaps a reflection of the immunohistological data. Thus we could show that MGC produce IL-6 *in vitro* and *ex vivo*. However, in contrast to the *in vitro* data, a distinction between FBMGC and LHMGC with respect to IL-6 production was not possible *ex vivo*, since IL-6-positive MGC in the sarcoid granulomas were predominantly of the LHMGC type. The varying intensity of staining for IL-6 in MGC may reflect a transient expression of IL-6 by these cells. Since elevated levels of IL-6 have been reported in sarcoidosis (Prior et al., 1996; Takizawa et al., 1997) and MGC were primarily present and positive for IL-6 in large, focused IL-6-positive granulomas, IL-6 may play an important role in granuloma promotion and sustenance.

To investigate this aspect, we utilized the *in vitro* granuloma model to uncover potential modulatory effects of IL-6 and an anti-IL-6 neutralizing antibody on MGC and granuloma formation by determining the cell numbers per *in vitro* granuloma, the number of MGC per *in vitro* granuloma, the size of the MGC formed, the fusion index and the morphology of the *in vitro* granuloma.

At time intervals of 1, 4, 7 and 14 days after coculture the specimens were fixed and stained with the Pappenheim method and evaluated under a light microscope.

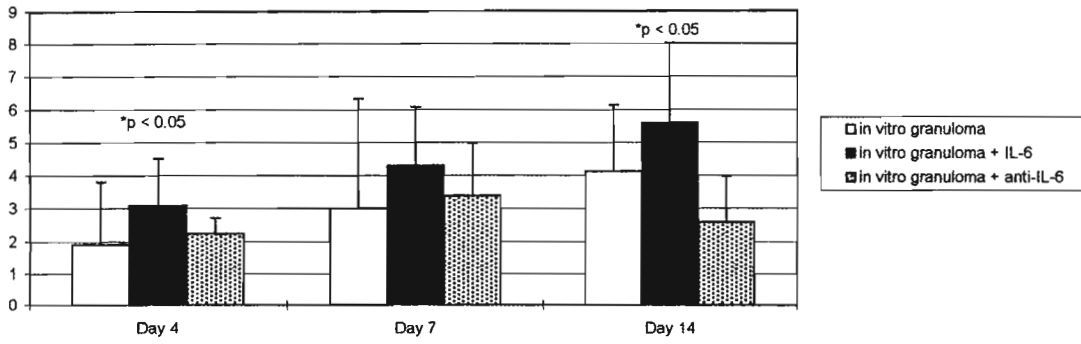
Experiments were performed in duplicate with triplicate samples each, of which a total of 10 *in vitro* granulomas were evaluated. Statistical analysis of the data was performed using the two-tailed student's t-test.

Results indicated that there were no significant differences in IL-6 levels in the supernatant of the control cultures over a period of 14 days, however with a trend to higher production from day 1 (9.8 ± 1.4 ng/ml) to day 4 (14.3 ± 0.7 ng/ml), and a decrease in production from day 4 to day 14 (7.4 ± 1.5 ng/ml). In the cultures supplemented with IL-6, a constant trend to higher numbers of MGC per *in vitro* granuloma over the entire time course from day 4 until day 14 of the cocultivation was observed, being significantly higher than in cultures treated with a neutralizing anti-IL-6 antibody at day 4 and day 14 (Fig. 8a). Evaluation of the number of nuclei per MGC was performed as a measure for the size of the MGC formed under the different incubation conditions. Interestingly, a significant difference was observed between the IL-6-supplemented and anti-IL-6-treated cultures at day 14, the MGC in the former culture exhibiting less nuclei per MGC than the culture treated with anti-IL-6 (Fig. 8b). Determination of the fusion index (fusion index (%) = (number of nuclei within MGC) / (total number of nuclei counted) x 100), a parameter for the extent of MGC formation, showed no significant difference between the three culture conditions on day 4 and day 7 (Fig. 8c). At day 14, the fusion index of the IL-6-treated cultures was significantly lower than the anti-IL-6-treated cultures, reflecting the results of the number of nuclei per MGC, even though a significantly higher number of MGC per

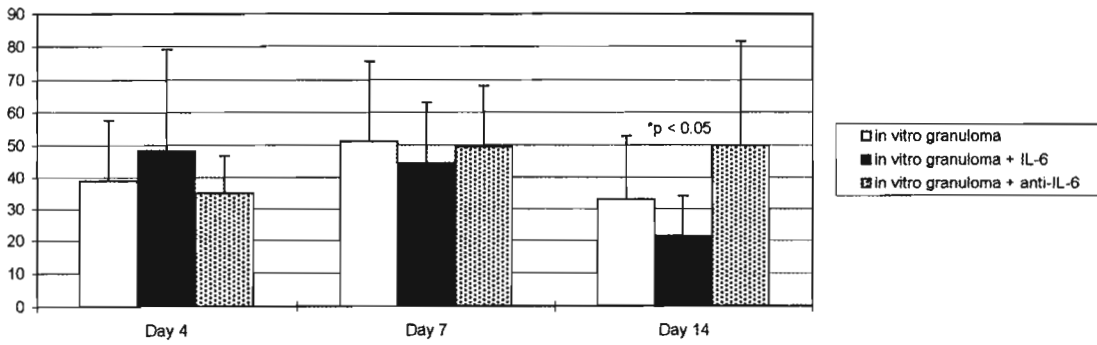
Table 1. Summary of the IL-6 stainings in seven different sarcoid granulomas.

SARCOIDOSIS CASE	GRANULOMA		MACROPHAGES/ EPITHELIOID CELLS	MGC	
	Morphology	IL-6-staining	IL-6-staining	Present	IL-6-staining
1	many focused entities	++	++/+++	yes	+++ cytoplasmic
2	many focused entities	+++	+++	yes	+ membranous +++ cytoplasmic some negative
3	focused and unfocused entities	- to +++	+++	yes	++ membranous +++ cytoplasmic some negative
4	small, focused entities	++	++	yes	++ cytoplasmic
5	diffusely distributed, unfocused entities	+++	+++	no	n.a.
6	very small, diffuse	+	++	no	n.a.
7	very small, diffuse	+	++	no	n.a.

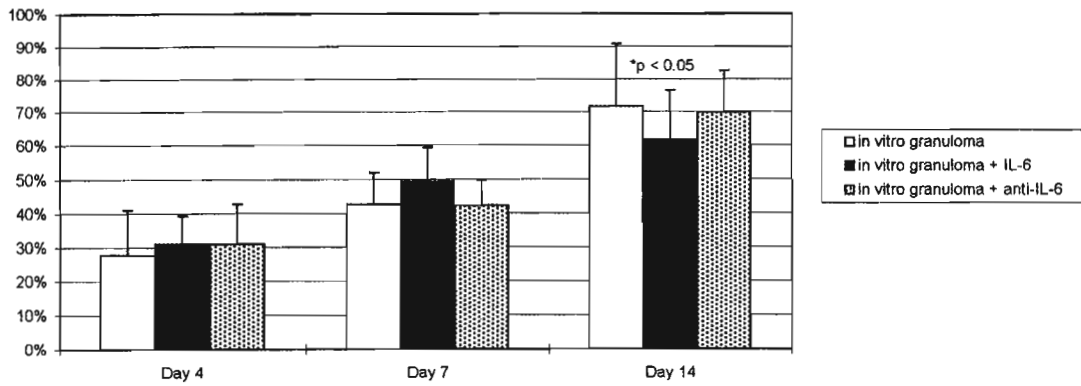
n.a.: not applicable.



b) Nuclei/MGC



c) Fusion Index



d) Cells/in vitro granuloma

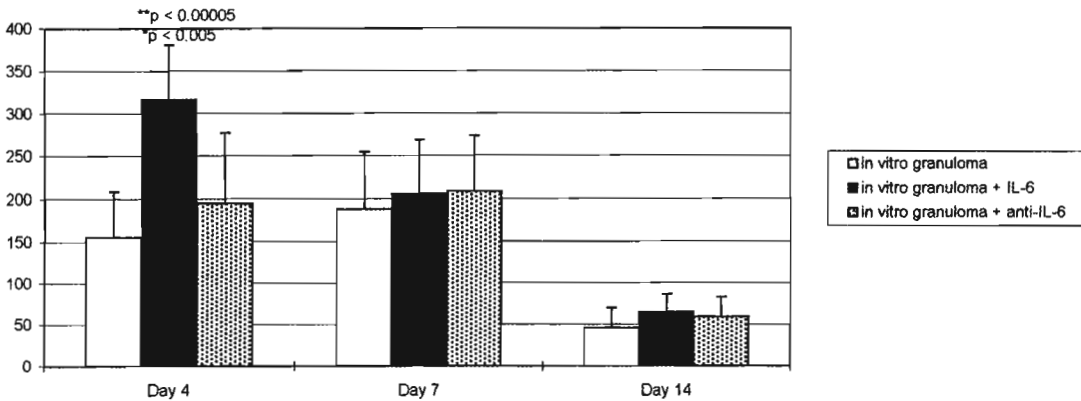


Fig. 8. Statistical analysis of modulatory effects of IL-6 on *in vitro* granuloma formation. *: IL-6-treated compared to anti-IL-6-treated culture; **: IL-6-treated compared to control culture.

granuloma in the IL-6 treated cultures was found for this timepoint (Fig. 8a). The most striking difference was found in the evaluation of cells per *in vitro* granuloma. At day 4 of the coculture, significantly more cells per granuloma were present in the IL-6-treated cultures than both in the untreated and anti-IL-6-treated cultures. In the continuation of the cocultures, this significant effect was no longer observed at day 7 and day 14. A noteworthy effect was also observed regarding the morphology of the cells. In the presence of IL-6, a considerable proportion of the cells exhibited large, elongated nuclei and oval shaped cytoplasm (Fig. 7a). In control cultures only a minor fraction of cells showed this morphological phenotype (Fig. 7b). Conceivably, elevated IL-6 levels exert a modulatory role in promoting the formation of granulomas with respect to the number of cells involved and in influencing distinct cell populations, as has been described previously for rat alveolar macrophages developing to MGC (Lemaire et al., 1996b). This effect, in turn, may influence the fusion events leading to MGC formation, resulting in more MGC per granuloma, but of a smaller size with respect to the number of nuclei present.

Concluding remarks

Unique morphological, handling and analytical features of the *in vitro* granuloma model described make it interesting for the study of granuloma formation. The cell types found, the differentiation of cells of the myeloid cell line, and the fact that granuloma formation, establishment and resolution, along with destruction of the granuloma-inducing agent, are observed makes this a model that closely mimicks events that take place in human granuloma formation. In combination with the handling features this model is accessible for the investigation of diverse aspects of granuloma formation, for the phenotyping and differentiation analysis of cells involved, and for functional analysis of granuloma-relevant cell types. It has the potential to deliver information pertinent to granuloma formation which may not be obtained with other *in vitro* models and thus may add to the knowledge of this important pathological process.

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