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Immunoregulatory Effects of Fas-Mediated Signalling

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During the past 2–3 years tremendous progress has been made in understanding the molecular and cellular basis for the autoimmune disease process in mice homozygous for either the *lpr* or *gld* genes. It is characterized by the accumulation of large numbers of lymphocytes with an aberrant cell surface phenotype ($CD3^+$, $CD4^-$, $CD8^-$, $B220^+$), lymphadenopathy, hypergammaglobulinemia, the production of antibodies to both single-stranded, and double-stranded DNA, rheumatoid factor, and glomerulonephritis (reviewed in 1). Mice homozygous for the *gld* mutation develop an autoimmune disease that is virtually identical to that observed in *lpr/lpr* mice (also reviewed in 1). However, genetic studies have clearly demonstrated that the *gld* and *lpr* mutations are distinct since they map to independently segregating chromosomes [2–5].

Of seminal importance in understanding the cellular basis for this disease process was the finding that the *lpr* locus contains a mutated form of the gene for the Fas/APO-1 protein [6], a member of a family of cell surface receptors related to TNFR and NGFR [7]. In *lpr/lpr* mice, insertion of an early transposable element into the second intron of the gene encoding the Fas protein leads to premature termination and aberrant splicing of Fas mRNA transcripts [8,9]. Interestingly, bone marrow transplantation studies suggested that the products of the *lpr* and *gld* genes were an interacting receptor-ligand pair [10]. The hypothesis that the *gld* phenotype is due to a mutation in the ligand for the Fas receptor has been confirmed by both functional assays [11,12] and direct molecular cloning and sequencing of the FasL gene from *gld* mice [13,14]. Thus, a defect in expression of either the Fas receptor or its cognate ligand can lead to the generation of autoimmune disease and the progressive accumulation of large numbers of lymphocytes in peripheral lymphoid tissue. However, the precise role played by Fas-mediated signals in regulating normal immune responses in vivo, and the mechanism(s) by which these signals are mediated, has not been established. These questions led us to begin a series of investigations designed to determine some of the biological properties of Fas/FasL expression and function.

The Fas protein was originally identified by monoclonal antibodies (mAb) that induced the rapid death of certain transformed target cells via apoptosis [15,16]. It is interesting to note, however, that whereas Fas is expressed by a wide variety of cells within freshly isolated peripheral blood (including activated and memory T cells, B cells, monocytes and neutrophils), these cell types do not undergo apoptosis when cultured with Fas-specific mAb [17]. Indeed, recent studies have shown that peripheral blood T cells (PBT) only become susceptible to Fas-mediated apoptosis following 5–6 days of stimulation in vitro [18,19]. These data indicate that induction of apoptosis via Fas is dependent upon the activation state of the cell. In fact, when viewed in a more global context, it seems likely that the consequences of signalling via the Fas receptor may be much more complicated than was originally envisioned.

BIOLOGICAL ASPECTS OF FasL EXPRESSION AND FUNCTION

Because most of the recently identified ligands for other members of the TNFR family (TNF, CD27, CD30, CD40, and 4-1BB) have been found to be type II integral membrane proteins [20–24], it was widely hypothesized that the ligand for Fas would also be membrane-bound. This was supported by the findings of Rouvier et al., who reported that the Ca^{2+}-independent component of cytotoxic activity mediated by either $H2^d$-specific peritoneal exudate

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lymphocytes or a PMA and ionomycin activated T cell hybridoma (PC60-d10S) lysed Fas positive, but not Fas negative, target cells [25]. Their findings suggested that this lytic activity might be mediated by a ligand for Fas.

After an extensive examination of a wide range of human and murine cell lines for cell surface expression of FasL, low-level binding of huFas.Fc to a long-term $CD8^+$ murine anti-tumor CTL line (B10-anti-B10.5; [26], was detected (Fig. 1). The CTL line also bound TNFR.Fc after stimulation with PMA and ionomycin, but not huIL-4R.Fc. These cells also failed to bind CD27.Fc, 4-1BB.Fc, CD40.Fc, huCD69.Fc, muOX40.Fc, or HSA.Fc (data not shown). Since unstimulated CTL did not bind detectable levels of huFas.Fc, we evaluated the kinetics of expression of the putative FasL after stimulation with PMA and ionomycin. A low but detectable increase in the mean fluorescence intensity (MFI) of cells stained with huFas.Fc was observed within 30 min of stimulation (Fig. 2A). The signal intensity rapidly increased with time, reaching maximal levels by 2 h. Expression of TNF by these cells, as detected using the huTNFR.Fc fusion protein, followed a similar kinetic profile although the magnitude of the signal was substantially greater than that observed with huFas.Fc (Fig. 2B). At no time was binding of huIL-4R.Fc to the activated CTL detected. Finally, expression of FasL by the CTL after stimulation was almost completely inhibited by inclusion of cycloheximide (10 μg/ml) into the culture medium during the stimulation period (Fig. 3). These results demonstrate that expression of FasL by cells after stimulation requires de novo protein synthesis.

Incubation of certain transformed cell lines (such as Jurkat) with the prototypic Fas-specific mAb CH-11 results in the apoptotic death of those target cells. We have recently generated a number of huFas-specific mAb that are able to completely inhibit this process in vitro [27]. Jurkat target cells were also found to be lysed by stimulated CTL, and this process was completely inhibited by huFas-specific mAb that abrogated Fas-mediated target cell lysis by the prototypic CH-11 mAb (Fig. 4A). The characteristic DNA laddering pattern associated with Fas-mediated apoptosis of Jurkat target cells was also induced by the activated CTL and was also

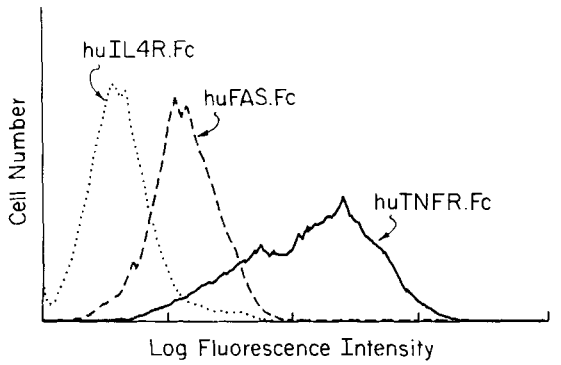

Fig. 1. Flow cytometric detection of FasL on a CTL line stimulated with phorbol ester and calcium ionophore. The CTL were stimulated for 2 h with PMA and ionomycin, and then stained with either huFas.Fc, TNFR.Fc, or huIL-4R.Fc followed by a biotinylated anti-hulgG1 and streptavidin-PE in a three-step staining protocol (data from reference 27).

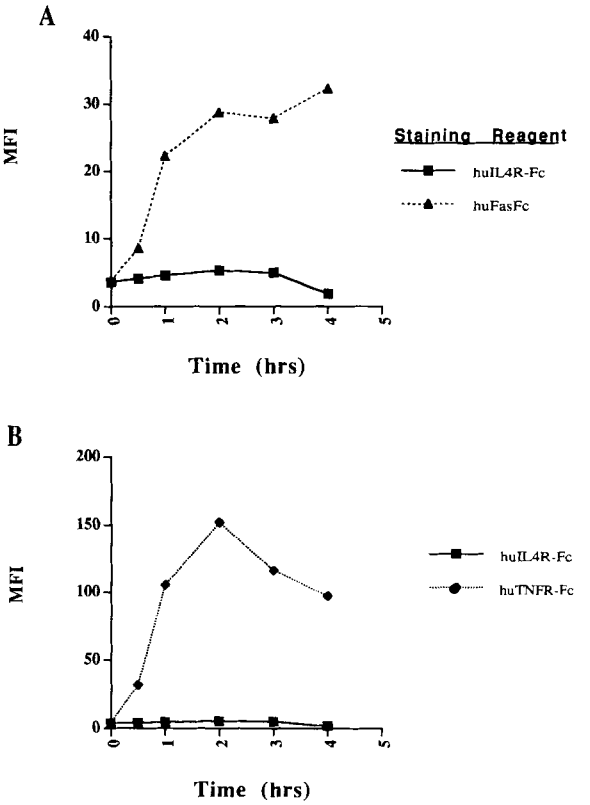

Fig. 2. Kinetics of expression of FasL on a CTL line after stimulation with phorbol ester and calcium ionophore. The CTL were stimulated with PMA and ionomycin for either 4, 3, 2, 1, 0.5, or 0.25 h prior to staining with huFas.Fc or TNFR.Fc. Unstimulated CTL served as a control. All cells were then stained with either huFas.Fc or TNFR.Fc using a three-step staining protocol and analyzed on a FACScan. MFI stimulated CTL stained with either huFas.Fc (▲, **A**) or huTNFR.Fc (◆, **B**) compared to huIL-4R.Fc (■) is plotted as a function of time of stimulation in culture.

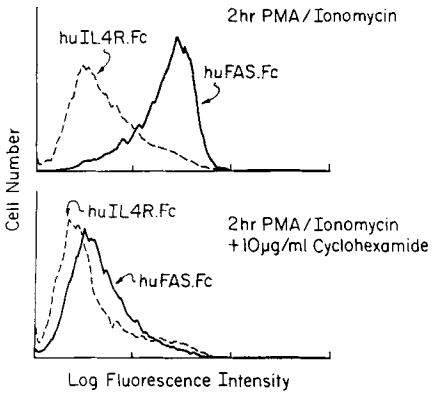

Fig. 3. Expression of FasL requires de novo protein synthesis. CTL were cultured for 30 minutes in either medium alone (A) or in medium containing cycloheximide (10 μg/ml, B) prior to stimulation with PMA and ionomycin. Following a 2-h stimulation period, cells were stained with either huIL-4R.Fc. or huFas.Fc using a three-step staining protocol, and analyzed on a FACScan.

completely abrogated by addition of the huFas M3 mAb to the bioassay (data not shown). Addition of a nonantagonistic huFas-specific mAb (such as huFas M31) failed to inhibit lysis by the activated CTL, thereby demonstrating the epitope specificity of the inhibitory effect of these mAb. Finally, CTL-mediated lysis of target cells was completely inhibited by the huFas.Fc fusion protein but not by huIL-4R.Fc, huTNFR.Fc, or CD30.Fc (Fig. 4B).

DIFFERENTIAL EXPRESSION OF FasL BY MURINE T_h1 AND T_h2 CELLS

Preliminary studies indicated that FasL could be expressed by both $CD4^+$ and $CD8^+$ T cells. In the course of examining a variety of cell types for the expression of functional FasL, we found that T_h1 but not T_h2 T cell clones were able to lyse Jurkat cells efficiently following stimulation with either PMA and ionomycin (Fig. 5A,B) or anti-TCR-α/β (data not shown). Confirmation that the mechanism was mediated through Fas was demonstrated by the ability of the huFas-specific M3 antibody to block cytolysis completely. Flow cytometric analysis showed that T_h1 clones, but not T_h2 clones, expressed significant levels of FasL as determined by Fas.Fc binding after stimulation with PMA and ionomycin (Fig. 5C,D). Interestingly, the expression of surface TNF, as detected by the binding of TNFR.Fc, was coordinately regulated with FasL (Fig. 5E,F).

ACTIVATION-INDUCED CELL DEATH IN T_h1 AND T_h2 CELLS IS LINKED TO FasL EXPRESSION

The ability of T cells to undergo activation-induced cell death (AICD) has been correlated with the expression of Fas and FasL in both murine [28] and human system [29]. Because T_h2 cells expressed low amounts of FasL, we hypothesized that these cells would not undergo AICD following direct TcR stimulation in the absence of antigen presenting cells. To test this hypothesis, T cell clones were cultured in the presence of either IL-7 alone or IL-7 and immobilized antibody to TcR-α/β for 18 h, harvested, stained with propidium iodide and Hoechst 33342, and the number of apoptotic cells quantitated by multiparameter flow cytometric analysis as described by Dive et al. [30]. A small fraction of cells were apoptotic even when cultured in the absence of TcR stimulation; however, this proportion increased dramatically in stimulated T_h1, but not T_h2 clones (Fig. 6).

Although the inability of T_h2 cells to undergo AICD after TcR ligation correlated with their inability to express significant levels of FasL, the possibility that they were insensitive to Fas-mediated lysis also existed. To address this possibility, T_h1 clones were labeled with PKH-2, a vital dye that can be detected by flow cytometry without interference from propidium iodide or Hoechst 33342. The labeled T_h1 cells and unlabeled T_h2 cells were then cultured separately or together and AICD induced by ligation of the TcR. After 24 hours in culture, T_h1 and T_h2 clones could be distinguished from one another by PKH-2 fluorescence. When stimulated separately, a large percentage of the T_h1 cells were apoptotic, and this was largely inhibited by inclusion of the Fas.Fc fusion protein into the culture medium (Fig. 7). In contrast, very few of the T_h2 cells were apoptotic. Mixing of the T_h1 and T_h2 cells resulted in an increase in the number of apoptotic T_h2 cells, and this was also significantly inhibited by the Fas.Fc fusion protein. Thus, the data indicate that the profound difference in the ability of T_h1 and T_h2 cells to express FasL is a principal basis for the relative abilities of these two cell types to undergo AICD.

Fas-MEDIATED COSTIMULATION OF T CELLS

The process of AICD is believed to play a critical role in controlling T cell expansion and preventing the accumulation of autoreactive

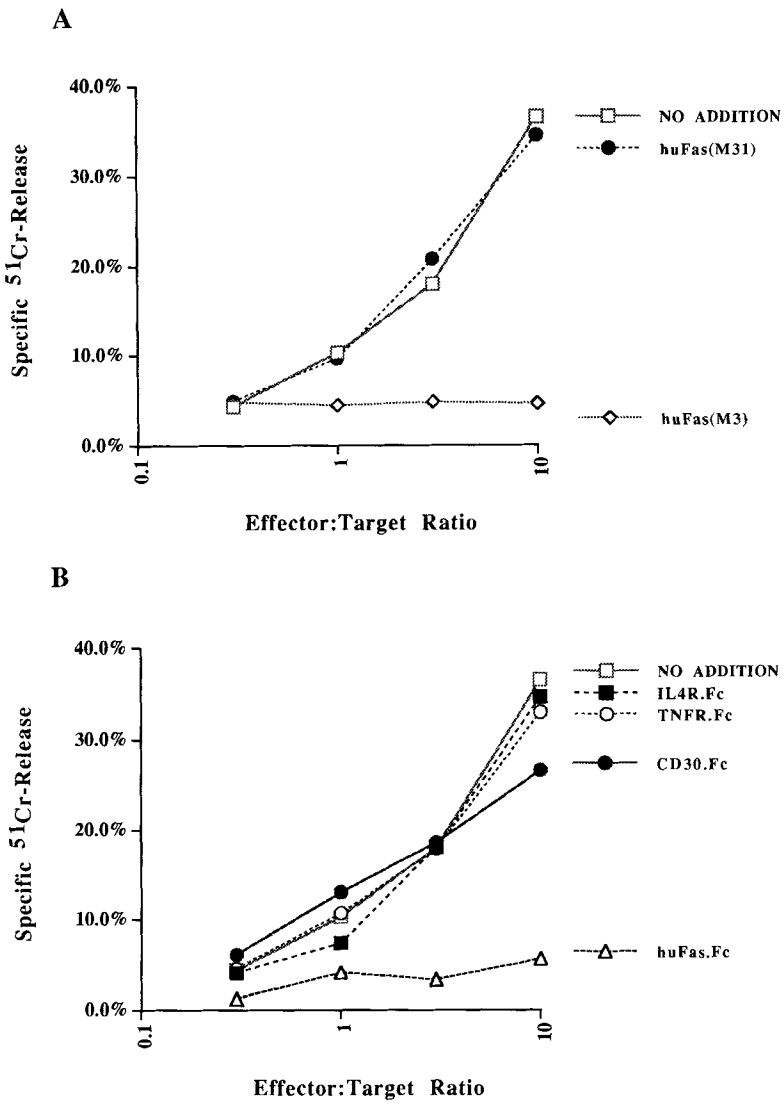

Fig. 4. Evaluation of the ability of hu-Fas-specific mAb and the huFas.Fc fusion protein to inhibit the lysis of Jurkat target cells by activated CTL expressing FasL. CTL were activated by incubation for 2 h in medium containing phorbol ester and calcium ionophore, followed by fixation (06.% formaldehyde for 1 min). **A:** Titrated numbers of the fixed, activated CTL were co-cultured overnight with ^{51}Cr-labeled Jurkat target cells in the presence of either medium alone (□), 10 μg/ml huFas M3 (◇), or 10 μg/ml huFas M31 (●). Specific ^{51}Cr-release from Jurkat target cells after overnight culture is shown. Spontaneous ^{51}Cr-release after overnight culture was 20.3%. **B:** Specific ^{51}Cr-release from Jurkat target cells after overnight culture with fixed, activated CTL in the presence of either medium alone (□), 25 μg/ml hulL-4R.Fc (■), huCD30.Fc (●), huTNFR.Fc (○), or huFas.Fc (△). (data in panel B taken from reference 27).

cells. However, it is also clear that susceptibility to Fas-mediated apoptosis is also dependent upon a number of other factors. As noted earlier, although many transformed cell lines and most chronically activated T cell clones are susceptible to Fas-mediated apoptosis, freshly isolated peripheral blood T cells expressing Fas are not [17]. Conversely, immobilized huFas-specific mAb in the presence of suboptimal CD3 mAb provide a potent costimulatory signal for peripheral blood T cells in an epitope-restricted and concentration dependent manner (Fig. 8). Fas-mediated costimulation also results in substantial increases in the production of IL-2, IFNγ, and TNF (Fig. 9). Additional studies have also indicated that Fas co-stimulation is a directly mediated effect that does not require the involvement of accessory cells or other cell types, en-

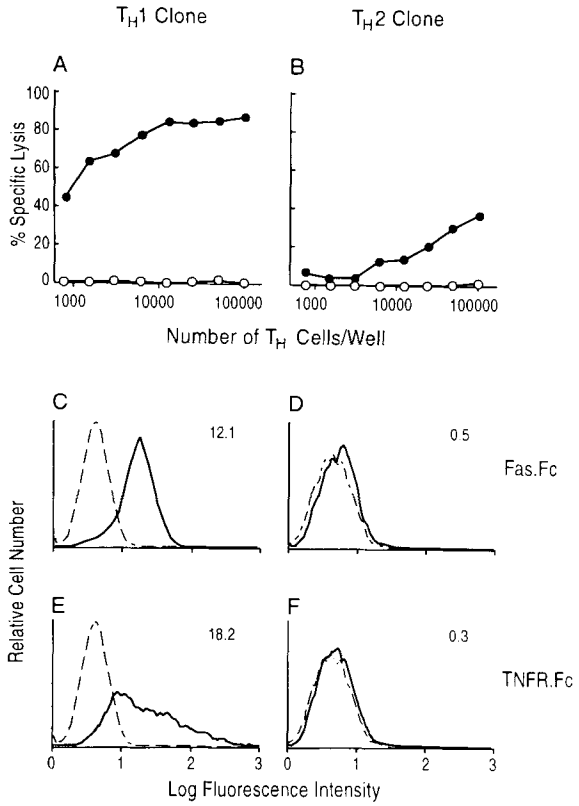

Fig. 5. T_h1 but not T_h2 cells are efficient at FasL-mediated cytolytic activity and express high levels of FasL following stimulation. Varying numbers of the T_h1 clone A.E7 **(A)** or the T_h2 clone E6 **(B)** were stimulated for 18 h with PMA (10 ng/ml) and ionomycin (500 ng/ml) in the presence of ^{51}Cr-labeled Jurkat target cells (10^4 cells/well). Cytolytic activity was measured in the presence (○) or absence (●) of anti-Fas M3 (5 μg/ml). Flow cytometric analysis was performed on T_h1 (A.E7) **(C,E)** or T_h2 (E6) **(D,F)** clones that were stimulated for 2 h with PMA (10 ng/ml) and ionomycin (500 ng/ml). Stimulated cells were then stained with IL-4R.Fc (dotted line), Fas.Fc, **(C,D)** or TNFR.Fc. **(E,F)**. Numbers within histograms represent the mean fluorescent intensity for the indicated reagent above the IL-4R.Fc control (compiled from data presented in reference 34).

hances other measures of T cell activation (e.g., CD-25 and CD-69 expression), and appears to act in an IL2-independent manner [31]. Thus, in addition to the role played by Fas and FasL in the regulation of the numbers of activated and/or autoreactive cells, this receptor/ligand pair may also play an important role in the activation and proliferation of naive T cells.

GENERAL CONCLUSIONS

The results of studies regarding the biological role of Fas/FasL interactions indicate unexpected levels of complexity. Although initially described as a cell-surface protein on transformed cells that mediated apoptotic death, Fas ligation on naive T cells costimulates cellular activation and proliferation and indicates that this receptor can mediate vastly different effects on T cells depending primarily on their state of

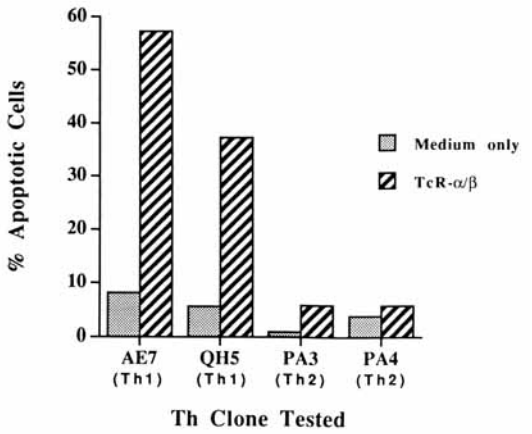

Fig. 6. T_h1 but not T_h2 cells undergo activation-induced cell death following TcR ligation. Cloned T_h1 (AE7 and QH5) and T_h2 (PA3 and PA4) cells were stimulated by culture in microtiter wells coated with mouse TcR α/β-specific mAb. Eighteen hours later cells were harvested and the proportion of apoptotic cells determined by multiparameter flow cytometry.

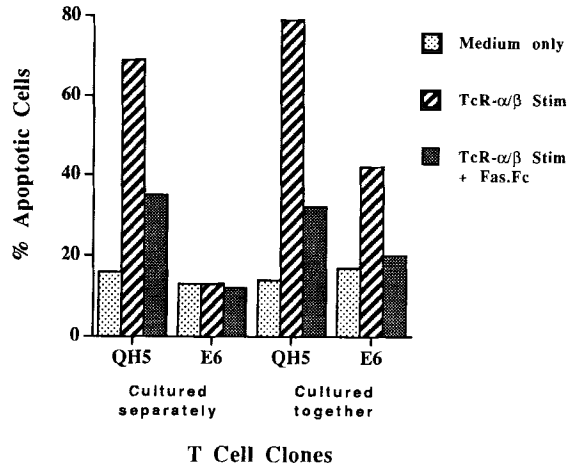

Fig. 7. FasL-expressing T_h1 cells can induce apoptosis of T_h2 cells. PKH-2 prelabeled QH5 (T_h1) cells and unlabeled E6 (T_h2) cells were cultured either separately or together in untreated wells or wells precoated with anti-TcR α/β. Fas.Fc fusion protein was added to some cultures as indicated. The fraction of QH5 and E6 cells induced to undergo apoptosis was determined 18 h later by multiparameter flow cytometry subsequent to appropriate electronic gating.

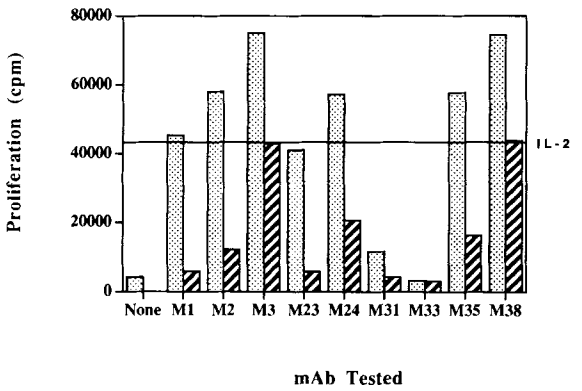

Fig. 8. Costimulatory activity of T cell proliferation by huFas-specific mAb. Purified human PBT were cultured with a suboptional concentration of immobilized CD3 mAb either in the presence or absence of immobilized huFas-specific mAb (wells coated with 1 or 10 μg/ml). Proliferation was determined 3 days later by ^{3}H-thymidine incorporation. The concentration of IL-2 used in some cultures for the purpose of comparison was 10 μg/ml.

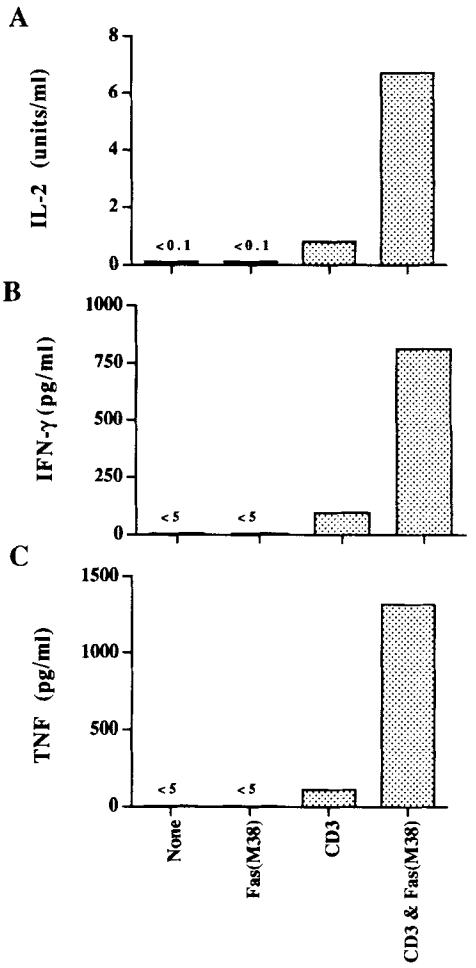

Fig. 9. Fas-mediated costimulation of T cells induces cytokine secretion. Purified human PBT were cultured in uncoated wells or wells coated with CD3 mAb, Fas (M38) mAb, or both. After 48 h of culture, supernatants were harvested and cytokine levels determined.

activation. Based on these findings it is likely that Fas-mediated signals through Fas play an important immunoregulatory role suggest that there may be pathological consequences of either too little or too much Fas or FasL function in vivo.

The consequences of a defective Fas/FasL signalling pathway is well documented in *lpr/lpr* and *gld/gld* mice. As a result of mutations in the structural genes for Fas and FasL in these mice, respectively, the ability to regulate the total number of lymphoid cells that have been generated in response to antigenic stimulation is substantially impaired and results in the progressive accumulation of lymphocytes and autoimmune disease. The opposite side of the regulatory coin (i.e., too much Fas/FasL signalling) may also result in pathological conditions. Although Fas-mediated apoptosis is not observed in freshly isolated PBT from normal, healthy donors, it can be induced if the cells are chronically activated [18,19]. Further, T cell activation also results in the expression of FasL. Thus, chronic activation of even normal T cells results in AICD mediated by Fas and FasL interactions. It is striking that freshly isolated T cells from a significant number of HIV$^+$ individuals undergo

AICD either spontaneously or subsequent to stimulation with T cell mitogens [32,33]. Although still circumstantial, the parallels suggest the possibility of a common underlying mechanism.

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