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CYCLOSPORIN A BLOCKS INDUCTION OF TUMOR NECROSIS FACTOR-alpha IN HUMAN B LYMPHOCYTES

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The effects of cyclosporin A (CSP) on tumor necrosis factor-alpha (TNF-α) RNA levels and protein production in human B cells and a B cell line were studied. The ability of CSP to block induction of RNA was compared to its inhibition of protein production in response to anti-IgM, phorbol ester (PMA) and platelet-activating factor (PAF). PAF is a phospholipid which has recently been found to activate human B cells. CSP blocks PAF-induced TNF-α RNA from the Ramos cell line, as well as inhibiting the enhancement of TNF protein production from both freshly isolated and Ramos B cells. CSP also blocks anti-Ig induced TNF-α RNA and protein but does not inhibit PMA-induced TNF-α. We conclude that B cells, like T cells, have CSP-independent and CSP-dependent signaling pathways and that PAF signaling is dependent upon a CSP-sensitive factor.

Since its discovery in 1976 as a lymphoid specific immunosuppressant, cyclosporin A (CSP) has become a mainstay of immunosuppressive therapy in preventing transplant rejection and treating autoimmune disease (1-3). In addition to its clinical usefulness, CSP has been used as a powerful tool to dissect signaling pathways in lymphocytes.

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For example, in T cells, anti-TCR-induced IL-2 production is blocked at the transcriptional level by CSP, whereas anti-CD28 signaling is relatively resistant to CSP. Some of the cellular targets and transcription factors impacted by CSP have been identified and have helped to differentiate the signaling mechanisms of various agonists (reviewed in 4). Although much attention has focused on the effects of CSP on T lymphocytes, CSP is also known to regulate B cell functions (5). Similar to what has been observed in T cells, CSP impacts most those B cell signaling pathways that are Ca^{2+}-dependent , such as anti-IgM and SAC (4,5).

Recently it has been reported that CSP blocks induction of the cytokine tumor necrosis factor-alpha (TNF-α) at the transcriptional level in both T and B lymphocytes (6-8). Anti-IgM-induced TNF-α RNA induction was CSP-sensitive in both tonsillar B cells and human B cell lines, whereas PMA-induced TNF-α was far less sensitive to CSP (6,7). Less is known about the effects of CSP on TNF-α protein production from B cells. We have undertaken to study the effects of CSP on TNF-α RNA levels and protein production after treatment of B cells with various agonists, including PAF, a phospholipid. Our laboratory and others have previously reported that PAF acts on B cells to generate a number of second messengers and increase the transcription of oncogenes, NF-kB, and TNF-α (9-14). In order to help delineate the signaling pathway of PAF in B cells we investigated the ability of CSP to block PAF-induced TNF-α RNA and protein production. Our goals were to compare the effects of CSP on TNF-α RNA and protein in B cells, and to assess the sensitivity of PAF-induced signaling to CSP.

Materials and Methods

Reagents. The following reagents were obtained from the listed sources: PAF (1-O-Hexadecyl-2-acetyl-3-glycerophosphocholine), lyso-PAF (1-O-Hexadecyl-sn-glycero-3-phosphorylcholine) (BIOMOL, Plymouth Meeting, PA), Anti-IgM (Cappel, Durham,NC), PMA (Calbiochem, La Jolla, CA), IL-2 (R&D Systems, Minneapolis, MN), cyclosporin A (TCH pharmacy, Houston, TX) RPMI 1640 and FCS (Gibco, Grand Island, NY), RNAzol B (Biotecx, Houston, TX), alpha-[^{32}P]- dCTP (DuPont NEN, Boston, MA), HB101 serum-free lymphocyte activation media (Irvine Scientific, Santa Ana, CA), anti-CD19 Dynabeads and Detach-a Bead (Dynal Inc., Great Neck, NY).
Cells. The cell line Ramos is an EBV-negative lymphoblastoid line derived from a Burkitt lymphoma. (ATCC, Rockville, MD). Peripheral B cells were isolated from leukopaks from normal donors (Gulf Coast Regional Blood Center, Houston, TX) by positive selection with anti-CD19 coated Dynabeads according to the manufacturer's instructions (15,16) as previously described (14).

Northern blots. Total RNA was isolated as previously described (13,14), size-fractionated on a 1% agarose gel and blotted onto Hybond-nylon membranes or directly slot-blotted onto membranes. All membranes were baked for 2 hours and hybridized to a ^{32}P-labelled TNF-alpha or GAPDH probe as described (14). The cDNA probe for G3PDH was obtained from ATCC (Rockville, MD) and the TNF-α probe from the natural sequence of TNF-α cloned into a pUC19 plasmid (R & D Systems, Minneapolis, MN). The size of the TNF-α transcript was 1.7 kB. Densitometry was performed using an LKB soft light densitometer and absorbance normalized to binding to GAPDH controls.

TNF -α and IL-6 Protein Determination. Cells were resuspended to 1 x 10^6 cells/mL and the appropriate reagents added. Immunoreactive TNF-α or IL-6 were quantitated using a sandwich-based ELISA kit (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. Triplicate samples were assayed in duplicate.

Results and Discussion

We have previously reported that PAF treatment alone upregulates TNF-α RNA levels in the human B cell line Ramos (14). Here we show that PAF-induced TNF-α RNA is blocked by increasing concentrations of CSP from 0.5 to 5 ug/mL, whereas PMA-induced TNF-α RNA is not (Fig. 1A). Induction of TNF-α in B cells is very rapid, with peak levels occurring within 2 hours in response to PAF in Ramos cells, and detectable levels by 1 hour in response to PMA or anti-IgM in peripheral B cells (data not shown). Increasing amounts of CSP, up to 5 ug/mL, did not inhibit PMA-induced TNF-α in Ramos cells in the 1.5 hour RNA experiments, however doses of 0.5 to 1.0 µg/mL appeared efficacious in blocking up to 90% of PAF-induced TNF-α RNA (Fig. 1A). We also found 1 µg/mL CSP to be effective in blocking anti-IgM-induced TNF-α in peripheral B cells by 60-90%, but again, PMA-induced TNF-α RNA was less sensitive to CSP (Fig. 1B). Goldfield *et al.* (7) previously reported that 1 µM CSP (1.2 µg/mL) blocked TNF-α RNA in B cells and B cell lines in response to anti-Ig, which agrees well with our findings, however, they did not assess its effect on PMA-induced TNF-α RNA. We do not detect TNF-α RNA after treatment of normal peripheral B cells with PAF alone, probably due to a lower level of receptor expression (15,16).

We next investigated the effects of CSP on TNF-α protein production from B cells. We have previously shown that PAF can enhance the secretion of TNF-α protein from Ramos cells and peripheral B cells in the presence of PMA (14). When Ramos cells were treated with 0.5 µg/mL CSP in the presence of PAF + PMA, TNF-α secretion was inhibited to the level of PMA treatment alone (Fig. 2). This effect was

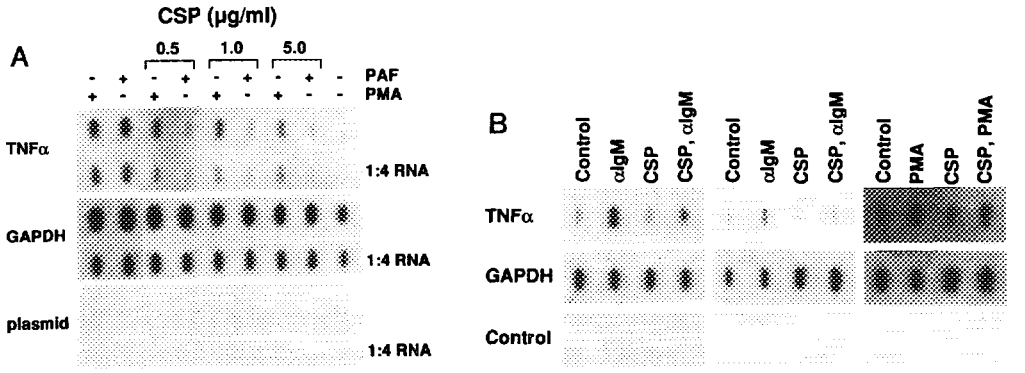

Figure 1. CSP blocks PAF and anti-IgM, but not PMA-induced TNF-α RNA.
(A) 30 x 10^6 Ramos cells were treated with CSP at the indicated concentrations for 15 min., then PMA (0.05 µM) or PAF (0.1 µM) was added. Cells were harvested at 1.5 hours, RNA was prepared and slot-blotted as described and blots were probed with TNF-α probe, a GAPDH control and a plasmid to control for nonspecific binding. The top row for each probe shows 5 µg of total RNA and the lower row a 1:4 dilution of the same RNA. CSP blocked PAF-induced RNA by 60, 75 and 90% at CSP 0.5, 1, and 5 µg/mL respectively, as assessed by densitometry. Represents one of four experiments. (B) Peripheral B cells were isolated as described and treated with the reagents indicated, anti-IgM (15 µg/ml), PMA (0.05 µM) and CSP (1 µg/mL) for 1.5 hours. 10-30 x 10^6 cells were used in each condition, RNA was prepared and 3 µg blotted as above. Each vertical panel represents one donor's B cells. CSP blocked anti-IgM induced-TNF-α 60% and 90% as assessed by densitometry. This figure represents three donors: similar results were obtained from two other donors for both anti-IgM and PMA treatment, including one experiment where all conditions utilized the same donor.

observed when CSP was included throughout the 18 hour culture as well as when the cells were pre-treated with CSP for 30 minutes, then washed and cultured for 18 hours. PAF alone induced comparatively little TNF-α from these cells (50 pg/mL) but this also was inhibited by CSP treatment (Fig. 2). In peripheral B cells we examined the effect of CSP on PMA-treated cells and PMA+PAF treated cells. As with the Ramos cells, CSP blocked the enhancement of TNF-α production seen with PAF (Fig 3A). This effect was dose-dependent from .1 to 1 µg/mL CSP (data not shown). As seen in Fig. 3A, 0.5 µg/mL CSP also seemed to partially block PMA-induced TNF-α secretion, up to 20% (Fig. 3A). Higher doses of CSP from 5 µg/mL to 10 µg/mL significantly inhibited PMA-induced TNF-α protein (up to 40%), suggesting that high dose CSP may be affecting this pathway as well (data not shown). Importantly, CSP did not appear to cause cell death in the 18 hour cultures as assessed by trypan blue exclusion at concentrations up to 5 µg/mL (data not shown). Interestingly, Sung *et al.*

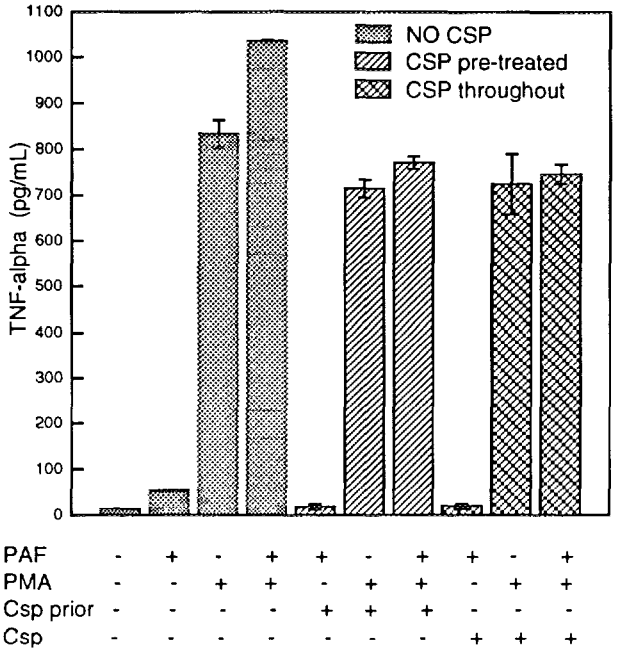

Figure 2. CSP blocks PAF-induced enhancement of TNF-α production from Ramos B cells. 1 x 10^6 cells/mL were incubated with the reagents indicated for 18 hours; PAF (0.1 μM) PMA (0.05 μM) and CSP (0.5 μg/mL). Supernatants were harvested and assayed for TNF-α as described. CSP was either added to the cells 10 min. before the other reagents or the cells were treated with CSP for 30 min., washed, and the other reagents added. Represents one of three similar experiments. Cells that were treated with CSP and PMA+PAF were significantly different than those without CSP, but not different than those treated with PMA alone ($p<0.5$).

(6) report that 5 μg/mL CSP did not block TNF-α RNA in tonsillar B cells after PMA treatment but they did not assess protein production from these cells. We also assessed the effect of CSP on TNF-α production from peripheral B cells after treatment with other B cell mitogens and cytokines. CSP blocked TNF-α production from B cells treated with SAC, anti-IgM, SAC+IL-2 and anti-IgM+BCGF (Fig 3B). We also found that CSP blocked IL-6 production from cells treated with PMA+PAF as well as other mitogens, in a manner similar to its inhibition of TNF-α production (data not shown).

These data indicate that B cell signaling pathways, like those in T cells, can be broadly divided into CSP-sensitive and CSP-insensitive. Furthermore, the effects of CSP on transcription of the TNF-α gene appear to correlate well with the effects on

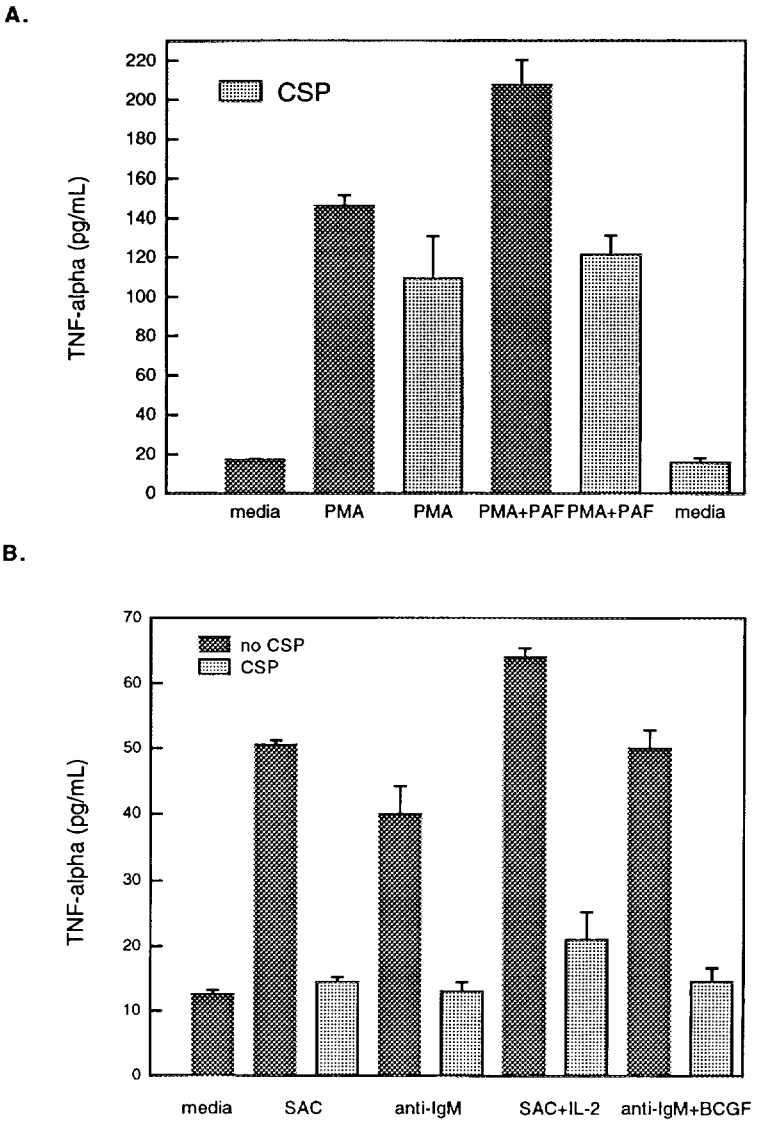

Figure 3. CSP blocks PAF-, anti-IgM- and SAC-, but not PMA-induced TNF protein production from peripheral B cells. (A) 1 x 10^6 cells/mL were incubated with the reagents indicated for 18 hours; PAF (0.1 μM) PMA (0.05 μM) and CSP (0.5 μg/mL). Supernatants were harvested and assayed for TNF-α as described. Cells treated with PAF alone produced 32 pg/mL TNF-α. Representative of three experiments. CSP significantly decreased TNF-α production from cells treated with PMA+PAF ($p<0.5$). (B) Concentration of cells was as before with SAC (0.1%), IL-2 (40 ng/mL), and BCGF (1:10,000). Representative of two experiments.

protein production of TNF-α, especially at low CSP concentrations. At high concentrations, we find some inhibition of PMA-induced TNF-α, suggesting that CSP may impact this signal in some way. Of interest, Goldfield *et al.* (8,17) have identified a

CSP-sensitive regulatory region within the TNF-α promoter whose binding factor resembles NFAT, is dependent upon calcineurin, and is blocked by CSP, implicating this transcription factor in the CSP responsiveness. These authors also report that calcineurin mediates TNF-α induction in a B cell line, though the mechanism is unknown (17,18). Additionally, Rieckmann *et al.* and Xia *et al.* (19,20) have reported that inhibition of cellular phosphatases with okadaic acid induces TNF-α RNA. The sensitivity of this pathway to CSP is not known, however, given the relative insensitivity of direct protein kinase C activation by PMA, inhibition of phosphatases activity may be similarly resistant.

Most importantly, our data indicate that PAF-mediated TNF-α production is sensitive to the effects of CSP in human B cells. CSP blocks both RNA increases and PAF-induced protein production, making it a physiologically relevant modulator of B cell function. Additionally these data aid in the description of the PAF-signaling mechanism. PAF has been reported to cause Ca^{2+} flux in human B cell lines and activation of MAP-kinases and tyrosine kinases, including PLC-gamma 1 (9,10,12). Our results implicate CSP-sensitive transcription factors in the PAF-induced increases in TNF-α RNA and further underscore the utility of CSP as an agent to study signaling pathways in B cells.

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