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# Synovial fluid antigen-presenting cells unmask peripheral blood T cell responses to bacterial antigens in inflammatory arthritis

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## SUMMARY

We and others have previously shown that synovial fluid (SF) mononuclear cells (MC) from patients with both reactive arthritis and other inflammatory arthritides proliferate *in vitro* in response to bacteria clinically associated with the triggering of reactive arthritis. In all cases, such SFMC responses are greater than the corresponding peripheral blood (PB) MC responses, often markedly so, and the mechanism for this is unclear. We have investigated this phenomenon by comparing the relative abilities of irradiated non-T cells derived from PB and SF to support autologous T cell responses to ReA-associated bacteria. Seven patients whose SFMC had been shown previously to respond to bacteria were studied. We demonstrate antigen-specific responses of PB T cells to bacteria in the presence of SF non-T cells which are in marked contrast to the minimal responses of either unfractionated PBMC or PB T cells reconstituted with PB non-T cells. We also show that PB, but not SF T cells respond strongly to autologous SF non-T cells in the absence of antigen or mitogen. These findings demonstrate that SF antigen-presenting cells (APC) are potent activators of PB T cells. We conclude that the contrasting responses of SFMC and PBMC to bacterial antigens may be accounted for at least in part by an enhanced ability of SF APC to support T cell proliferative responses.

**Keywords** inflammatory arthritis antigen presenting cells bacterial antigens

## INTRODUCTION

Reactive arthritis (ReA) is characterized by sterile joint inflammation following infection of the gastro-intestinal or genito-urinary tracts by a range of bacteriologically dissimilar organisms including *Salmonella*, *Campylobacter*, *Yersinia* and *Chlamydia* (Aho, Leiresalo-Repo & Repo, 1985). Previous studies have demonstrated that mononuclear cells (MC) isolated from the inflamed joints of ReA patients respond preferentially to the organism that had caused the infection (Ford, da Roza & Schulzer, 1982, 1985; Ford, da Roza & Ward, 1984; Ford, 1986; Sheldon, 1985). Similar findings have been reported in Lyme arthritis patients in that synovial fluid (SF) MC (SFMC) respond specifically to the known aetiologic bacterium, *Borrelia burgdorferi* (Sigal et al., 1986). We have also reported that the SFMC from many patients with inflammatory arthritidies other than ReA can respond to ReA-associated bacteria (Gaston *et al.*, 1989); this may be due to the recognition of cross-reactive antigens such as heat shock proteins that are highly conserved amongst bacteria. The 65-kD antigen of mycobacteria has been studied in this context (Gaston *et al.*, 1988; Res *et al.*, 1988). SFMC responses to bacterial antigens are inhibited by cyclosporin A or monoclonal antibodies specific for class II major histocompatibility complex (MHC) antigens and are not induced using optimal concentrations of bacterial lipopolysaccharide (LPS) (Gaston *et al.*,1989). These findings indicate that such SFMC proliferation represents a class II MHC-restricted T cell response.

A consistent finding of studies investigating MC responses to bacterial antigens is that peripheral blood MC (PBMC) responses are invariably lower than those of corresponding SFMC and often minimal (Ford *et al.*, 1985; Sigal *et al.*, 1986; Gaston *et al.*, 1989). This observation may be explained by two hypotheses that are not mutually exclusive. Firstly, increased SFMC responsiveness may reflect an increased frequency of bacteria-specific T cells in the joint relative to the peripheral blood. Secondly, there may be a preferential activation of bacteria-specific T cells by antigen-presenting cells (APC) in the synovial compartment relative to peripheral blood.

The aim of this study was to compare directly the abilities of APC derived from peripheral blood and from SF to support T cell responses to ReA-associated bacteria in order to establish whether increased SFMC responsiveness to such antigens were a consequence of enhanced function of APC within the inflamed joint.

## MATERIALS AND METHODS

*Patients*

Seven patients with inflammatory arthritis whose SFMC had

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previously been shown to respond to bacterial antigens were studied. Maximal responses were to either *Yersinia* or *Salmonella*, and none had a significant response to *Chlamydia* (although the chlamydial antigens used in this study had been shown to be stimulatory for SFMC obtained from other patients with inflammatory arthritis). Four of the patients had active, classical rheumatoid arthritis (RA), two had clinically established ReA following gastroenteritis, and one had Whipple's disease. All patients required therapeutic aspiration of SF from inflamed knee joints; 20 ml of peripheral blood were donated at the time of aspiration.

*MC separation*

SF and peripheral blood were heparinized and SF was also incubated for 30 min at 37°C with hyaluronidase (10 U/ml). MC were obtained by centrifugation over Ficoll–Paque and washed twice in serum-free RPMI 1640 (Flow). A proportion of the SFMC and PBMC was resuspended in complete medium (CM)—RPMI 1640 supplemented with 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10% heat-inactivated fetal calf serum (FCS) and 1% heat-inactivated $A^+$ human serum—and used as unmanipulated control populations. For two patients 10% heat-inactivated autologous serum replaced FCS in CM.

*Isolation of T cell and non-T cell enriched populations*

T cell enriched populations were prepared from PBMC and SFMC by rosetting with 1% v/v neuraminidase-treated sheep erythrocytes (SRBC). After centrifugation over Ficoll–Paque the cell pellet containing rosetted T cells was incubated for 10 min at room temperature with tris/$NH_4Cl$, pH 7.2, to lyse SRBC. Following two washes in serum-free RPMI the T cell-enriched populations were resuspended in 10 ml of CM and incubated at 37°C for two 45-min periods in 9-cm plastic Petri dishes (Nunc, Roskilde, Denmark) to further deplete for contaminating adherent cells. This protocol routinely yielded populations which contained >95% positive cells when stained with anti-CD3 monoclonal antibodies using standard immunoperoxidase techniques.

Non-rosetting cells were harvested from the Ficoll interface and washed twice in serum-free RPMI. Immunoperoxidase staining of these populations revealed <5% CD3 positive and >90% class II MHC positive cells. These non-T cell populations were then subjected to 3000 rad of gamma irradiation from a $^{60}$Co source in order to prevent cell proliferation, washed twice and used as APC.

*Proliferation assays*

PBMC and SFMC ($5 \times 10^4$) were cultured separately in triplicate U-bottomed 200-μl wells; $5 \times 10^4$ peripheral blood and SF T cells were cultured either alone or with $5 \times 10^3$ irradiated autologous peripheral blood or SF non-T cells at a ratio (T: non-T) of 10:1 (additional ratios were investigated for two patients). Limiting cell numbers prevented the investigation of a full range of cell combinations in some patients and cultures of SFMC alone for one patient were technically unsatisfactory. In some instances, control cultures of $5 \times 10^4$ irradiated peripheral blood and SF non-T cells were also set up.

Cells were cultured for 6 days in CM alone or with suspensions of stimulatory or non-stimulatory bacteria. In two of the patients whose SFMC responded preferentially to *Salmonella*, cultures of PBMC and SFMC were also set up using a soluble *Salmonella* preparation at 10 μg/ml (previously determined to be optimal) or phytohaemagglutinin (PHA) at 150 μg/ml. For the last 18 h of culture, 0·15 μCi of $^3$H-thymidine (Amersham International, Amersham, UK) was added to each well. The cells were then harvested and thymidine incorporation measured using a liquid scintillation counter. Results were recorded as the mean distintegrations per min (d/min) of at least three replicate cultures.

*Preparation of bacterial suspensions*

*Salmonella agona*, isolated from a patient with ReA, was obtained from Dr G. Clarke, Department of Microbiology, University of Birmingham, and killed using gamma irradiation. Heat-killed *Yersinia enterocolitica* 0:3 was obtained from Dr K. Granfors, University of Turku, Finland. *Chlamydia trachomatis* serotype E elementary bodies, purified from McCoy cells, was supplied by Dr J. Pearce, Department of Microbiology, University of Birmingham. The concentration of each of these organisms was determined by counting in a Thoma chamber and stock solutions stored at −20°C prior to use. *Salmonella* and *Chlamydia* were used at $2 \times 10^8$ organisms per ml and Yersinia at $2 \times 10^6$ per ml, concentrations previously determined to be optimal.

*Preparation of soluble* Salmonella *antigen*

A soluble preparation of *Salmonella agona* antigens was prepared by treating $4 \times 10^{11}$ killed bacteria with 0·1% sodium dodecyl sulphate for 1 h at 37°C. Following centrifugation in an Eppendorf airfuge, the supernatant was filtered (0.22 μm) and dialysed for 24 h against 2 *l* of 0·01 M phosphate-buffered saline (PBS). Protein concentration was estimated using the Lowry technique and the preparation stored at −20°C until use.

*Statistical analysis*

All data were analysed using Wilcoxon ranked pairs test for non-parametric data.

## RESULTS

*Proliferative response to CM*

Figure 1 shows the results obtained when MC, T cells alone and T cells co-cultured with autologous non-T cells were cultured for 6 days in CM only. Neither SFMC or PBMC proliferated spontaneously, except in the case of one patient's SFMC as shown. Following a subsequent SF aspiration from this patient, a similar spontaneous SFMC response was observed on culture in 10% autologous serum. Reconstitution of SF T cells with SF non-T cells elicited proliferative responses equivalent to those of SFMC (median 608:323 d/min, not significant; $n=6$). In contrast, when peripheral blood T cells were co-cultured with SF non-T cells, highly significant proliferative responses were observed in comparison to unfractionated PBMC (median 6500:636 d/min, $P<0·01$; $n=7$).

*Proliferative response to bacterial suspensions*

Figure 2 shows the results obtained when MC, T cells alone and T cells reconstituted with autologous non-T cells were cultured

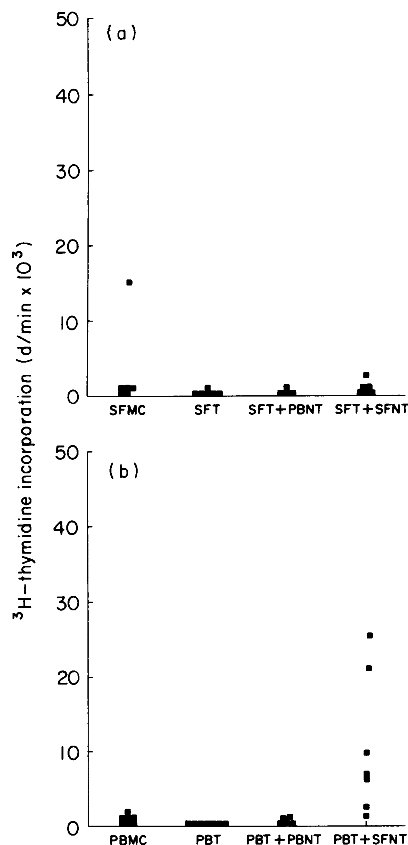


**Fig. 1.** Proliferative responses of synovial fluid (SF) and peripheral blood (PB) mononuclear cells (MC), T cells alone and T cells co-cultured with autologous, irradiated non-T (NT), cells for seven patients. Cells were cultured for 6 days in medium alone. a, Results of responder cells isolated from SF; b, results of responder cells isolated from peripheral blood. A T:NT ratio of 10:1 was used in co-culture experiments.

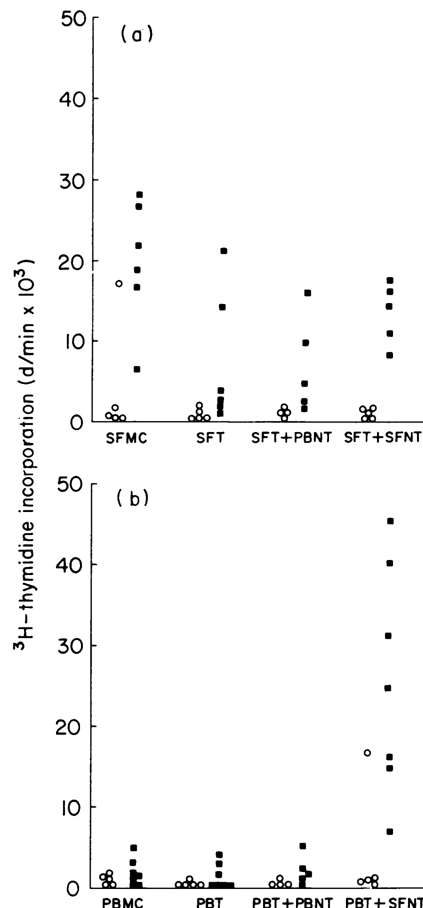


**Fig. 2.** Proliferative responses of synovial fluid (SF) and peripheral blood (PB) mononuclear cells (MC), T cells alone and T cells co-cultured with autologous, irradiated non-T (NT) cells for seven patients. Cells were cultured for 6 days with an optimal concentration of stimulatory bacteria (*Salmonella agona* or *Yersinia enterocolitica*) (■) or a non-stimulatory control (*Chlamydia trachomatis*) (○). a, Results of responder cells isolated from SF; b, results of responder cells isolated from PB. A T:NT ratio of 10:1 was used in co-culture experiments.

for 6 days with an optimal concentration of stimulatory bacteria (either *Salmonella* or *Yersinia*) and a non-stimulatory control organism (*Chlamydia*). Responses in control cultures of irradiated APC alone were consistently $<500$ d/min (data not shown). PBMC responses were significantly less than those of the corresponding SFMC (median 1888:20 262 d/min, $P<0{\cdot}01$; $n=6$). SF T cells alone gave significantly diminished proliferative responses compared with unfractionated SFMC controls (median 3321:20 262 d/min, $P<0{\cdot}05$; $n=6$). SF T cell responsiveness was restored more effectively by co-culture with SF non-T cells than with peripheral blood non-T cells. Neither unfractionated PBMC nor peripheral blood T cells reconstituted with peripheral blood non-T cells gave significant responses. In contrast, co-culture of peripheral blood T cells with SF non-T cells resulted in striking proliferative responses which were significantly greater than those of non-antigen pulsed cultures (median 22 992:6500 d/min, $P<0{\cdot}05$; $n=7$) antigen-pulsed unfractionated PBMC (median 22 992:1888 d/min, $P<0{\cdot}01$; $n=7$) and of the same magnitude as those of unfractionated SFMC (median 20 566:20 262 d/min, not significant; $n=6$).

*Proliferative response to soluble bacterial antigens*

Since the enhanced function of SF APC compared with peripheral blood APC may be explained by a reduced capacity of the latter population to phagocytose intact bacteria prior to antigen processing and presentation, MC responses to a soluble bacterial preparation which can be endocytosed were compared. Table 1 shows the results obtained in two patients where SFMC and PBMC responses to a soluble *Salmonella* preparation were compared with responses to an optimal concentration of whole killed *Salmonella*. The lack of PBMC responsiveness to *Salmonella* was observed with both the intact and soluble *Salmonella* preparations. In contrast, both preparations were highly stimulatory for SFMC.

*Titration of SF non-T stimulation of peripheral blood T cell responses*

Figure 3 shows the results obtained for an individual patient whose SFMC had been shown in prior assays to respond well to *Yersinia*, but not to *Chlamydia*. Peripheral blood T cells were co-cultured with autologous non-T cells at ratios from 50:1 to 2·5:1 in CM alone, or with optimal concentrations of either

**Table 1.** Proliferative responses of synovial fluid (SF) and peripheral blood (PB) mononuclear cells (MC) from two patients

| | $^3$H-thymidine incorporation (d/min) | | | |
|---|---|---|---|---|
| | Patient 1 | | Patient 2 | |
| Stimulus | SFMC | PBMC | SFMC | PBMC |
| Control (medium) | 15 136 | 1681 | 5542 | 94 |
| *Salmonella* ($2 \times 10^8$/ml) | 27 019 | 2174 | 15 711 | 44 |
| Soluble *Salmonella* (10 $\mu$g/ml) | 30 049 | 497 | 11 921 | 57 |
| Phytohaemagglutinin (150 $\mu$g/ml) | 24 157 | 47 361 | 34 362 | 17 691 |

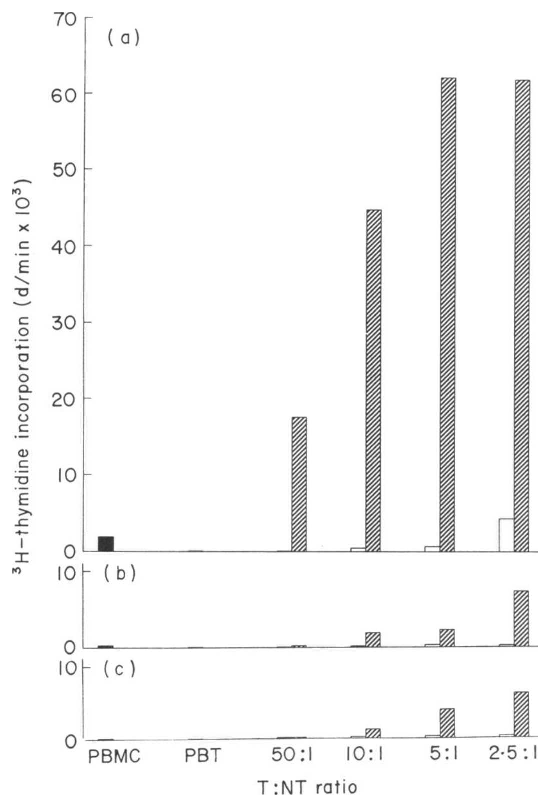


**Fig. 3.** Proliferative responses of peripheral blood (PB) mononuclear cells (MC), PB T cells and PB T cells co-cultured with autologous, irradiated PB (□) or synovial fluid (SF) (■) non-T (NT) cells at the ratios indicated for one patient. Cells were cultured for 6 days with (a) *Yersinia enterocolitica*; or (b) *Chlamydia trachomatis* (both at optimal concentrations); or (c) medium alone.

*Yersinia* or *Chlamydia*. The PBMC response to *Yersinia* was considerably lower than that of SFMC (1888:21 621 d/min); the proliferative response of peripheral blood T cells alone was minimal. Reconstitution using peripheral blood non-T cells at ratios (T:non-T) of 50, 10, 5 and 2·5:1 resulted in significant peripheral blood T cell proliferation at the highest ratio only. In marked contrast, however, co-culture with SF non-T cells elicited a striking peripheral blood T cell response to *Yersinia* at a T:non-T ratio as low as 50:1. Proliferation was found to increase with increasing T:non-T ratios with a maximum at a T:non-T ratio of 5:1 (62 185 d/min, corresponding responses to CM and *Chlamydia* being 3915 and 2243 d/min, respectively). This peripheral blood T cell response to *Yersinia* was considerably greater than that of both unfractionated SFMC and SF T cells reconstituted with SF non-T cells (21 621 and 15 728 d/min, respectively). Similar results were obtained using *Salmonella* in one other patient (data not shown).

## DISCUSSION

Inflammatory arthritis is characterized by an extensive mononuclear cell infiltration of synovium and SF. Such infiltrates contain abundant class II MHC-expressing APC in addition to large numbers of T lymphocytes, many of which express markers of activation (Burmester *et al.*, 1982; Lindblad *et al.*, 1983).

We and others have previously reported that whereas the SFMC of patients with inflammatory arthritis proliferate in response to ReA-associated bacteria *in vitro*, the corresponding PBMC responses to the same antigens are much lower or even absent (Ford *et al.*, 1985; Sigal *et al.*, 1986; Gaston *et al.*, 1989). The hypothesis offered by these workers to explain this phenomenon proposes that there is an accumulation of bacteria-specific T cells in the inflamed joint, compared with the peripheral circulation. Immunohistological studies have detected bacterial antigens within cells taken from the joints of patients with ReA (Keat *et al.*, 1987; Granfors *et al.*, 1989) and it is possible that such antigens attract antigen-specific T cells into the joint, where they could then undergo local activation and expansion. It has also been proposed that 'self' antigens within the joint cross-react with bacterial antigens. This hypothesis is supported by studies of adjuvant arthritis in the rat which have shown that shared T cell stimulatory epitopes exist between the arthritis-inducing immunogen and cartilage proteoglycan (Van Eden *et al.*, 1988). The presence of such cross-reactive antigens could contribute to T cell accumulation and thus perpetuate the inflammatory response.

However, there is a lack of direct evidence, such as limiting dilution analyses of T cell precursor frequency in peripheral blood and SF, for an accumulation of antigen-specific T cells within the joint in inflammatory arthritis. The results of this study suggest an alternative explanation for the enhanced responses of SFMC relative to PBMC. We have been able to demonstrate that purified peripheral blood T cells proliferate vigourously in response to ReA-associated bacteria when cultured with autologous SF APC, although no significant response can be elicited from the corresponding, unmanipulated PBMC. These responses were found to be of equal magnitude with those of SFMC (or of SF T cells reconstituted with SF APC) and were specific in that only the organism recognized by the corresponding SFMC elicited a peripheral blood T cell response. Furthermore, for two patients, an optimal concentration of *Salmonella*-derived LPS did not elicit peripheral blood T cell proliferative responses greater than background (data not shown). This implies that the observed antigen stimulation is specific rather than simply a mitogenic effect involving LPS.

One possible explanation for these results would be the relative deficit of an APC subpopulation in peripheral blood compared with SF. However, in two patients a range of T:non-

T ratios were investigated. It was shown that while marked antigen-specific peripheral blood T cell responses could be stimulated by SF APC at a ratio of 50:1, no significant response could be elicited from peripheral blood T cells reconstituted with peripheral blood APC at a ratio of 2·5:1. These findings would suggest that the difference in APC function between SF and peripheral blood is likely to be qualitative rather than quantitative. To address this point in more detail, we are currently studying the relative stimulatory capacities of APC subpopulations (e.g. dendritic cells and macrophages) from both peripheral blood and SF.

Throughout this study, assays were performed using killed, intact bacteria. The relative lack of of peripheral blood antigen-presenting ability compared with that of SF-derived APC could have been explained by a reduced capacity to phagocytose particulate antigens. To address this possibility we investigated PBMC and SFMC responses to a soluble preparation of *Salmonella*, since it is well recognized that peripheral blood APC are capable of processing and presenting soluble mycobacterial antigens in the form of purified protein derivative (PPD). While soluble antigen was highly stimulatory for SFMC, the PBMC response was minimal and indistinguishable from that produced with intact antigen (Table 1, and other data not shown). This finding suggests that reduced PBMC responses to bacterial antigens cannot simply be explained by a reduced capacity to phagocytose the particulate antigens used in this study. In further support of this view we have found that PBMC can proliferate in response to particulate *Candida* (P. F. Life, unpublished data).

It is recognized that T cell recognition of antigen is influenced by the density of class II MHC molecules on the APC surface (Matis *et al.*, 1983). Studies have demonstrated that SF APC express high levels of class II MHC antigen relative to those in peripheral blood (Firestein & Zvaifler, 1987a; Teyton *et al.*, 1987). In a separate study, we have unsuccessfully attempted to augment PBMC responsiveness to ReA-associated bacteria by upregulating APC class II MHC antigen expression using recombinant human interferon-gamma (IFN-$\gamma$) in concentrations ranging from 10 to 1000 U/ml (P. F. Life, unpublished data). This suggests that class II MHC molecule density alone is unlikely to explain the differences in function between peripheral blood and SF APC. However, it has been suggested that IFN-$\gamma$ is unlikely to be a major activating factor for synovial APC, since low levels are found in the joint (Firestein & Zvaifler, 1987b). One cytokine that has been found in significant concentrations in inflamed joints is granulocyte–macrophage colony-stimulating factor (GM–CSF) (Xu *et al.*, 1989) and this has been shown to exert a maturation effect on APC function (Falk, Wahl & Vogel, 1988; Heufler, Koch & Schuler, 1988). Work is in progress to determine whether GM-CSF can be used to augment peripheral blood APC function *in vitro*.

The marked proliferation of peripheral blood T cells when co-cultured with autologous SF non-T cells in the absence of added antigen or mitogen was a consistent finding throughout this study. Such proliferation was observed regardless of whether FCS or autologous serum was used to supplement culture medium, suggesting that recognition of xenoantigens in FCS is an unlikely explanation for these results. An alternative explanation would be that the peripheral blood T cells were responding to antigens acquired *in vivo* and presented by SF non-T cells, but the lack of SF T cell proliferation when cultured with SF non-T cells suggests that this is also unlikely. Possibilities currently under investigation are: (i) that the observed peripheral blood T cell response is stimulated by high level surface expression of an adhesion molecule, such as ICAM-1 (Altmann *et al.*, 1989), by SF non-T cells; and (ii) that the lack of a corresponding SF T cell response is due to reduced numbers of $2H4^+$ $CD4^+$ T cells within the synovial compartment (Pitzalis *et al.*, 1987), since members of this T cell subset have been shown to be the principal responders in autologous mixed lymphocyte responses (Morimoto *et al.* 1986) and the major interleukin-2 (IL-2) producing subset (Salmon *et al.*, 1988). It is possible that IL-2 secreted by $2H4^+$ $CD4^+$ peripheral blood cells in response to autologous SF non-T cells is utilized by activated antigen-specific $2H4^-$ $CD4^+$ T cells in the peripheral blood T cell population. This may explain the increased peripheral blood cell responses to antigen compared with those of SF T cells observed in some patients.

We have shown that SF APC are potent activators of peripheral blood T cells, eliciting 'autoreactive' responses and unmasking responses to bacterial antigens which were not apparent in unfractionated PBMC. We conclude that the contrasting responses of SFMC and PBMC to bacterial antigens may be accounted for at least in part by an enhanced ability of SF APC to support T cell proliferative responses. Thus, APC within the synovial compartment may play an important role in sustaining the local inflammatory response by activating T cells which traffic into the synovium.

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