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Comparative Analysis of CD137 and LPS Effects on Monocyte Activation, Survival, and Proliferation

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CD137 (ILA/4-1BB), a member of the TNF receptor family, regulates activation, survival and proliferation of primary human monocytes. Here we compare the activities of lipopolysaccharide (LPS), a classical and potent monocyte activator to that of CD137. LPS is a more potent activator of monocytes, as evidenced by a stronger induction of the proinflammatory cytokine IL-8. However, CD137 could further increase maximal cytokine induction by LPS, which points to separate signaling pathways for LPS and CD137. Also, expression of myc was only induced by the combination of CD137 and LPS. Expression of macrophage colony-stimulating factor is induced more potently by CD137, but an additive effect is obtained by the combination of CD137 and LPS. Monocyte/macrophage survival and proliferation is only induced by CD137. LPS counteracts both activities of CD137 via activation induced cell death. While LPS has a role in activation of monocytes in innate immunity, the CD137 receptor/ligand system seems to deliver an activating signal to monocyte in acquired immunity. © 2000 Academic Press

Monocytes/macrophages are key regulators of immune responses. They are part of the first defense line, the innate immunity, against invading microorganisms, viruses and transformed cells. By releasing cytokines and by presenting antigens monocytes also initiate the second defense line, the lymphocyte mediated acquired immunity (1).

Monocytes can aquire multiple physiological and morphological states and forms and perform wide variety of functions. The type and strength of their response will be determined by an interplay of monocyte internal factors, such as the activation and differentiation state, with the external milieu, type, numbers and activation states of surrounding cells and the presence and activity of soluble factors (2, 3). The factors regulating monocyte activation and their diverse functions are only partially understood. Dysregulated monocyte activation has been implicated in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematousus as well as in several immune deficiencies (1). Therefore, the ability to purposefully modulate monocyte activation and functions would be of eminent therapeutic significance.

A molecule with potent effects on monocyte activities is CD137, a member of the TNF receptor family (4–6). CD137 induces activation of primary monocytes with enhanced expression of proinflammatory cytokines and activation markers and a reduction of the antiinflammatory cytokine IL-10 (7). CD137 also promotes adherence and substantially prolongs survival of monocytes *in vitro* (8). Further, CD137 induces a profound and widespread proliferation and endomitosis in primary monocytes (9). These activities of immobilized CD137 are mediated by crosslinking of a CD137 ligand which is constitutively expressed on monocytes (7–9).

The CD137 receptor is expressed by activated T lymphocytes, and its crosslinking delivers a costimulatory signal to T lymphocytes. The CD137 ligand and agonistic anti-CD137 antibodies enhance T cell proliferation and cytotoxicity *in vitro* and anti-tumor and anti-transplant immune responses *in vivo* (10–14). Therefore, the CD137 receptor ligand system exerts potent immunostimulary activities by activating T lymphocytes a well as monocytes via bidirectional signalling.

In order to achieve a more detailed understanding of the physiological role of CD137 on monocytes we compared the activities of CD137 on monocytes to that of LPS, a well established monocyte activator. We find that LPS is a more potent activator than CD137, while CD137 provides mainly a survival signal. Proliferation of monocytes can be initiated solely by CD137 but not by LPS. Both compounds utilize separate but interacting signaling pathways.

MATERIAL AND METHODS

Reagents. CD137-Fc protein was purchased from Alexis (Grünberg, Germany). Human IgG_1 Fc protein was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). LPS (*Salmo-*

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nella abortus equi) was kindly provided by C. Galanos (Max-Planck-Institut, Freiburg, Germany).

Cells and cell culture. Primary monocytes were isolated by elutriation (15). Elutriated monocytes were more than 95% pure and contaminating T lymphocytes were less than 3% as estimated by morphology and antigenic phenotype (CD14, CD3, CD4 and CD8 expression). Cells were cultured in polystyrene dishes (Becton Dickinson, Franklin Lakes, NJ) in RPMI 1640 supplemented with 5% FCS at a concentration of 10^6/ml. 96 well plates were used, unless indicated otherwise.

RT-PCR. Total RNA was isolated using RNAzol B (Tel-Test, Inc., Friendswood, TX) and up to 5 μg RNA were reverse-transcribed in a 20 μl volume, using random Hexanucleotide primers (50 μg/ml), 25 μM dNTP, 10 mM DTT, 200 units SuperScriptII RNaseH-RT (BRL, Eggenstein, Germany) and 20 units RNAsin (Roche, Mannheim, Germany) for 60 min at 42°C.

2 μl of the RT reaction served as template for the subsequent PCR, which was performed in a 20 μl volume with 1 unit Taq DNA polymerase (Roche, Mannheim, Germany), 200 μM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris pH 8.3, 50 mM KCl and 10 μM of each primer. After a 5 min denaturation step at 94°C, the reaction proceeded in 30 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, followed by 10 min at 72°C.

Primers used were: myc sense, 5′ TCTCGTAGACGAGCTTGGCGG; myc antisense, 5′ GTTAGCTTCACCAACAGGAAC; cyclophilin sense, 5′ GTCCAGCATTTGCCATGGAC; cyclophilin antisense, 5′ GACAAGGTCCCAAAGACAGC.

ELISA. ELISA kits were purchased from R&D Systems (Wiesbaden, Germany) and performed according to the manufacturer's instructions. Cytokine concentrations were determined in triplicates and are expressed as mean ± standard deviation.

Cell survival and apoptosis. For determination of the number of living cells, the monocyte cultures were treated with trypsin for 30 min at 37°C. Using a hemacytometer cells in four representative fields were counted. Dead and live cells were distinguished by trypan blue exclusion.

Apoptosis was determined via DNA fragmentation by the 'Cell Death Detection ELISA', (Boehringer Mannheim, Germany) following instructions provided by the manufacturer. Measurements were performed in triplicates.

Cell proliferation. Proliferation of cell populations was determined in 96 well microtiter plates. 10^5 monocytes per well were pulsed for 24 h with 0.5 μCi ^{3}H-thymidine, harvested and evaluated on the TopCount microplate scintillation counter (Packard, Meriden, CT). Each condition was performed in triplicate and results are depicted as means ± standard deviation.

RESULTS

Additive Effects of CD137 and LPS on Monocyte Activation

Monocytes were cultured on tissue culture dishes coated with a fusion protein consisting of the extracellular domain of CD137 and the constant domain of human immunglobulin G1 (Fc). Coating was performed with a solution of 1 μg/ml protein in PBS at 4°C overnight unless otherwise indicated. Untreated and Fc protein coated plates were used as controls. CD137 induced low (around 5 ng/ml), but significant levels of IL-8 (Fig. 1A). No IL-8 was detectable in control cultures. LPS at 10 ng/ml induced about six-fold higher levels of IL-8. No further increase in IL-8 concentrations were obtained when the monocytes were activated with 50 or 100 ng/ml of LPS, indicating that at 10 ng/ml monocyte activation by LPS was already maximal. Interestingly, the combination of LPS and CD137 further enhanced IL-8 expression even after maximal LPS activation of the monocytes (Fig. 1A). This implies separate signaling pathways for CD137 and LPS.

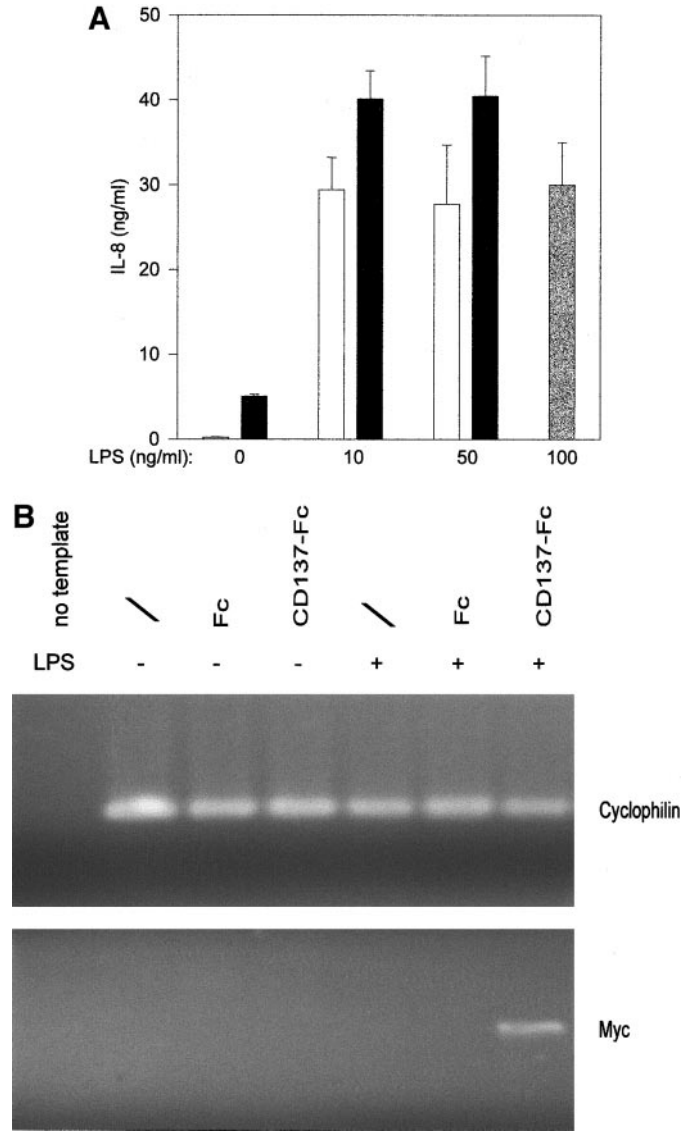

FIG. 1. Induction of monocyte activation by CD137 and LPS. (A) Expression of IL-8. 10^5 primary monocytes were cultured on 1 μg/ml of immobilized Fc (white bars) or CD137-Fc protein (black bars) with increasing concentrations of LPS. Gray bar: LPS only. Supernatants were harvested after 16 h and concentrations of IL-8 were determined by ELISA. Similar results were obtained in three separate experiments. (B) Expression of myc. 10^6 primary monocytes were cultured on untreated 6 well plates (control) or on plates with immobilized Fc or CD137-Fc protein (1 μg/ml) in the absence and presence of LPS (10 ng/ml). Expression of myc and cyclophilin were evaluated by RT-PCR. Cyclophilin served as a control for comparable amounts of cDNAs.

A sensitive marker for cellular activation is the expression of myc (16). No induction of myc mRNA ex-

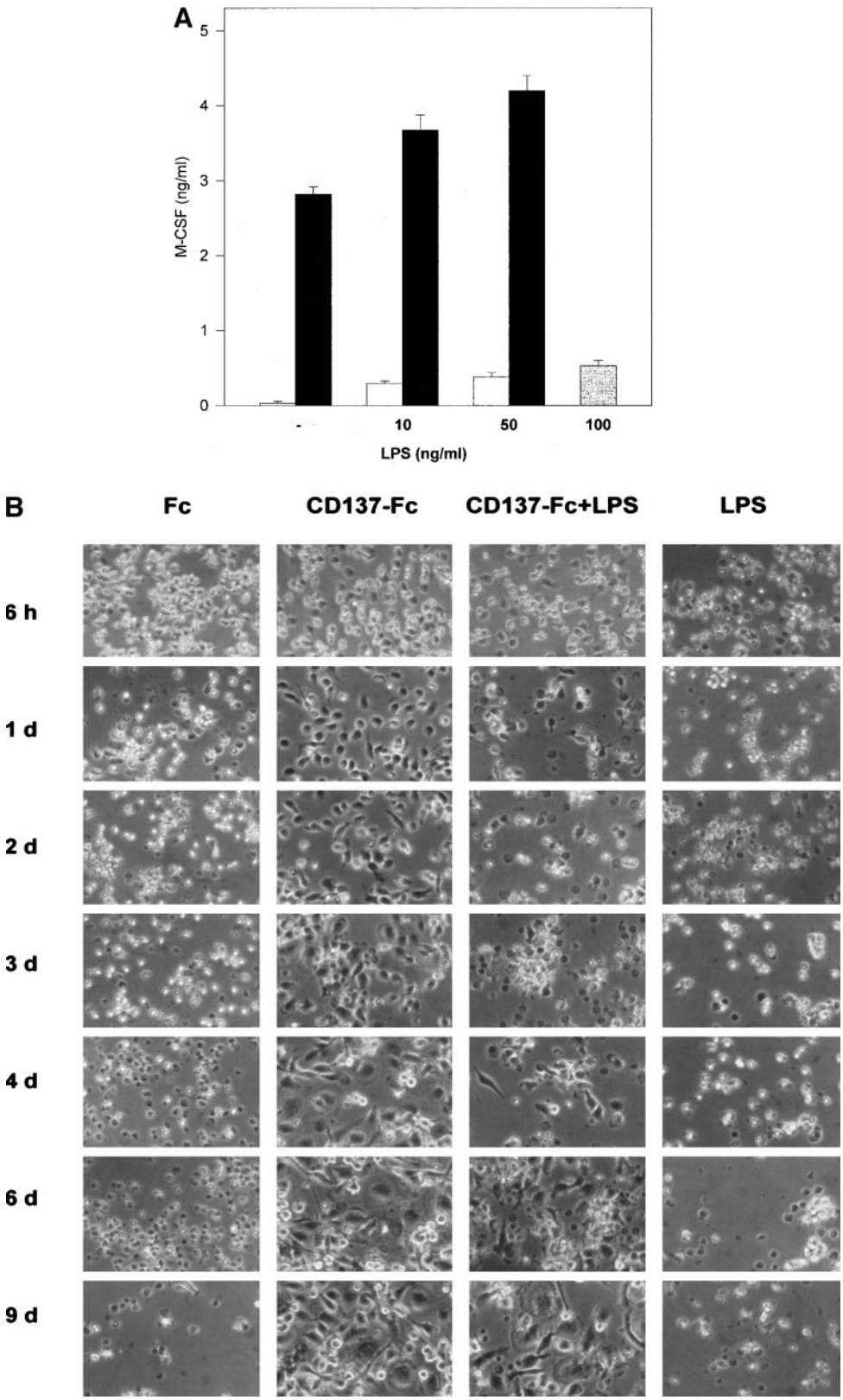

FIG. 2. Regulation of monocyte survival by CD137 and LPS. (A) Induction of M-CSF. 10^5 primary monocytes were cultured on immobilized Fc c; white bars) or CD137-Fc protein (1 μg/ml; black bars) with increasing concentrations of LPS. Gray bar: LPS only. Supernatants were harvested after 3 days and concentrations of M-CSF were determined by ELISA. Similar results were obtained in three separate experiments. (B) 10^5 primary monocytes were cultured on immobilized Fc (1 μg/ml) or CD137-Fc protein (1 μg/ml), or LPS (10 ng/ml), or a combination of LPS and CD137. Photographs were taken at indicated times at a magnification of 250×. This experiment was repeated five times with cells from different donors and produced similar results.

pression could be detected in monocytes activated by either CD137 or LPS alone (Fig. 1B). The fact that the combination of both activators did induce myc expression also argues for an additive effect of CD137 and LPS on monocyte activation and for the utilization of separate signaling pathways by the two compounds.

Antagonistic Effects of CD137 and LPS on Monocyte Survival

An additive effect of LPS and CD137 was also observed in induction of the essential monocyte survival factor monocyte/macrophage colony-stimulating factor (M-CSF). Contrary to the case of IL-8, CD137 was by far more potent than LPS in inducing M-CSF expression (Fig. 2A).

CD137 also mediates adherence and prolongs the survival of monocytes over several days (Fig. 2B). LPS induced monocyte adherence only during the first hours of culture and no extension of survival time could be observed. On the contrary, when monocytes were treated simultaneously with LPS and CD137, LPS reduced CD137-induced adherence and survival (Fig. 2B).

This negative contribution of LPS to monocyte survival was surprising because LPS further augmented CD137-induced expression of M-CSF. An explanation for this discrepancy could be activation induced cell death (AICD). Upon activation, a certain percentage of the cells die by apoptosis and the extent of this cell death correlates with the degree of the cellular activation (17). Since CD137 and LPS together induce a stronger activation than either stimulus alone, as evidenced by the additive effect on cytokine induction, a higher incidence of AICD could be expected. We verified this hypothesis by measuring internucleosomal DNA fragmentation, a typical occurrence of an apoptotic cell death. CD137 as well as LPS alone did induce apoptosis in monocytes, but as predicted by the concept of AICD, significantly higher levels of apoptosis were induced by the combination of the two activators (Fig. 3).

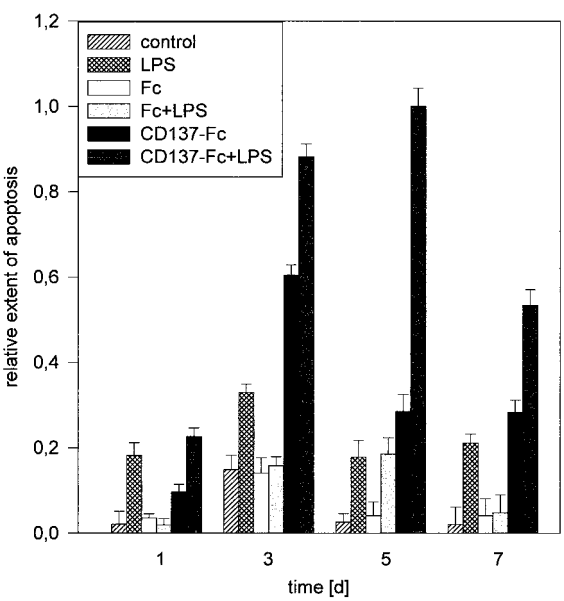

FIG. 3. Induction of monocyte apoptosis by CD137 and LPS. 10^5 primary monocytes were cultured on immobilized Fc (0.5 μg/ml) or CD137-Fc protein (1 μg/ml) or on untreated plates (control) or activated by 10 ng/ml LPS. The extent of apoptosis was evaluated at day 1, 3, 5 and 7 and the extent of apoptosis (x-axis) is given as OD values. Error bars indicate standard deviation. The differences in rates of apoptosis and the number of living cells between Fc and CD137-Fc treated cultures were significant starting at day 3 and 5, respectively, with P values < 0.05. Comparable results were obtained in three separate experiments.

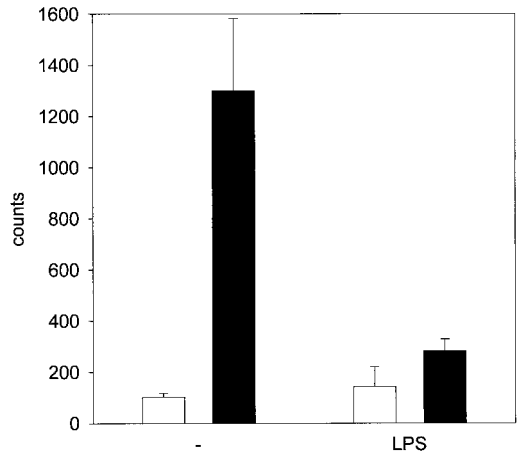

FIG. 4. CD137, but not LPS induces proliferation of peripheral monocytes. 10^5 peripheral monocytes were cultured on immobilized Fc (0.5 μg/ml; white bars) or CD137-Fc protein (1 μg/ml, black bars) and/or activated by 10 ng/ml LPS. Proliferation was determined at day 10 by ^{3}H-thymidine incorporation. Identical results were obtained in three independent experiments.

Work from several laboratories has shown convincingly that the ability of monocytes to attach to a solid support strongly increases their survival *in vitro* (18, 19). Our results are in line with these findings. CD137 which promoted monocyte survival also induced cell attachment while LPS promoted neither attachment nor cell survival (Fig. 3).

CD137 but Not LPS Induces Proliferation in Monocytes

CD137 can induce proliferation and endomitosis in primary monocytes (9). When monocytes were cultured for eight days on immobilized CD137 it induced an increase in the proliferative rate by more than an order of magnitude (Fig. 4). The Fc control protein or LPS did not elicit monocyte proliferation. As in the case of monocyte survival, LPS counteracted the CD137-induced monocyte proliferation and inhibited it almost completely (Fig. 4).

DISCUSSION

CD137 and LPS induced activation of monocytes as evidenced by an increased expression of the proinflammatory cytokine IL-8. LPS was found to be the stronger activator for monocytes. As a component of the cell wall of gram-negative bacteria, LPS signals to monocyte the imminent danger of a bacterial infection. Since monocytes are part of the innate immune response and the first barrier to invading microorganisms, their strong and fast activation by LPS is vital.

Under which physiological conditions CD137 initiates monocyte activation is not yet fully understood. CD137 is expressed on activated T lymphocytes while CD137 ligand is constitutively expressed as a cell surface protein on monocytes and B lymphocytes (10, 20). Both proteins interact at cognate T lymphocyte-monocyte interactions and both transduce an activating signal into the cell they are expressed on (7–10, 12). This suggests a role for the CD137 receptor/ligand system in the coordinated activation of the acquired immunity.

The different functions of CD137 and LPS in the regulation of immune responses is reflected in separate signal transduction pathways. Two lines of evidence support this notion: (1) Maximal induction of IL-8 by LPS could be further increased by CD137; (2) myc was induced only by the combination of CD137 and LPS, and, (3) expression of M-CSF was induced additively by CD137 and LPS.

LPS, in spite of delivering the stronger activating signal than CD137, did not promote monocyte survival. Further, in spite of enhanced expression of M-CSF when monocytes were activated by the combination of CD137 and LPS, less cells survived compared to monocytes which were treated solely with CD137.

Activation and proliferation are intimately and inseparably connected with the apoptotic program of a cell (16). This connection provides a safeguard against uncontrolled cell division and is maintained by the transcription factor myc. The transcriptional activity of myc is essential for and correlates with the degree of proliferation and activation. Simultaneously, myc induces proapoptotic genes/proteins and lowers the threshold for apoptosis. Under physiological conditions apoptosis in activated cells is blocked by survival factors. Therefore, the degree of activation also correlates with the need for survival factors.

Since LPS induces a strong activation, but little survival factor, LPS activated monocytes die. CD137 induces a weaker activation, accompanied by a profoundly higher M-CSF expression, and therefore the monocytes can survive. This situation in monocytes is similar to that of lymphocytes, where engagement of the T cell receptor induces activation, and engagement of the costimulatory receptor CD28 ensures survival (21, 22). The activating signal through T cell receptor alone leads to activation induced cell death (23). *In vivo,* monocyte survival upon encounter of LPS is likely mediated by factors which are absent at *in vitro* culture conditions.

This study found LPS to be the more potent monocyte activator compared to CD137. While CD137 also induces activation of monocytes it is mainly a survival and proliferation factor. These activities of CD137 on monocytes may be especially important and therapeutically applicable for improvement of conditions were large numbers of moncytes/macrophages are beneficial, such as wound healing.

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