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Monocytes Stimulated by 110-kDa Fibronectin Fragments Suppress Proliferation of Anti-CD3-Activated T Cells[1]

Holly H. Birdsall,[2]*†‡ Wendy J. Porter,*† JoAnn Trial,*§ and Roger D. Rossen*‡§

One hundred ten to 120-kDa fragments of fibronectin (FNf), generated by proteases released in the course of tissue injury and inflammation, stimulate monocytes to produce proinflammatory cytokines, promote mononuclear leukocytes (MNL) transendothelial migration, up-regulate monocyte CD11b and CD86 expression, and induce monocyte-derived dendritic cell differentiation. To investigate whether the proinflammatory consequences of FNf are offset by responses that can suppress proliferation of activated T lymphocytes, we investigated the effect of FNf-treated MNL on autologous T lymphocytes induced to proliferate by substrate-immobilized anti-CD3. FNf-stimulated MNL suppressed anti-CD3-induced T cell proliferation through both contact-dependent and contact-independent mechanisms. Contact-independent suppression was mediated, at least in part, by IL-10 and TGF-β released by the FNf-stimulated MNL. After 24–48 h exposure to FNf, activated T cells and monocytes formed clusters displaying CD25, CD14, CD3, and CD4 that were not dissociable by chelation of divalent cations. Killing monocytes with L-leucine methyl ester abolished these T cell-monocyte clusters and the ability of the FNf-stimulated MNL to suppress anti-CD3 induced T cell proliferation. Thus, in addition to activating MNL and causing them to migrate to sites of injury, FNf appears to induce suppressor monocytes. ***The Journal of Immunology,*** **2005, 175: 3347–3353.**

When parenchymal tissues are injured by ischemia-reperfusion or by infection, proteases released by the damaged parenchymal cells and infiltrating leukocytes degrade matrix proteins and produce fragments of fibronectin with novel biologic properties (1–3). Among these are the 110- to 120-kDa VLA-5 (CD29/CD49e)-binding fibronectin fragments (FNf)[3] that signal MNL to produce TNF-α and that stimulate both T cells and monocytes to migrate across vascular endothelial barriers (4). FNf also induces cell surface display of proteinase-3 on tissue-infiltrating monocytes. Proteinase-3 cleaves cell surface CD49e, reducing the ability of phagocytes to migrate within fibronectin-rich tissue matrices and causing them to accumulate at the site of injury (1). In addition, FNf up-regulates monocyte and monocyte-derived dendritic display of CD18/CD11b, an integrin involved in formation of immunologic synapses, and CD86, a molecule that provides critical costimulatory signals to activated T cells (5). These observations raised the possibility that signals provided by these proinflammatory cytokines and FNf-stimulated dendritic cells (DC) could induce appropriately activated T cells to proliferate. Alternatively, products of FNf-stimulated inflammatory cells may contribute to the regulatory networks that suppress the proliferation of activated T cells. To evaluate these alternatives, we have studied the effects of FNf-treated MNL on the proliferation of T lymphocytes in response to anti-CD3.

Materials and Methods

Reagents

Purified human fibronectin enriched for the 110- to 120-kDa chymotrypsin fragment, hereafter called FNf, was obtained from Upstate Biotechnology and its purity documented by SDS-PAGE as described previously (5). Controls for the FNf included intact fibronectin (Chemicon International) and the connecting segment fragment of fibronectin (CS-1) (6) (a gift from R. Baughn, Baylor College of Medicine, Houston, TX). Recombinant IL-10, TGF-β, anti-TGF-β1, -β2, and -β3 (clone 1D11), anti-IL-10 (clone 23738), and ELISA kits to quantify IL-10, and TGF-β were purchased from R&D Systems. Media, fibronectin reagents, cytokines, and Abs used in these experiments contained <0.03 EU/ml endotoxin as determined by the Limulus amoebocyte assay (Associates of Cape Cod).

Mononuclear leukocytes (MNL)

Healthy normal subjects donated blood under protocols approved by the Institutional Review Board. MNL were isolated from heparinized peripheral blood by Ficoll/Hypaque density gradient centrifugation. MNL were treated with 5 μg/ml FNf unless otherwise specified. Treated MNL were cultured in Teflon jars because monocytes become highly adhesive within minutes of exposure to FNf (4). Because of this adhesiveness of activated monocytes, we used siliconized tubes (Sigmacote; Sigma-Aldrich) for cell purification and flow cytometric analyses and added 0.1% gelatin (Sigma-Aldrich) to Dulbecco's PBS wash solutions after Ab staining.

Proliferation studies

To quantify proliferation, responder cells were preloaded with CFSE, a cytoplasmic dye whose fluorescence intensity decreases as it is partitioned among daughter cells. CFSE (Molecular Probes) was dissolved in DMSO to make a 50 μM stock solution and stored under nitrogen. MNL at 10×10^6/ml in RPMI 1640 with 10% FCS were incubated with 1.4 μM (final) CFSE for 15 min at 23°C in the dark. An equal volume of FCS was added, and cells were incubated for an additional 40 min at 37°C in 5% CO_2. Labeled MNL were washed twice in RPMI 1640 with 10% FCS, and 0.5 million MNL were added to 24-well plates. Plates were precoated overnight with 0.1 μg of anti-CD3 (OKT3; Immunotech) or mouse IgG (Sigma-Aldrich) in 1 ml of Dulbecco's PBS (Invitrogen Life Technologies) and washed twice with 1 ml of RPMI 1640/FCS before use. In some experiments, CFSE-labeled cells were treated directly with FNf or with the culture supernatant of FNf-treated (regulatory) MNL. In other experiments,

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[3] Abbreviations used in this paper: FNf, 110–120 kDa fibronectin fragment; DC, dendritic cell; CS-1, connecting segment-1 of fibronectin; MNL, mononuclear leukocyte; LME, L-leucine methyl ester; T_{reg}, regulatory T.

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0.5×10^6 CFSE-loaded fresh responder MNL were mixed with an equal number of pretreated MNL. Cell mixing experiments were always done with autologous cells to avoid allogeneic stimulation. Unless specified otherwise cultured cells were maintained in 5% CO_2 and humidified air at 37°C. After 3–6 days, cultured cells were collected, stained with anti-CD3 to identify T cells, and analyzed by flow cytometry (Fig. 1, *top panel*). Using CFSE-loaded MNL incubated in murine IgG-coated wells, we defined the gate required to encompass the nonproliferating cells. We then defined a series of nonoverlapping gates with constant width (i.e., number of channels) to estimate the intervals of decreasing fluorescence intensity associated with cells that proliferated through one or more cycles (7) (Fig. 1, *middle* and *lower panels*). Percent suppression was calculated as the fold reduction in the total number of proliferating T cells in the FNf-treated samples vs the sham-treated samples exposed to medium alone.

Putative regulatory MNL were induced with 5 μg/ml FNf, or medium alone, for 48 h in Teflon jars and then washed before addition to responder cells. Preliminary dose response studies demonstrated that 5 μg/ml FNf reliably induced the regulatory phenotype described in this report, whereas the CS-1 fragment of fibronectin and intact fibronectin had no effect. In some experiments, MNL were incubated with FNf for only 1 h, washed to remove FNf (<0.02 μg/ml carryover of FNf), and cultured for the remaining 47 h in RPMI 1640 with FCS. The pulse-treatment protocol was particularly important when generating a cell-free supernatant that contained the soluble products released in response to FNf without carrying over FNf itself. In some experiments, we sought to block the effects of agents that suppress T cell proliferation by adding 10 μg/ml anti-TGF-β (to neutralize up to 25,000 pg/ml TGF-β) or 1–10 μg/ml anti-IL-10 (to neutralize up to 125,000 in initial studies and 1,250,000 pg/ml IL-10 in later studies).

Monocytes were removed with anti-CD14-coated iron beads (Dynal Biotech) at a ratio of 20 beads/monocyte for 30 min at 37°C. Bead-coated monocytes were removed with a magnet, and the cycle was repeated. The resulting population contained <1% (often <0.1%) $CD14^+$ cells by flow cytometry. A similar approach was used to deplete $CD25^+$ cells with anti-CD25-coated Dynal beads, except that the ratio was decreased to 10 beads/cell, and incubation was conducted at 4°C. In other studies, we used anti-CD14-coated beads obtained from Miltenyi Biotec, according to the manufacturer's directions, to prepare CD14-enriched and CD14-depleted populations. Bead preparations and buffers used in these studies were all endotoxin-free. To lyse the monocytes, we treated 5×10^6 MNL in RPMI 1640 without FCS, with 5 mM (final) L-leucine methyl ester (LME; Sigma-Aldrich) using a 20× stock in calcium-magnesium-free HBSS (8). MNL were incubated for 40 min at 23°C and washed twice with RPMI 1640. MNL were cultured an additional 24 h in RPMI 1640 with 10% FCS to allow the monocytes to lyse. Treated populations contained less then 0.1% $CD14^+$ cells by flow cytometry.

Flow cytometry

Flow cytometric analyses were performed as previously described (5, 9) on a Beckman Coulter Epics XL cytometer. mAbs from Beckman Coulter included anti-CD25 (clone B1.48.9), anti-CD11b (CR3, clone Bear1), CD49e (clone Sam1), CD45 (clone J.33), anti-CD4 (clone SFCI12T4D11), anti-CD3 (clone UCHT1), and anti-CD54 (clone 84H10). Isotype control Abs were obtained from BD Biosciences. Flow count fluorospheres purchased from Beckman Coulter were used as internal references to enumerate cells. Cells to be stained were preincubated for 5 min with human plasma (20% v/v) to block FcR before addition of Ab. Backgrounds were determined for each analysis using the same cells stained with isotype controls, and delimiters in each analysis were set to exclude <2% of the events.

Statistical analysis and graphic representations of data were performed using Statistica 6 (StatSoft).

Results

Addition of 5 μg/ml 110-kDa FNf decreased T cell proliferation in response to immobilized anti-CD3 by 58 ± 5% (mean ± SEM, six experiments, $p = 0.05$ paired t test) (Figs. 1 and 2). The failure to proliferate in response to anti-CD3 was not due to cytotoxicity as T cells proliferated in the presence of FNf (average 2.2 ± 0.6-fold increase in the number of proliferating T cells) but to a significantly lower extent than in the absence of FNf ($p = 0.02$, paired t test) (data not shown). Furthermore, the fraction of T cells undergoing apoptosis, based on display of Apo 2.7, or were nonnonviable, based on uptake of propidium iodide, were lower and not higher in the FNf-treated cultures (Fig. 3).

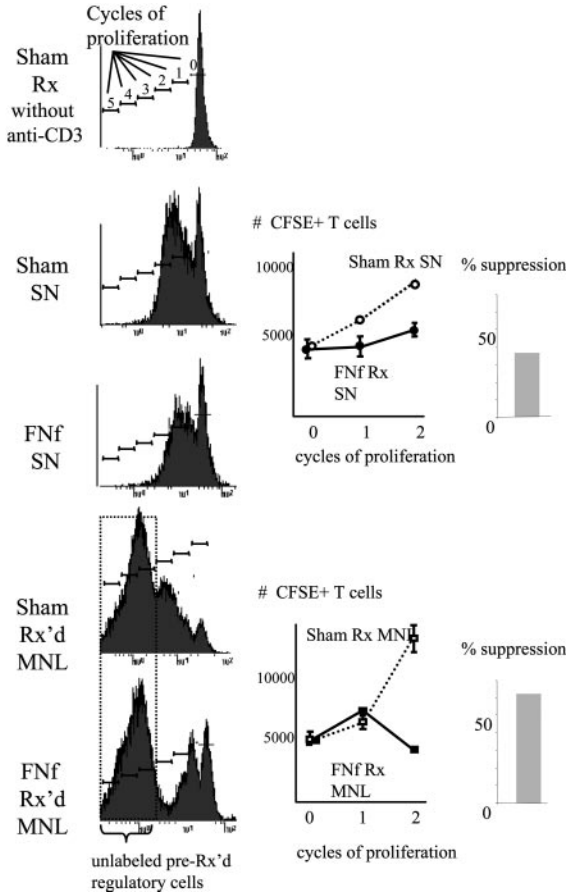

FIGURE 1. Suppression of proliferation by FNf-treated MNL and their supernatant. FNf-pretreated MNL or their soluble products were added to CFSE-labeled fresh responder MNL was stimulated with immobilized anti-CD3, and proliferation was measured after 3–4 days. *Top panel*, The histogram for CFSE-labeled responder cells, alone, without anti-CD3 stimulation; this condition is used to define the delimiter for the nonproliferating population. *Middle panel*, CFSE-labeled cells were cultured on immobilized anti-CD3, in the presence of the supernatant of FNf-treated or sham-treated MNL. The data from the histograms on the *left* were converted to a line drawing in the *middle* that shows the numbers of $CD3^+$ T cells that proliferated through zero, one, or two cycles. The decrease in the total number of proliferating T cells was used to calculate the percent suppression, which is shown in the bar graph on the *right*. *Lower panel*, Experiments in which MNL pretreated with FNf or not were mixed with fresh CFSE-labeled MNL. The boxed area in the histogram encloses the unlabeled pretreated MNL that are excluded when calculating the numbers of proliferating CFSE-labeled MNL.

Previously, we found that FNf-treated MNL produce TNF-α, fibroblast growth factor, leukemia inhibitory factor, and insulin-like growth factor (5, 9). Therefore, it seemed likely that FNf-treated MNL might also produce immunosuppressive cytokines. Indeed, FNf-treated MNL produced increased quantities of both IL-10 and TGF-β (Table I). Because both of these cytokines suppress the proliferation of activated T cells (10–15), we attempted to reverse the suppressive effects of FNf using blocking Abs to these cytokines. Supernatants of MNL that were pulse treated with FNf, washed to remove the FNf, and cultured for an additional 24 h suppressed proliferation when added to fresh MNL (Fig. 4). The suppression could be partially blocked with anti-TGF-β or anti-IL-10. When these Abs were added together, they almost completely reversed the suppressive effect of supernatants from FNf-treated MNL (Fig. 4).

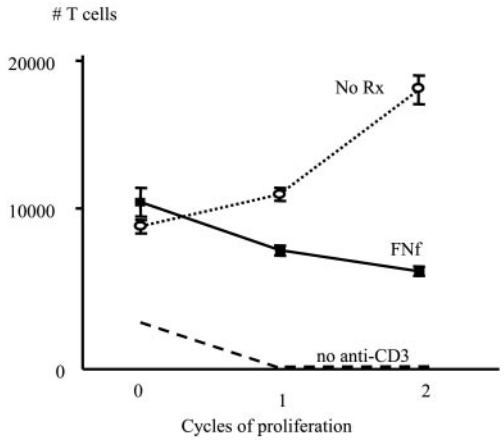

FIGURE 2. Effect of FNf on T cell proliferation. Fresh CFSE-labeled MNL were stimulated with immobilized anti-CD3 in the presence of 5 μg/ml FNf (■ and solid line) or medium (no Rx, ○ and dotted line). Control cultures were incubated with nonspecific mouse IgG in lieu of anti-CD3 (dashed line). Data shown are the number of $CD3^+$ T cells that were divided none, once, or twice as assessed by the intensity of the CFSE signal. Error bars note the mean and SD of replicates. Results are representative of three donors.

However, these same Abs were unable to block the suppressive effects of FNf added directly to MNL while undergoing stimulation with anti-CD3 (Fig. 5). This suggested that there were two types of suppression induced by FNf: one that was contact independent and mediated by secreted TGF-β and IL-10 and another that was contact-dependent and not blocked by Abs to TGF-β or IL-10. To test this hypothesis, we pretreated MNL with FNf for 48 h, washed them to remove the FNf, and evaluated their ability to suppress proliferation of anti-CD3-stimulated T cells in fresh autologous MNL. To evaluate the need for contact, we compared FNf-treated MNL in direct contact with the responder cells and FNf-treated MNL separated from the responder cells by a semipermeable membrane (Transwell) (Fig. 6). When in contact, FNf-treated MNL suppressed 67% of the proliferating T cells, whereas when separated by a semipermeable membrane, there was only 40% suppression (Fig. 6). The suppressive effect of FNf-pretreated MNL in direct contact with the responder cells was not blocked by Abs to TGF-β or IL-10 (Fig. 7).

Both monocytes and regulatory T (T_{reg}) cells can suppress proliferation of Ag-activated T cells (16, 17). To determine which cell

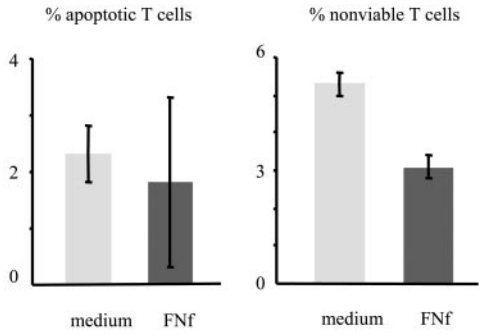

FIGURE 3. Effect of FNf on T cell viability. MNL stimulated with immobilized anti-CD3 in the presence of FNf (dark bars) or medium (light bars) were analyzed after 3 days. Bars indicate the percentage of $CD3^+$ cells staining with Apo 2.7 to identify apoptotic cells (*left panel*) or propidium iodide (*right panel*) to identify nonviable cells. Error bars indicate the SD of replicates, and results are representative of two donors.

Table I. *Cytokines produced by FNf-treated MNL*[a]

Cytokine	Sham-Treated MNL	FNf-Treated MNL
IL-10	4 ± 7 pg/ml	87 ± 21 pg/ml
TGF-β	1687 ± 240 pg/ml	2392 ± 136 pg/ml

[a] MNL from three donors were incubated with 5 μg/ml FNf, and the quantity of cytokine released into the supernatant was measured after 24 h. Results shown are mean ± SD three donors. Quantities of cytokine produced after FNf were significantly greater than sham-treated MNL $p = 0.05$; Mann-Whitney U test.

type was responsible for suppressing T cell proliferation in these experiments, we depleted specific populations from the FNF-treated MNL using Ab-coated magnetic beads (Dynal Biotech). Removal of $CD14^+$ cells completely abrogated the suppressive effect of the FNF-treated MNL (Fig. 8); in fact, it enhanced the proliferation above that was seen after addition of sham-treated unfractionated MNL. Removal of $CD25^+$ cells, a marker typically associated with T_{reg} cells, also greatly decreased the suppressive effect of the FNf-pretreated MNL (Fig. 8). However, careful examination of the MNL population after FNf treatment revealed that CD25 was expressed on several types of cells (Fig. 9). CD25 was found on $CD3^-/CD14^+$ activated monocytes, on $CD3^+/CD14^-$ activated T cells, and on large numbers of cells that expressed both CD14 and CD3, suggesting that they were incorporated in clusters of T cells with monocytes—one or both of which expressed CD25 (Fig. 10). Incubation with equimolar quantities of intact fibronectin or the CS-1 fragment of fibronectin had no effect on MNL expression of CD25 or their ability to suppress proliferation of anti-CD3-stimulated T cells (data not shown).

The presence of these clusters called to question the interpretation of the experiment shown in Fig. 8. If the T cell:monocyte clusters were induced by FNf display CD14, then using anti-CD14-coated beads to deplete monocytes would also remove T

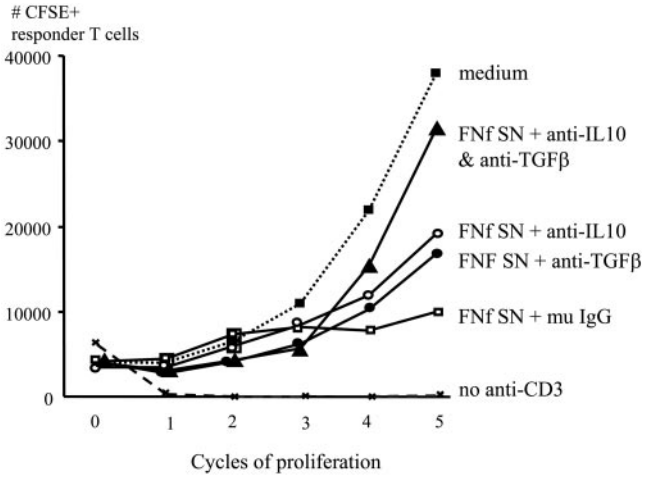

FIGURE 4. Effect of blocking IL-10 and TGF-β on the suppression of proliferation by soluble products of FNf-treated MNL. Fresh CFSE-loaded MNL cultured in the presence of medium (dotted line) or supernatant collected from MNL pulse treated with FNf (FNf SN) 48 h previously (solid line). To the FNf SN were added nonspecific mouse IgG (muIgG, 10 μg/ml; □), murine anti-IL-10 (10 μg/ml; ○), murine anti-TGF-β (10 μg/ml; ●), or a mixture of both Abs (▲). CFSE-labeled cells were stimulated with immobilized anti-CD3 in all wells, except for the baseline control (dashed line). After 5 days, responder $CD3^+$ T cells were counted by flow cytometry, and the number of proliferative cycles was estimated from CFSE intensity. Results obtained with FNf SN alone (data not shown) were no different from FNf SN with nonspecific murine IgG. Results are representative of two donors.

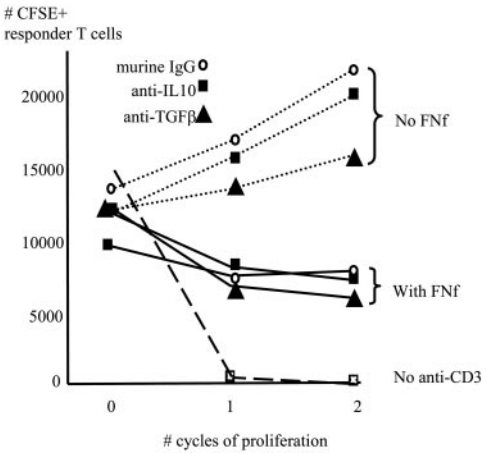

FIGURE 5. Effect of blocking IL-10 and TGF-β on the suppression of proliferation by FNf. CFSE-labeled fresh MNL were exposed to purified FNf (solid lines) or medium (dotted lines) in combination with nonspecific murine IgG (○), 1 μg/ml anti-IL-10 (■), or 10 μg/ml anti-TGF-β (▲). After 3 days, responder T cells were counted by flow cytometry, and the numbers of proliferative cycles were estimated from the CFSE intensity. Baseline proliferation in the absence of immobilized anti-CD3 is shown with a dashed line.

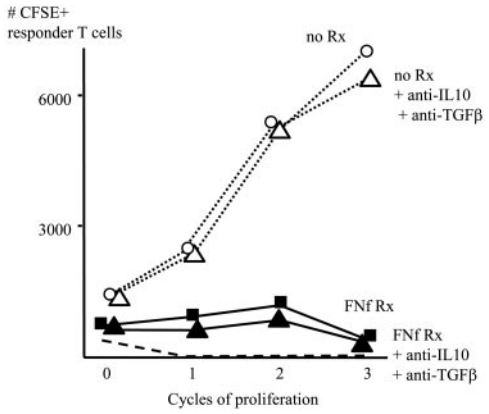

FIGURE 7. Effect of blocking IL-10 and TGF-β on the suppression of proliferation by FNf-pretreated MNL. MNL were treated with FNf (FNf Rx, filled symbols and solid lines) or medium (no Rx, open symbols and dotted lines) for 48 h, washed, and added to fresh CFSE-labeled responder MNL stimulated with immobilized anti-CD3. Some cultures also received 10 μg/ml anti-IL-10 plus 10 μg/ml anti-TGF-β (triangles). After 3 days, responder $CD3^+$ T cells were counted by flow cytometry, and the numbers of proliferative cycles were estimated from the CFSE intensity. Baseline proliferation in the absence of immobilized anti-CD3 is shown with a dashed line. Results are representative of two donors.

cells that could be responsible for or contribute to the suppressive effect. Similarly, treatment with anti-CD25 beads would also remove $CD25^+$ monocytes. To determine whether monocytes or T cells were mediating the suppression, we separated T cells and monocytes before FNf treatment to prevent cluster formation and treated each population alone. Purified $CD14^+$ cells treated with FNf were even more effective than FNf-treated unfractionated MNL at suppressing the proliferation of anti-CD3 activated T cells (Fig. 10). However, the CD14-depleted population did not suppress; in fact, it enhanced the proliferation of responder T cells (Fig. 10). Even when the CD14-depleted population was treated with FNf while exposed to FNf-treated monocytes separated by a semipermeable membrane, the T cells did not suppress (data not shown). The limitation of this experimental design was that it prevented potentially important cell-to-cell signaling between T cells and monocytes in contact with each another as they were activated by the FNf. Therefore, we attempted to dissociate the clusters of monocytes and T cells after they had been exposed to FNf. However, the clusters of T cells and monocytes were resistant to all strategies designed to separate them, including incubation in calcium-magnesium-free medium and exposure to 5 mM EDTA. Because we were unable to dissociate the clusters, we used LME to selectively lyse the monocytes (8). MNL were pretreated with FNf for 24 h and then incubated with LME for 40 min and washed. The subsequent osmotic lysis of monocytes required an additional 24 h incubation, after which the cells were washed again and assayed for their ability to suppress proliferation of fresh MNL. Removal of monocytes from FNf-treated MNL completely abrogated the suppressive effect (Fig. 11) without affecting the viability of the remaining T cells. The FNf-activated T cell-enriched population remaining after LME treatment actually enhanced the proliferation of the fresh responder cells.

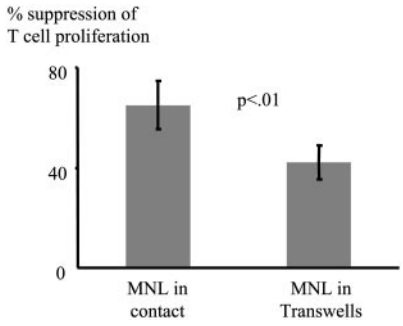

FIGURE 6. Contact dependence of suppression by FNf-treated MNL. MNL were pretreated for 48 h with FNf or medium and then cultured with fresh CFSE-labeled responder cells. The pretreated MNL were either in direct contact with the CFSE-labeled responder cells or suspended in Transwells above the responder cells. Three days later, $CD3^+$ T cells were counted by flow cytometry, and proliferating cells were identified by their CFSE signal. Percent suppression was based on the reduced number of T cells that proliferated in the present of FNf-treated MNL compared with sham-treated MNL. Results are representative of three donors; the value of p was estimated by a paired t test.

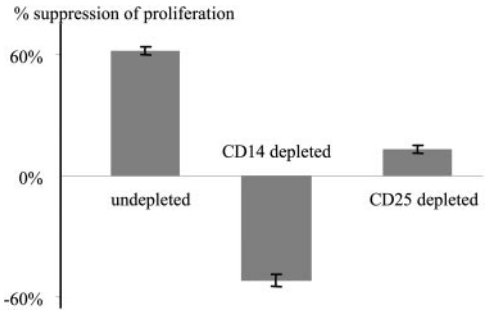

FIGURE 8. Role of $CD14^+$ and $CD25^+$ cells in the suppression of proliferation by FNf-treated MNL. MNL were exposed to FNf or medium for 48 h and then $CD14^+$ or $CD25^+$ cells were depleted using Ab-coated magnetic beads. Depleted populations contained $<0.3\%$ $CD14^+$ cells or $<1\%$ $CD25^+$ cells, respectively. The treated MNL populations were washed and placed with fresh CFSE-labeled responder cells in anti-CD3-coated wells. After 4 days, responder T cells were counted by flow cytometry, and the number of proliferative cycles was estimated from the CFSE intensity. Percent suppression was based on the reduced number of T cells that proliferated in the presence of undepleted FNf-treated MNL, CD14-depleted, FNf-treated MNL, or CD25-depleted, FNf-treated MNL compared with sham-treated MNL.

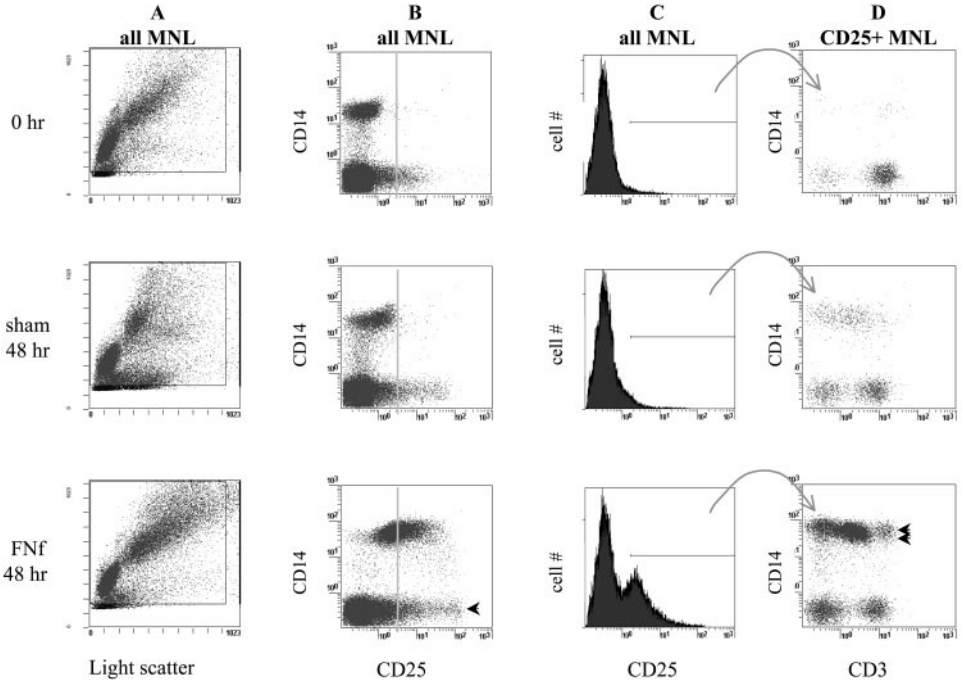

FIGURE 9. FNf induces CD25 and monocyte: T cell clusters. Normal donor MNL were cultured in Teflon jars with medium or 5 μg/ml FNf for 48 h. The original population was assayed at time 0. Results are representative of those seen with three donors. *A*, The forward and side scatter for the three populations. *B*, Gated on all MNL and shows the staining for CD25 (*x*-axis) and CD14 (*y*-axis); the vertical delimiter was set to include <1% positive monocytes when stained with nonspecific IgG. After exposure to FNf, a subset of $CD14^-$ cells becomes CD25 bright (arrowhead), with a maximum fluorescence intensity 1-log greater than $CD14^-$ cells cultured in medium alone. CD25 is also increased on the $CD14^+$ population. *C*, The histogram for CD25 expression on all MNL. *D*, Gated from the $CD25^+$ population shown in *C* and shows the distribution of CD14 and CD3 on the $CD25^+$ cells. In circulating blood (0 h), the only $CD25^+$ cells are $CD14^-/CD3^+$ T cells. After 48 h of culture in medium alone (sham), CD25 is also found on a few $CD14^+$ cells and a few $CD14^-/CD3^-$ cells that may be NK or B cells. However, after FNf treatment, a large number of $CD14^+$ cells begin to express CD25. In addition, $CD25^+$ is found on double-positive cells that express both CD3 and CD14 (double arrowheads).

Discussion

Tissue matrix and plasma fibronectin are broken down by proteolytic enzymes released in the course of host responses to infectious agents (2, 3) and ischemic injury (1). The 110- to 120-kDa fragment of fibronectin, which we refer to as FNf, is not normally present in the circulation but can be found in the plasma of up to two-thirds of HIV-infected individuals (5) and in the plasma of patients with heart failure (unpublished observations). Particularly high concentrations of FNf are found in the lymph-draining infarcted myocardium after ischemia-reperfusion injury (1). In vitro models suggest that generation of FNf will be followed by inflammation. For example, FNf induce monocytes and T cells to produce TNF-α, migrate across endothelial barriers, and accumulate just below the endothelium (4). Because FNf also up-regulates DC surface expression of CD86, one might postulate that the presence of FNf at the site of an inflammatory response would help to activate and induce proliferation of T cells in contact with these activated DC. However, the data presented here suggest that such an outcome is thwarted by the ability of FNf-stimulated MNL to suppress proliferation of activated T cells through both contact-dependent and contact-independent mechanisms. It is clear that T cells were not killed by FNf-stimulated MNL because the numbers of viable and nonapoptotic T cells were, if anything, higher in the FNF-treated cultures. Notably, FNf suppressed, but did not abolish, T cell proliferation in response to immobilized anti-CD3.

Blocking Abs to TGF-β and IL-10, together, negated the suppressive effects of supernatants collected from FNf-treated MNL, indicating that contact-independent suppression by FNf was mediated by both of these two cytokines. The failure of anti-TGF-β and anti-IL-10 to block contact-dependent suppression does not unequivocally mean that these cytokines are not involved. Surface-bound TGF-β can mediate contact-dependent suppression (18). However, it is possible that in our system the tight complexes formed between T cells and monocytes after FNf activation prevented blocking Abs from reaching the surface TGF-β.

Depletion of $CD25^+$ cells removed the suppressor activity from FNf-treated MNL, indicating that activated cells were involved. However, removal of the $CD25^+$ cells did not tell us whether the suppression resulted from the actions of T cells or monocytes because both expressed increased quantities of CD25 after FNf. Indeed, four cell populations displayed CD25: $CD14^+/CD3^-$, $CD14^+/CD3^{dim}$, $CD14^+/CD3^+$, and $CD14^-/CD3^+$ cells. We postulated that the $CD3^+/CD14^+$ cells represented T cell:monocyte clusters. This interpretation is supported by the fact that these $CD3^+/CD14^+$ cells were found among the largest populations of cells identified by the flow cytometer, i.e., those with the greatest forward and side scatter (data not shown). The dual positive cells were depleted, as were the $CD14^+CD3^-$ cells, by incubating the FNf-treated MNL on plastic (data not shown). The tendency of FNf-treated monocytes and T cells to aggregate was no doubt enhanced by the fact that monocyte and T cell surface expression of ICAM-1 (CD54) doubled 3 h after exposure to FNf, and expression of monocyte CD11b, a counterligand for CD54, increased over 5-fold within 5 min after addition of FNf (data not shown).

The tendency of monocytes and T cells to form clusters after FNf treatment made it challenging to determine which of the two was responsible for the suppressor activity. By separating monocytes and T cells before FNf exposure, we could demonstrate that treatment with FNf had no impact on the regulatory behavior of isolated T cells. However, preventing contactual interactions of T

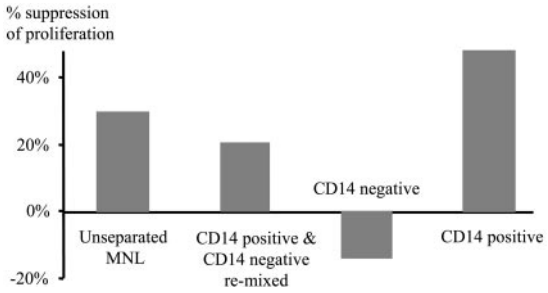

FIGURE 10. Role of $CD14^+$ cells in the suppression of proliferation by FNf-treated MNL. MNL were separated into $CD14^+$ and $CD14^-$ subsets using Ab-coated magnetic beads (Miltenyi Biotec). Controls included unfractionated MNL and a remixed population of $CD14^+$ and $CD14^-$ cells in a 1:5 ratio. Each of the populations was treated with FNf or medium for 48 h in Teflon jars, washed, and added to fresh CFSE-labeled responder cells in anti-CD3-coated wells. After 4 days, responder CD3 T cells were counted by flow cytometry, and the number of proliferative cycles was estimated from the CFSE intensity. Percent suppression was based on the reduced number of T cells that proliferated in the presence of FNf-treated cells compared with sham-treated cells of the same type.

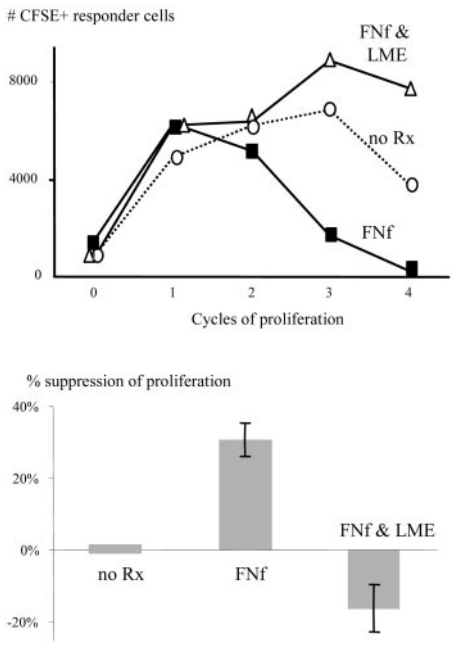

FIGURE 11. Effect of removing monocytes on the suppression mediated by FNf-treated MNL. MNL were treated with FNf or medium alone. After 24 h, some of the FNf-stimulated MNL were treated with LME to lyse monocytes. After an additional 24 h, pretreated MNL were washed and placed with fresh CFSE-labeled responder cells in anti-CD3-coated wells. After 4 days, responder CD3 T cells were counted by flow cytometry, and the number of proliferative cycles was estimated from the CFSE intensity. Percent suppression was based on the reduced number of T cells that proliferated in the presence of FNf-treated MNL or monocyte-depleted, FNf-treated MNL compared with sham-treated MNL. Error bars are the SEM of quadruplicates.

cells and monocytes before treatment with FNf eliminated any possibility of studying the effects of monocyte:T cell signaling induced by FNf that might modify the subsequent behavior of the T cells. Indeed, preliminary experiments (data not shown) demonstrated that $CD25^+$ was not up-regulated on T cells when stimulated with FNf in the absence of monocytes nor when monocytes were present but separated by a semipermeable membrane. This suggested that cell-cell signaling induced by FNf may be required to alter the functions of cells incorporated in the monocyte-T cell aggregates.

Once stimulated with FNf, it proved impossible to separate T cells from monocytes in the aggregates. Therefore, we used LME to selectively lyse the monocytes after the MNL had been exposed to FNf for 24 h. LME is freely diffusible into cells, metabolized to leucine, concentrated within lysosomes, and ultimately causes osmotic lysis of monocytes. The remaining T and B lymphocytes are unaffected (8). Less than 0.1% of the $CD14^+$ cells remained after LME, and the depleted population had no ability to suppress T cell proliferation. Although we cannot be absolutely sure that LME did not affect the T cell partner in the monocyte:T cell cluster in some manner, we demonstrated that the total number of T cells recovered from LME-treated cultures was not diminished and their viability remained >98%.

Regulatory monocytes were induced by as little as 5 μg/ml FNf. By way of comparison, FNf reaches concentrations as high as 100 μg/ml in plasma of HIV-infected patients (5) and 1500 μg/ml in lymph draining canine cardiac tissue after ischemia-reperfusion injury (1). In pulse-treatment studies, monocyte regulatory activity was induced after exposure to FNf for no more than 1 h. Thus, transit through the circulation, when the plasma contains sufficient FNf, would provide more than adequate time for monocytes to be activated by FNf. In addition, monocytes migrating into inflamed tissues would encounter even more FNf.

There is increasing interest in understanding how the immune system down-regulates responses to maintain homeostasis and prevent autoimmune responses. Much attention has been paid to the roles of T_{reg} cells (10–14) in inhibiting autoimmune and allergic diseases (10) as well as immune responses to bacterial and viral infections (11–13). "Naturally occurring" T_{reg} cells have a high expression of CD25, tend to be specific for self-Ags, and require cell-to-cell contact to suppress proliferation of activated T cells (14). Their targets include both effector T cells and APCs. "Induced" T_{reg} cells appear after chronic Ag stimulation, may be specific for self or foreign Ags, have variable CD25 expression, and secrete soluble cytokines such as IL-10 and/or TGF-β that suppress Ag-activated T cell proliferation.

Suppressor monocytes have been found in a variety of conditions characterized by chronic inflammation, including tuberculosis (19, 20), HIV (21), cancer (22, 23), and sarcoidosis (24); they are also present in peripheral blood stem cells mobilized with growth factors (25). In some systems, these monocytes induce T cell apoptosis (26), whereas in other systems, T cell proliferation is inhibited without evidence of cytotoxicity (27, 28). Similar to T_{reg} cells, regulatory monocytes can suppress T cell proliferation through production of TGF-β (20, 29) or IL-10 (23) and may require direct contact (30). The data in this report suggest that 1-h exposure to one particular fibronectin degradation product can induce suppressor functions in normal donor peripheral blood monocytes. By way of contrast, induction of T_{reg} cells from peripheral blood typically require 5 or more days of stimulation with cytokines in vitro (31). We do not know whether the $CD25^+CD4^+$ T cells that were induced by 24-h exposure to FNf may develop regulatory activity when cultured for longer periods of time. However, it is clear that FNf can induce suppressor monocytes very quickly, and these may be the first regulatory cells to initiate a return toward homeostasis after the onset of an inflammatory response.

Disclosures

The authors have no financial conflict of interest.

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