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Early cellular responses to intradermal injection of Kveim suspension in normal subjects and those with sarcoidosis

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SUMMARY In a detailed controlled study of the cellular response to Kveim suspension in vivo we used immunohistological and histochemical methods to examine cryostat sections of immature Kveim biopsy specimens in subjects with sarcoidosis and normal controls. Changes seen at 48 hours, at which time papular reactions have sometimes been reported, are described. Eight cases of sarcoidosis previously confirmed by a positive Kveim test were studied, in five of whom the test remained positive; plus two subjects with sarcoidosis studied prospectively; and four healthy controls.

There were two main features of the 48 hour response: collagen disruption with associated histiocytes, which showed increased acid phosphatase activity; and perivascular infiltrates of lymphocytes and small groups of dendritic cells. The T4:T8 ratios in the infiltrates were similar to those found in the peripheral blood of the subjects, and few lymphocytes showed evidence of activation. T lymphocytes were also seen free in the dermis and migrating to the epidermis. Small juxtacapillary clumps of dendritic cells, identified by NA1/34 (= OKT6; Langerhans' cells) and RFD1 (interdigitating cell) monoclonal antibodies, were found. The Langerhans' cells in the epidermis were, however, normal in number and distribution. These features, which were found in all groups, are not consistent with pre-existing hypersensitivity to Kveim suspension in sarcoidosis. Subsequent differences between sarcoid and normal subjects in the development of granulomas in the Kveim response may therefore relate to the different handling of the foreign material by the cells affected, rather than to differences in the early non-specific recruitment of the cells to the test site.

The Kveim test is a useful diagnostic test for sarcoidosis in which a suspension validated for diagnostic selectivity derived from a sarcoid spleen is injected intradermally. In many cases of the disorder an epithelioid granuloma develops at the injection site over the course of several weeks. The mechanism of this response remains unknown. Its selectivity for sarcoidosis has led to the suggestion that Kveim suspensions contain an antigen or antigens to which the subject with sarcoidosis is specifically sensitised[1] and that the reaction reflects a form of cellular hypersensitivity. The timing of the reaction, however, differs from classical cutaneous delayed type hypersensitivity, which is maximal at 48–72 hours. Nevertheless, the first report of a diagnostic skin test in sarcoidosis by Williams and Nickerson in 1935[2] described papular reactions occurring in subjects with sarcoidosis from 36 hours, and since then various reports[3–5] have alluded to an early component of the response. Chase,[6] using partially purified suspensions, claimed to have been able to predict the outcome of the test at three to four days. In view of the impaired expression of cutaneous delayed type hypersensitivity common in sarcoidosis this hypothesis implies a paradox, and other authors[7] considered that reactions occurring in the first few days were non-specific. Moreover, the putative antigen(s) have defied various attempts to isolate them[8 9] and in vitro cellular responses to Kveim suspension[10–12] show no consistent evidence of hypersensitivity in sarcoidosis.

Despite the long interval required for the production of epithelioid granulomas histological

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Table 1 *Patients with sarcoidosis*

Subject	*Age and sex*	*Presentation*	*Months since original Kveim test*	*Most recent Chest radiograph stage*	*Current Kveim test*
RL	29M	Optic neuritis	29	0	Positive
JL	34M	BHL, arthralgia	9	1	Equivocal
FC	47F	EN, malaise	3	2	Positive
TM	30M	Pulmonary mottling	11	3	Negative
CD	31M	BHL, uveitis, malaise	6	1	Positive
NM	31F	Pulmonary mottling	2	3	Positive
RA	35M	BHL	6	1	Negative
CP	28M	BHL	1	1	Positive
CN	55F	Fever, cough, chest pain	0*	2	Positive
MP	24M	BHL, EN, arthralgia	0*	1	Positive

BHL = Bilateral hilar lymphadenopathy; EN = erythema nodosum; Chest radiograph stage 0 = normal; 1 = bilateral hilar lymphadenopathy and mottling; 3 = mottling alone.
*Studied prospectively.

confirmation of their presence in biopsied Kveim test sites is the only satisfactory criterion of a positive test. Nevertheless, the processes that occur during this interval are likely to be relevant to the formation of the granulomas in sarcoidosis: these have been little examined to date. Recent developments in the use of monoclonal antibodies to examine normal and pathological tissues have permitted more precise analysis of their cellular content. We used such techniques to study early reactions after intracutaneous injection of Kveim suspension in those with sarcoidosis and healthy control subjects, looking particularly for evidence of delayed type hypersensitivity, or other differences between responders and non-responders.

Material and methods

Eight subjects with clinical features compatible with sarcoidosis and who had previously yielded a positive granulomatous response to a Kveim test consented to have further tests. Two additional subjects with clinically confirmed sarcoidosis were studied prospectively. Table 1 shows their details. Kveim tests were also given to four healthy male clinical and laboratory workers aged 27–40 to examine normal responses. In each case the current Kveim tests were performed using a suspension from a spleen (K12), which has been extensively validated in sarcoidosis, other diseases, and normal controls.[13] Intracutaneous tests, each of 0·15 ml, were injected into sites on the ulnar aspect of the forearm of each subject. The epidermis overlying the test site was marked with inert Pelikan ink to ensure correct identification for biopsy. In 10 of the 14 subjects peripheral blood was drawn for the determination of T4:T8 ratios of the lymphocytes circulating when the Kveim test was inserted. After 48 hours the full thickness of the test site was removed under local anaesthesia with a 4 mm skin punch, mounted in OCT compound (Titertek) and snap frozen in isopentane cooled by liquid nitrogen. In the subjects with sarcoidosis a second adjacent test site was harvested at four to six weeks, routinely processed, and assessed blind by a histologist experienced in reading Kveim tests. Biopsies of three positive Mantoux responses to 10 tuberculin units of purified protein derivative at 48 hours in healthy subjects were included in the study as examples of classical delayed type hypersensitivity.

Cryostat sections of the biopsies were fixed in chloroform-acetone (1:1) and stored at −20°C until

Table 2 *Monoclonal antibodies used in this study*

Antibody	*Specificity*	*Source*	*References*
RFT "cocktail"	Most E rosetting cells	Royal Free Hospital School of Medicine	15
OKT4*	T helper cell	Ortho Diagnostics	16
RFT8	T suppressor cell	Royal Free Hospital School of Medicine	15
Leu-9	Interleukin 2 receptor	Becton Dickinson	17
Anti Tac			37
RFB4+6	Most B cells	Royal Free Hospital School of Medicine	18
RFD2	Most myeloid cells		19
RFD7	Macrophages		Poulter *et al* (unpublished observation)
RFD1	Interdigitating cells		19
NA 1/34	Langerhans' cell; cortical thymocytes	A McMichael Oxford	20
RFDR2*	Class II histocompatibility antigens	Royal Free Hospital School of Medicine	18

*IgM; all others IgG_1 or IgG_2.

use. Haematoxylin and eosin preparations of multiple sections of each biopsy were examined by a histopathologist unaware of the source of the specimen, and the extent of various features, including dermal and perivascular histiocytic and lymphoid infiltration, were scored on an arbitrary scale of 0–10. In studies of the reproducibility of scoring 88% (63 of 72) of individual scores on duplicate specimens were within one point of the original score.

Other sections were examined using monoclonal antibodies and immunoperoxidase (Dako) and single and double immunofluorescence techniques, as well as a histochemical technique for acid-phosphatase activity (ACP), as described by Poulter *et al.*[14] Table 2 lists the monoclonal antibodies used in the study. Sections of normal skin and tonsil were used as positive controls. At least three sections from separate parts of each biopsy were examined with each antibody or a combination of antibodies. To quantify T4:T8 ratios an IgM OKT4 antibody (the gift of Ortho Diagnostics) was used in combination with IgG RFT8, and the localisation of these first layers was recognised with affinity purified goat antibodies to mouse immunoglobulins G and M, conjugated to fluorescein and rhodamine, respectively (Southern Biotechnology). The proportions of cells positive for each marker were counted with a Zeiss fluorescence microscope. The coefficient of variation for T4:T8 ratios of 200 T cells in each of 10 consecutive sections of the same biopsy was 11%. The same antibody combination was used for determining blood T4:T8 ratios, which were measured in suspensions of mononuclear cells separated by centrifugation on Lymphoprep (Nyegaard); the single sample coefficient of variation was 15%. The incidence of the activation markers in tissue sections (Leu-9+, Tac+) was determined by again using the IgM OKT4, but in combination with the relevant IgG antibody. To estimate the proportion to total T cells the resulting percentage was adjusted using a formula derived from the T4:T8 count. These activation markers were present only in small proportions, and the coefficients of variation of the counts, 25% and 32%, respectively, were therefore relatively large in the available samples.

Results

Five of the patients with sarcoidosis who had repeat Kveim tests gave a positive Kveim response at four to six weeks; of the remainder, one was read as equivocal and two as negative. The two subjects studied prospectively were both Kveim positive. For the purposes of analysis the patients with sarcoidosis were divided into unequivocal Kveim positive patients and others; normal controls comprised a third study group.

The macroscopic features of the reactions at two days were variable, slight erythema (0–3 mm) and just palpable induration (1–2 mm) being found in all groups. The histological appearance of all reactions was similar, and no feature distinguished the groups. The lesions consisted of an amorphous mass containing disrupted collagen, which was interspersed with and surrounded by dermal histiocytes. There were also pericapillary infiltrates of mononuclear cells, which occurred at sites without collagen disruption,

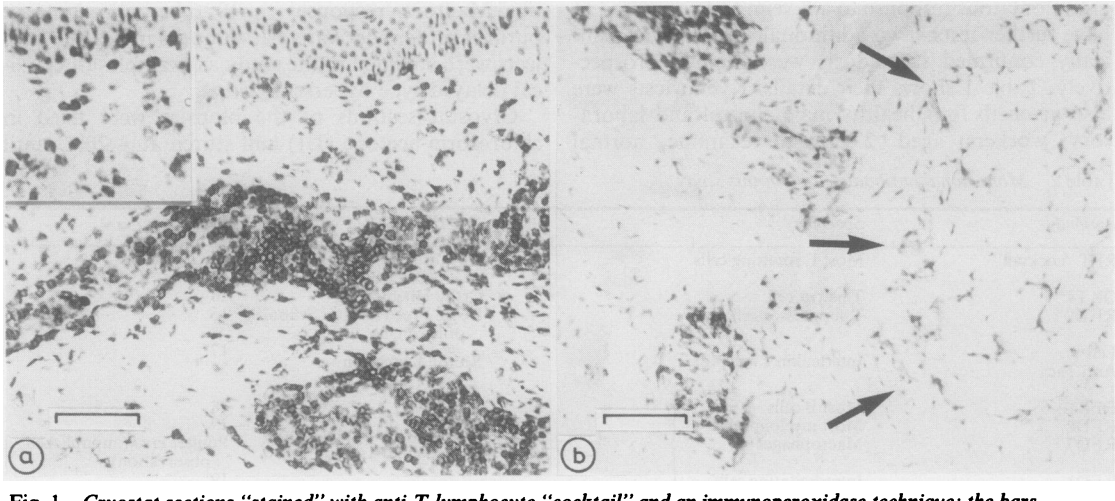

Fig. 1 *Cryostat sections "stained" with anti-T-lymphocyte "cocktail" and an immunoperoxidase technique; the bars represent 100 microns. (a) 48 hour positive Mantoux response: note dense infiltrates with (inset) free T cells scattered in dermis and epidermis (b) 48 hour response to Kveim test in sarcoid subject. Edge of area of collagen disruption caused by test is arrowed. Elsewhere, mononuclear perivascular infiltrates containing T cells are again seen, but few are free in dermis.*

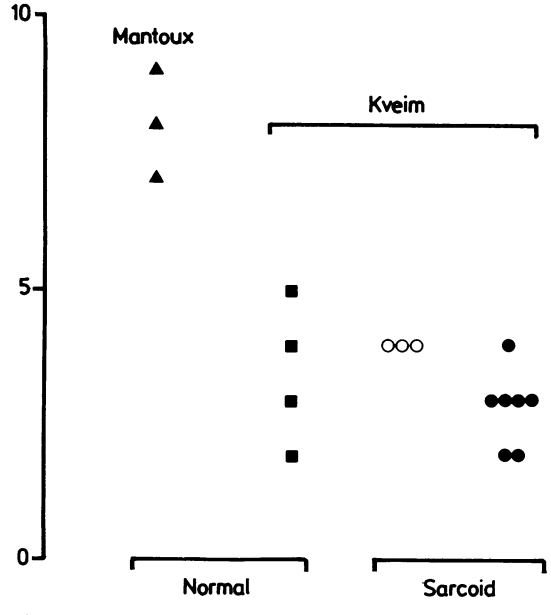

Fig. 2 *Histological scores for perivascular lymphoid infiltrates in haematoxylin and eosin sections of positive Mantoux reactions at 48 hours (▲), Kveim responses at the same time in normal subjects (■), and currently Kveim negative (○) and Kveim positive (●) subjects with sarcoidosis.*

in the superficial dermis and adjacent to hair follicles and sweat glands. Although similar mononuclear infiltrates were seen in positive Mantoux responses, these were denser and more extensive. Fig. 1 shows the presence of T lymphocytes in Mantoux and Kveim responses at 48 hours, and Fig. 2 compares the scores for one of the histological variables (degree of lymphoid infiltration) in these reactions.

The histiocytes surrounding the amorphous material at the test site had the monoclonal antibody defined phenotype of tissue macrophages: they were recognised both by RFD2 (which identifies cells of the myeloid lineage) and RFD7 (tissue macrophages). The Kveim associated macrophages, however, contained increased acid-phosphatase (ACP) activity compared with those in normal dermis. Many also expressed class II major histocompatibility complex (MHC) antigens (RFDR2 positive). No lymphocytes were identifiable in these areas at this stage.

In the perivascular infiltrates the lymphocytes were mostly T cells, and only an occasional B cell was seen. In all cases cells of helper phenotype (OKT4 positive) predominated over those of suppressor phenotype (RFT8 positive), although the T4:T8 ratios varied between subjects from 1·1:1 to 3·3:1. A similar range was seen in the peripheral blood of the subjects, but there was no correlation between the blood and tissue ratios in individual subjects (Table 3): the prevalence of Tac and Leu-9 positive cells is also shown. There was no evidence of greater activation in the infiltrates of the Kveim positive sarcoid group than in those of the Kveim negative group or control subjects. In general, the proportions of cells displaying the Tac marker were also similar to those in the larger infiltrates of the Mantoux response, but there were relatively high numbers of Leu-9+ cells in two of the Mantoux responses. Non-lymphoid mononuclear

Table 3 *Incidence of T4:T8 ratios, activation markers, and Langerhans' cells*

Subjects	*Current Kveim test*	*T4:T8 ratio**		*Tac+* (%)*	*Leu-9+† (%)*	*Langerhans' cells (NA/34+)/ 100 basal layer cells*
		Blood	*Infiltrate*			
Sarcoidosis:						
RL	+	—	—	—	—	8·3
JL	±	—	—	—	—	3·0
FC	+	2·6	1·1	7	5	5·3
TM	−	1·4	2·6	12	12	4·0
CD	+	1·5	2·6	5	4	6·7
NM	+	2·8	1·5	2	3	5·3
RA	−	2·8	2·0	3	6	7·0
CP	+	1·8	3·3	3	4	7·0
CN	+	—	—	2	1	4·0
MP	+	—	—	5	3	3·7
Mean		2·2	2·2	5	5	5·4
Controls:						
DC		1·3	2·7	2	5	6·7
CM		2·0	1·4	5	7	6·0
LP		3·0	1·6	9	3	7·0
PD		1·0	2·2	4	4	5·3
Mean		1·8	2·0	5	5	6·3
Mantoux responses:						
DC			2·1	7	11	8·7
CM			1·4	4	21	5·0
VA			1·1	7	8	7·3
Mean			1·5	6	13	7·0

* = OKT4 positive/RFT8 positive; † = positive cells as % of T4 positive, x (T4 + T8)/T4

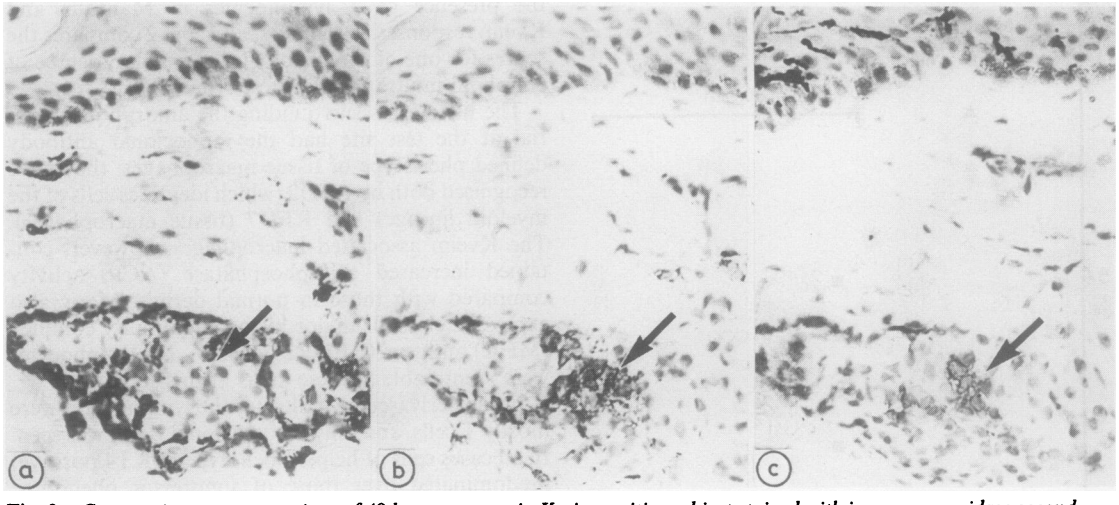

Fig. 3 *Consecutive cryostat sections of 48 hour response in Kveim positive subject stained with immunoperoxidase second layer, using as first layer (a) RFD7 (tissue macrophages) (b) RFD1 (interdigitating cells) and (c) NA1/34 (Langerhans' cells). Note RFD7 negative, RFD1 positive, NA1/34 positive dendritic cells in infiltrate (arrowed): epidermal Langerhans cells are RFD1 negative.*

cells in the Kveim infiltrates included RFD7 positive cells with weak acid-phosphatase activity. These tended to be in the outer regions of the infiltrates, whereas other non-lymphoid cells, identified by markers associated with dendritic cells (RFD1; NA1/34), were seen in small clumps adjacent to the capillaries (Fig. 3). These RFD7 negative and ACP negative cells were strongly RFDR2 positive.

Some T lymphocytes were seen free in the superficial dermis and in the basal layers of the epidermis, perhaps migrating to or from the epidermis. These were a more common feature of the Mantoux response (Fig. 1). Both helper and suppressor phenotypes were present, and activation markers were common among these cells. Table 3 shows the number and distribution of NA1/34 positive Langerhans' cells in the epidermis in the various groups. In combination immunofluorescence studies virtually all expressed class II MHC antigens.

Table 4 shows overall comparisons, including the findings in positive Mantoux responses. In none of the respects described above was there a demonstrable qualitative or quantitative difference in the response to Kveim suspension between the groups, and no marker predicted the outcome of the test.

Discussion

Since its first description in 1941, only two reported studies of the Kveim test have attempted to use the evolution of the reaction to explore the cellular mechanisms of granuloma formation in the disorder. Rogers and Haserick[21] examined evolving responses at various intervals in two normal and two sarcoid subjects, but in this small group they were unable to identify conclusive differences. Siltzbach[22] described the morphological evolution of Kveim granulomas, using conventional histological preparations, but his study was not controlled by the inclusion of normal subjects. With the aid of monoclonal antibody tech-

Table 4 *Comparison of Mantoux and Kveim reactions at 48 hours*

Feature	*Mantoux*	*Kveim*	
		Sarcoid subjects	*Normal subjects*
Erythema	++/+++	−/±	−/±
Induration	++	−	−
ACP positive cells	−	++	++
Perivascular infiltrates:	++	+	+
T lymphocytes	++	+	+
Activated T cells	±/+	±	±
Mean T4:T8 ratio	1·5	2·2	2·0
Accessory cells	++	+/++	+/++
Free T cells dermis and epidermis	++/+++	+	+

niques, therefore, further study of developing responses may yet help to elucidate the mechanisms of the Kveim reaction.

Despite the failure to isolate an antigen or show consistent in vitro sensitivity to Kveim suspensions evidence from other disorders suggests that cell mediated immunity may contribute to a positive response. Delayed type hypersensitivity has been implicated in granulomatous diseases in man, such as specific sensitivity to beryllium[23]and zirconium,[24] and studies on animals suggest that the formation of granulomas is augmented by delayed type hypersensitivity.[25 26] T lymphocytes are found in and around sarcoid granulomas[27 28] and in the structurally similar Kveim test granuloma,[29] with a central predominance of T4 positive (helper) cells. Despite depressed cutaneous cellular responses T cells at sites of disease are activated.[30 31] Features suggestive of cell mediated responses in early reactions to Kveim suspension would therefore be an important clue to the mechanism of Kveim granuloma formation.

Non-lymphoid cells whose phenotype indicates that they are activated tissue macrophages are the central feature of the early response to Kveim suspension. They are found equally in all groups and are not associated with lymphocytes. This predictable reaction, however, is accompanied by perivascular infiltrates that do contain lymphocytes but which are less common than the mononuclear infiltrates seen in the Mantoux reaction. We confirmed previous observations[32 33]—namely, that the brisk infiltrates in positive Mantoux responses contain many T cells and that the ratios of helper to suppressor phenotypes in these reflect the ratios in peripheral blood. The mean ratios in the lesser infiltrates of the Kveim response were comparable; a precise correlation with peripheral blood ratios in each subject was unlikely in view of the sometimes considerable circadian variation in the peripheral blood ratios.[34] "Activated" lymphocytes are present in responses to Kveim material, and their incidence, relative to total T cells, is comparable to that in the Mantoux responses. Owing to the larger infiltrates, there are more activated T cells in Mantoux responses. The small and similar *proportion* of T cells in perivascular infiltrates, which bear activation markers in either reaction at this stage, however, suggests that, with the possible exception of Leu-9 in the Mantoux response, their presence may be a non-specific phenomenon. In the Kveim test, as T4:T8 ratios in the infiltrates are variable and are not correlated with the outcome of the test, and because evidence of lymphocyte activation in all groups is comparable, neither selective recruitment nor selective stimulation of a subgroup of lymphocytes seem to be part of this early stage of the response.

Although there is no evidence of hypersensitivity, the subsequent course of the reaction may depend on cells recruited in the early response. The mediation of cellular immune responses entails antigen presentation to T cells by macrophages or by specialised accessory cells of the so called dendritic cell lineage.[35] In the dermal response to Kveim suspension we found in all groups macrophage like cells whose phenotype (RFD1+, RFDR2+, NA1/34+, ACP−) strongly suggested that they were dendritic cells. Similar cells, which were also prominent in the Mantoux responses, were only occasionally present in normal and unaffected sarcoid skin. ACP negative cells form a large proportion of the initial macrophage like population in the Mantoux response at six hours,[32] and may have a role in recruiting other cells. The dendritic cell group also includes epidermal Langerhans' cells, and Fox *et al*[36] reported that considerably fewer Langerhans' (OKT6+) cells occur in sarcoid epidermis, even when unaffected, than in normal skin. They speculate that this might in some way reflect the cutaneous anergy seen in the disease. Using NA1/34, however, which recognises the same antigen as OKT6,[20] we found no consistent reduction in incidence of epidermal Langerhans' cell in our group with sarcoidosis. In view of the appearance of NA1/34 positive cells in the dermis early in the response to Kveim suspension in all subjects changed numbers of accessory cells at this stage are unlikely to be responsible for the differences in outcome of the test. Furthermore, despite a reduced T cell infiltrate, RFD1 positive cells are present in the impaired Mantoux response in sarcoidosis.[28]

We conclude that in comparison with healthy controls there is no evidence of pre-existing hypersensitivity to a component of Kveim suspension in subjects with sarcoidosis; neither is there any other manifestation of changed immunological reactivity unique to them at this stage. The development of small perivascular infiltrates containing lymphocytes and non-lymphoid cells, whose phenotype implies the capacity to mediate immune responses, is probably due to a non-specific irritant reaction. Nevertheless, a changed pattern of handling of the material by the cells evoked in this initial phase may determine the subsequent variations in responses to the intradermal deposition of Kveim suspension. The immunohistological approach to the study of both lymphoid and non-lymphoid mononuclear cells at later stages of the developing Kveim response may disclose the existence of such changed mechanisms and thus be of relevance to the elucidation of the reasons for the formation of granulomas in sarcoidosis.

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