

## **Engagement of Major Histocompatibility Complex Class II Molecules by Superantigen Induces Inflammatory Cytokine Gene Expression in Human Rheumatoid Fibroblast-like Synoviocytes**

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### **Summary**

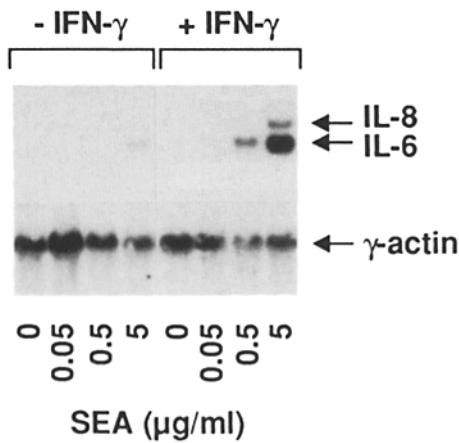
Cells in the rheumatoid synovium express high levels of major histocompatibility complex (MHC) class II molecules *in vivo*. We have therefore examined the ability of engagement of MHC class II molecules by the superantigen Staphylococcal enterotoxin A (SEA) to activate interleukin 6 (IL-6) and IL-8 gene expression in type B synoviocytes isolated from patients with rheumatoid arthritis. SEA had a minimal or undetectable effect on the expression of either gene in resting synoviocytes, as determined by Northern blot and specific enzyme-linked immunosorbent assay. However, induction of MHC class II molecule expression after treatment of synoviocytes with interferon  $\gamma$  (IFN- $\gamma$ ) enabled the cells to respond to SEA in a dose-dependent manner, resulting in an increase in both the level of steady-state mRNA for IL-6 and IL-8, and the release of these cytokines into the supernatant. IFN- $\gamma$  by itself had no effect on the expression of either cytokine. Pretreatment of the cells with the transcription inhibitor actinomycin D prevented the increase in cytokine mRNA induced by SEA, whereas cycloheximide superinduced mRNA for both cytokines after stimulation by SEA. Taken together, these results indicate that signaling through MHC class II molecules may represent a novel mechanism by which inflammatory cytokine production is regulated in type B rheumatoid synoviocytes, and potentially provides insight into the manner by which superantigens may initiate and/or propagate autoimmune diseases.

Rheumatoid arthritis (RA) is a chronic articular inflammatory disorder in which the synovial environment is characterized by intense immunological activity (1). In particular, the macrophage-like (type A) and fibroblast-like (type B) synoviocytes of the hyperplastic lining layer exhibit an activated phenotype. Both cell types express a high level of MHC class II molecules (1), which are involved in the initial step of the immune response (2), and these cells are a major source of several inflammatory cytokines, which also are believed to play a crucial role in the pathogenesis of RA (1).

Using superantigens such as Staphylococcal enterotoxins A or B (SEA or SEB), toxic shock syndrome toxin 1 (TSST-1), or mAbs that bind to various epitopes on the MHC class II molecule (3, 4), a number of reports have addressed the possibility that upon engagement, MHC class II molecules on APC (B cells and monocytes) may act as signal transduction molecules. For example, activation of B cells with an anti-MHC class II antibody stimulated translocation of pro-

tein kinase C (PKC) and a rise in intracellular cAMP levels (5). Binding of TSST-1 to MHC class II molecules also triggers human B cell proliferation and differentiation via MHC class II-unrestricted cognate T-B cell interaction (6). We have also reported that TSST-1 induces sustained LFA-1-dependent adhesion of MHC class II-positive cells (7) and delivers a mitogenic signal to human B cells primed with PMA or anti-IgM (8). In addition, it has recently been reported that treatment of human monocytes and monocytic cell lines with TSST-1 and SEB induces an upregulation of the genes for IL-1 and TNF- $\alpha$  (9).

In view of the potentially important role of IL-6 and IL-8 in the pathogenesis of rheumatoid arthritis (1), and of their immunological effects *in vitro* and *in vivo*, we have characterized the ability of engagement of MHC class II molecules on synoviocytes to modulate the expression of these two cytokines. Our results indicate that the interaction of SEA with MHC class II molecules on synoviocytes results in the



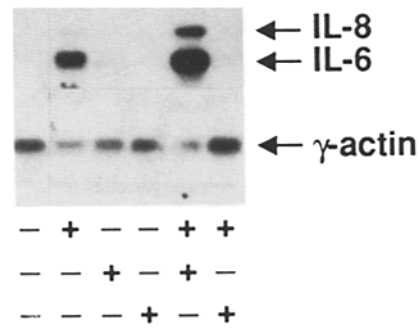
**Figure 1.** The effect of SEA on IL-6 and IL-8 mRNA levels in human rheumatoid synoviocytes. Subconfluent synoviocytes were incubated for 48 h with 200 U/ml IFN- $\gamma$  or its diluent followed by increasing concentrations of SEA for a further 6 h. The cells were then harvested, cytoplasmic RNA was purified, and Northern blots were performed to detect mRNA for IL-6 and IL-8. The filters were also hybridized with a  $\gamma$ -actin probe as a control. This autoradiogram is from one experiment, which is representative of three others performed with similar results.

upregulation of IL-6 and IL-8 gene transcription and protein secretion, and this regulation occurs independently of prior protein synthesis.

### Materials and Methods

**Reagents.** SEA and SEB were purchased from Toxin Technology (Madison, WI). No LPS was detected when the preparation was tested by the *Limulus amoebocyte* Lysate (LAL) test (sensitivity, <1 ng/ml). mAb L243, directed against a monomorphic determinant on human HLA-DR molecules, was generated from hybridoma cells (American Type Culture Collection, Bethesda, MD). Human rIFN- $\gamma$  was obtained from Amgen Biologicals (Thousand Oaks, CA).

**Synoviocyte Culture.** The synoviocyte cultures used in this study were derived from different patients with rheumatoid arthritis undergoing total knee replacement due to joint deterioration essen-

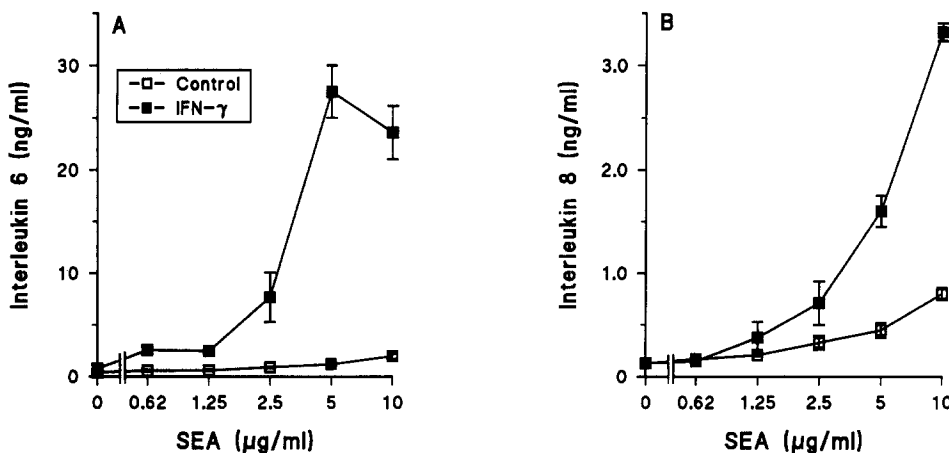


**Figure 2.** The effect of actinomycin D and cycloheximide on SEA-induced IL-6 and IL-8 mRNA in human rheumatoid synoviocytes. Subconfluent synoviocytes were pretreated with IFN- $\gamma$  for 48 h, followed by a 1-h treatment with either cycloheximide (10  $\mu$ g/ml) or actinomycin D (1  $\mu$ g/ml) before being stimulated for 6 h with SEA (2.5  $\mu$ g/ml). The cells were then harvested, cytoplasmic RNA was purified and Northern blots were performed as described in the legend to Fig. 1. This autoradiogram is from one experiment, which is representative of three others performed with similar results.

tially as previously described (10). All patients were diagnosed as suffering from either definite or classical RA according to the American Rheumatism Association 1987 criteria (11). Cells used during this study were obtained after four to eight passages, morphologically, resembled the previously reported "fibroblast-like" synoviocytes, and were Mo-1 and MHC class II negative, the latter indicating the absence of type A or "macrophage-like" synoviocytes. The cells were cultured in DMEM containing 10% FCS.

**ELISAs for IL-6 and IL-8.** Synoviocytes were grown in 96-well microtiter plates and treated as described in the figure legends. IL-6 was measured using a standard sandwich ELISA purchased as a kit from R&D Systems (Minneapolis, MN). IL-8 levels were determined using a sandwich ELISA developed at Genentech Inc. (South San Francisco, CA). All experiments were performed in triplicate and repeated on at least three occasions on different cultures.

**Northern Blot.** The synoviocytes were grown in tissue culture flasks and treated as described in the figure legends. Cytoplasmic RNA was purified using a previously described method (12). 10  $\mu$ g of RNA was loaded onto 1% agarose gels. Equal loading and RNA integrity were confirmed by ethidium bromide staining. The



**Figure 3.** The effect of SEA on the synthesis of IL-6 and IL-8 by human rheumatoid synoviocytes. Subconfluent synoviocytes grown in 96-well microtiter plates were incubated as described in the legend to Fig. 1 and stimulated with increasing concentrations of SEA for 12 h. The supernatants were then collected and analyzed for the presence of immunoreactive IL-6 and IL-8. Results are the mean  $\pm$  SD of triplicate determinations from one representative experiment.

RNA was then transferred onto Hybond-N filter paper and hybridized with random primer-labeled cDNA probes for IL-6 and IL-8. The human IL-8 cDNA probe was a 244-bp fragment representing the coding region of the IL-8 cDNA from nucleotides 49–293 (13). A 1.2-kb human IL-6 cDNA probe was a generous gift from the Genetics Institute (Cambridge, MA). Equal loading of RNA was also confirmed by hybridization with a  $\gamma$ -actin cDNA probe (a generous gift from Dr. J. Thibodeau, CHUL Research Center, Quebec, Canada). All washes were performed under stringent conditions. The mRNA hybridizing with the two cytokine cDNA probes was visualized by autoradiography.

## Results and Discussion

Subconfluent human rheumatoid synoviocytes were pretreated with either 200 U/ml IFN- $\gamma$  or its diluent (0.1% BSA) for 48 h. Standard FACS<sup>®</sup> analysis for the surface expression of MHC class II molecules using a mAb directed against HLA-DR revealed maximal induction of MHC class II antigens under these conditions, as had previously been reported (14). This treatment has no detectable effect on cell proliferation (14). After preincubation with IFN- $\gamma$  or its diluent, the cells were treated with increasing concentrations of SEA for a further 6 h, and the level of steady-state mRNA for IL-6 and IL-8 was determined (Fig. 1). More detailed time course studies revealed maximal induction of mRNA at this time point (data not shown). No mRNA for IL-8 was detected in response to SEA when the cells were not pretreated with INF- $\gamma$ . In contrast, a low level of IL-6 mRNA was detected in non-IFN- $\gamma$ -treated synoviocytes when the cells were incubated with 5  $\mu$ g/ml of SEA, a result supporting the fact that a minor expression of MHC class II was observed in resting synoviocytes. However, after treatment with INF- $\gamma$ , a dose-dependent stimulation by SEA of the level of mRNA for both IL-6 and IL-8 was observed. The failure of SEA to induce a significant response in untreated synoviocytes supports the hypothesis that MHC class II molecule expression is required for cellular activation by SEA. This conclusion is further supported by the fact that stimulation of INF- $\gamma$ -treated synoviocytes with the anti-MHC class II mAb L243 or with a related enterotoxin (SEB) induced the same response (McCull et al., manuscript in preparation).

Studies using the transcription inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide were also conducted in order to determine the level at which SEA was activating the cytokine genes. The cells were therefore pretreated with IFN- $\gamma$  for 48 h, followed by a further incubation with either actinomycin D, cycloheximide, or diluent. They were then incubated with either SEA or diluent for 6 h and the cells were harvested and cytoplasmic RNA was purified for Northern blot analysis (Fig. 2). No mRNA for either IL-6 or IL-8 was observed in the synoviocytes that were not treated with SEA. In addition, actinomycin D completely inhibited the induction of mRNA by SEA, whereas cyclo-

heximide superinduced the mRNA for both IL-6 and IL-8 when the cells were stimulated with SEA. Taken together, these results indicate that SEA activates IL-6 and IL-8 gene expression by stimulating transcription of the respective genes for these two cytokines and suggest that translation of the mRNA is also occurring.

To confirm the latter point, we conducted experiments to determine the level of secretion of IL-6 and IL-8 in response to SEA. Synoviocytes were therefore plated into 96-well microtiter plates and pretreated for 48 h with either IFN- $\gamma$  or its diluent. They were then stimulated with increasing concentrations of SEA for 12 h. The supernatants were then collected and analyzed for IL-6 and IL-8 content by specific ELISA (Fig. 3). Very little release of either IL-6 or IL-8 was observed in the non-IFN- $\gamma$ -treated synoviocytes. In contrast, SEA stimulated a dose-dependent release of both IL-6 and IL-8 when the synoviocytes were pretreated with IFN- $\gamma$ . A consistent stimulatory effect of SEA was first observed at 2.5  $\mu$ g/ml, and increased to be maximal at between 5 and 10  $\mu$ g/ml for IL-6 release, whereas IL-8 release was still increasing with 10  $\mu$ g/ml of SEA, the highest concentration used in these experiments. Pretreatment of the cells with either cycloheximide or actinomycin D inhibited the release of both IL-6 and IL-8 in SEA-stimulated fibroblasts (data not shown).

In the present study, incubation of rheumatoid synoviocytes with SEA, a specific MHC class II ligand, had little or no detectable effect in resting cells. However, induction of MHC class II molecule expression on the synoviocyte surface rendered them responsive to activation by SEA, leading to upregulation of the genes for IL-6 and IL-8. These results provide evidence for a role of MHC class II molecules in mediating intracellular signaling events. In addition, the fact that cytokine gene expression in rheumatoid synoviocytes was upregulated in response to MHC class II receptor ligation indicates that the latter molecules could play an important role in amplifying the inflammatory cycle in RA. Recent information suggests that the modulation of IL-6 and IL-8 gene expression by MHC class II molecules is likely to occur in vivo. Firstly, RA is a disease in which the cells of the synovium exhibit a high level of MHC class II molecule expression (1). Second, recent studies have indicated that molecules that could activate MHC class II-mediated signal transduction events may also be present in the synovial environment. For instance, the presence of a superantigen that is responsible for the V $\beta$ 14 T cell oligoclonality observed in RA has recently been reported (15). In addition, antibodies directed against the Epstein-Barr virus gp110 glycoprotein, which also recognize specific sequences in the  $\beta$  chain of HLA-DR4 MHC class II molecules, have been found in patients with RA (16). Therefore, engagement of MHC class II molecules may represent an important mechanism by which cytokine gene expression is controlled in the rheumatoid synovium.

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