Macrophages Have Cell Surface IL-10 That Regulates Macrophage Bactericidal Activity[1]

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IL-10, which is secreted by multiple cell types, has regulatory effects on macrophages, including decreasing their ability to kill some microorganisms. The experiments presented here test the hypothesis that endogenous IL-10 inhibits the ability of macrophages to kill the facultative intracellular bacterium, *Listeria monocytogenes*. We show that the nonbactericidal macrophage hybrid, H36.12j (12j), can kill *Listeria* after incubation for 3 days with anti-IL-10 mAb. IL-10 was not detected in 12j macrophage supernatants by ELISA. However, flow cytometric analysis revealed high levels of IL-10 on the 12j cell surface. This indicates that macrophages that fail to secrete IL-10 may express IL-10 on the cell surface, and this IL-10 appears to suppress listericidal activity. Surface IL-10 is not unique to the 12j macrophage hybrid and may correlate with the absence of bactericidal activity in other macrophages. For instance, nonlistericidal resident and thioglycolate-elicited peritoneal exudate cells have 24 to 72% IL-10-positive macrophages. In contrast listericidal proteose peptone-elicited peritoneal exudate cells contained <5% IL-10-positive macrophages. Whether this IL-10 is an integral membrane protein or is bound to IL-10 receptors is not yet known. However, the IL-10 does not elute with acid as other passively bound molecules do, nor does exogenous IL-10 bind to macrophages. In either case, since anti-IL-10 induces nonbactericidal macrophages to become bactericidal, the surface IL-10 is biologically active, and it appears to regulate macrophage bactericidal activity. *The Journal of Immunology*, 1996, 156: 1143–1150.

I L-10 was originally identified as a Th2 cytokine that inhibits the secretion of IFN-γ by Th1 (1). This inhibition is macrophage dependent, although the mechanism is unknown (2). IL-10 is also secreted by B cells, Langerhans cells, and human monocytes (3–5). Not only does IL-10 inhibit Th1 cells, but it also affects the function of several other cell types, including macrophages (6–10). Specifically, IL-10 treatment of macrophages decreases both MHC class II expression and the transcription of multiple cytokine genes (2, 3, 11, 12). It has been suggested that by inhibiting IFN-γ secretion, IL-10 inhibits the ability of macrophages to kill intracellular parasites, including *Schistosoma mansoni* (13), *Toxoplasma gondii* (14), and *Trypanosoma cruzi* (15, 16). It has also been suggested that IL-10 enhances the growth of the facultative intracellular bacteria *Brucella abortus* (17), *Mycobacterium avium* and *tuberculosis* (18–20), and *Listeria monocytogenes* (21–23).

Macrophages play a significant role in host defense against *L. monocytogenes*. During a *Listeria*-induced inflammatory response, the macrophage population is heterogeneous; some macrophages can kill *Listeria*, while others are not listericidal (24, 25). For example, proteose peptone-elicited peritoneal exudate macro-

phages kill *Listeria*, while thioglycolate-elicited macrophages do not (24, 25). In permissive thioglycolate-elicited and resident peritoneal macrophages, *Listeria* can invade the macrophage, replicate within it, and then spread to other macrophages. Because of the heterogeneity of the macrophage response to *Listeria*, this bacterium is frequently used as a model for studying macrophage bactericidal activity.

Based on reports that not all macrophages can kill *Listeria* and that IL-10 may inhibit the host response to *Listeria*, we tested whether IL-10 was responsible for the inability of some macrophages to kill *L. monocytogenes*. The data presented here show that the nonlistericidal macrophage hybridoma, H36.12j (26), does not secrete IL-10 detectable by ELISA. However, immunoreactive IL-10 is present on the cell surface of 12j cells. In addition, nonbactericidal resident peritoneal macrophages and thioglycolate-elicited macrophages have immunoreactive IL-10 on their cell surfaces. Moreover, this cell surface IL-10 has biologic activity, since treating IL-10-bearing nonlistericidal 12j macrophages with anti-IL-10 mAb induces them to express bactericidal activity, and the surface IL-10 down-regulates IL-10 production by unstimulated macrophages.

Materials and Methods

Cell lines

H36.12j (12j) is a clonally derived macrophage precursor hybrid produced by fusion of drug-selected P388D$_1$ macrophages with Percoll-separated, proteose peptone-elicited macrophages, as described previously (26). The 12j hybrid is a nonadherent macrophage hybrid that has previously been found to be nonlistericidal when treated with IFN-γ, TNF-α, LPS, or combinations of these macrophage stimulators (data not shown). The TNF-α-sensitive WEHI 164.13 fibrosarcoma cell line (provided by A. Zlotnik, DNAX, Palo Alto, CA) and the nonadherent 12j hybrid were cultured in RPMI 1640 (Life Technologies, Grand Island, NY), 2 mM L-glutamine (Life Technologies), 50 μM 2-ME, and 10% FCS (Hyclone Laboratories Inc., Logan, UT). The presence of gentamicin in culture medium can inhibit the growth of *Listeria* in macrophages (27); therefore, all cells were routinely cultured, and assays were performed in the complete absence of antibiotics. The B cell lymphoma, CH12.LX (graciously provided by

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Dr. J. Hagman, National Jewish Center, Denver, CO), was grown in RPMI 1640, 5% heat-inactivated FCS, 5% heat-inactivated calf serum (Life Technologies), 2 mM L-glutamine, 50 μM 2-ME, 100 mM nonessential amino acids (Life Technologies), and 1 mM sodium pyruvate (Irvine Scientific, Santa Ana, CA) (28).

Bacteria

Log phase cultures of *L. monocytogenes*, strain EGD, were inoculated into 5 ml tryptose phosphate broth (Difco, Detroit, MI), cultured overnight at 37°C, and used in the study.

Mice

Female, 3- to 5-mo-old, (C57Bl/6 $\times$ DBA$_2$)F$_1$ mice were obtained from Taconic Farms (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME) and kept in the specific pathogen-free animal facility at the National Jewish Center.

Cytokines, antibodies, and reagents

Recombinant mouse IL-10 was obtained from Genzyme (Cambridge, MA) and had a specific activity of approximately 5×10^5 U/mg. The manufacturer determined the activity using the MC-9 cell proliferation assay. IgG1 anti-mouse IL-10 Abs were purchased from either Genzyme or PharMingen (San Diego, CA). The IgG1 rat isotype control Ab was purchased from PharMingen. FITC-labeled F(ab')$_2$ fragments of goat anti-rat Ig used for fluorescent staining were purchased from Jackson ImmunoResearch (West Grove, PA). *R*-Phycoerythrin-conjugated, rat anti-mouse CD11b (M1/70.15) and normal rat Ig were obtained from Caltag Laboratories (San Francisco, CA). The F4/80 mAb was kindly provided by Dr. Pieter Leenen (Eramus University, Rotterdam, Holland). For cytokine stimulation, *Escherichia coli* 055:B5 LPS was purchased from Sigma Chemical Co. (St. Louis, MO) and used as a positive control.

Peritoneal macrophages

Resident peritoneal cells were obtained as described previously (25) by peritoneal lavage with 20 ml of ice-cold balanced salt solution (BSS).[4] Proteose peptone-elicited peritoneal exudate cells (PEC) were obtained by peritoneal lavage 48 h after the injection of 1 ml of sterile 10% proteose peptone solution (Difco). Thioglycolate-elicited (Difco) PEC were obtained 4 days after the injection of 1.5 ml of sterile 4% thioglycolate medium.

Phagocytosis

Phagocytosis was measured as described previously with slight modifications (24, 25). Briefly, equal numbers of 12j cells and *Listeria* (1×10^6) were rotated for 20 min in 500 μl of BSS containing 10% normal mouse serum (NMS). The cells were washed three times to remove extracellular bacteria, and cytocentrifuge preparations were made. At the end of these washes, approximately 90% of the bacteria were intracellular (25). Phagocytosis was determined by counting under oil immersion the number of bacteria in 100 to 150 macrophages/slide. The phagocytic index was calculated as (% macrophages containing ≥ 1 bacterium) $\times$ (mean bacteria/positive cell).

Bactericidal assay

The bactericidal assay used was modified slightly from that described previously (24). Briefly, the cells and bacteria were incubated for 20 min as in the phagocytosis assay. The cells were then washed three times with 1 ml of BSS to remove extracellular bacteria. Cells were resuspended in BSS with 5% NMS and incubated for 90 min at 37°C. At the beginning and the end of the 90-min incubation, 0.1 ml was removed from the suspension, diluted in sterile water, and plated on trypticase soy agar (Remel, Lenexa, KS) in duplicate. Bacteria colonies were counted 24 h later, and the log$_{10}$ *Listeria* CFUs at 0 and 90 min were determined and are reported as the log$_{10}$ *Listeria* CFUs. Using this assay, a decrease in the log$_{10}$ *Listeria* CFUs after the 90-min incubation period shows killing of the bacteria by the macrophages, while an increase in log$_{10}$ *Listeria* CFUs reflects intracellular growth of bacteria.

Fluorescence staining and analysis

After being washed in PBS, 1×10^6 cells were incubated on ice for 15 min in 100 μl of staining buffer (PBS containing 2-5% FCS and 0.2% sodium

azide) and 2 μg of either an IgG1 anti-mouse IL-10 mAb or an IgG1 rat isotype control Ab. The cells were washed three times with staining buffer and then incubated for 15 min on ice with FITC-labeled F(ab')$_2$ fragments of goat anti-rat Ig. After an additional three washes in 150 μl of staining buffer and resuspension in 1 ml of staining buffer, the cells were analyzed using an Epics C flow cytometer (Coulter Electronics, Inc., Hialeah Park, FL) equipped with a profile flow cell and an argon ion laser tuned to 488 nm and run at 15 milliwatts, a FACScan flow cytometer (Becton Dickinson, San Jose, CA), or an ELITE flow cytometer (Coulter Electronics). The log fluorescence intensity of 5,000 to 10,000 macrophages from each sample was displayed as a frequency distribution histogram. To reduce background staining levels, peritoneal macrophages were incubated with 5% NMS for 10 min before staining with the primary Abs, and the secondary F(ab')$_2$ fragments were preincubated with an equal amount of NMS for 10 min and then brought to the final volume in staining buffer. After staining with the FITC-labeled secondary Ab, the cells were washed twice with staining buffer, incubated with normal rat Ig for 5 min, centrifuged, and then incubated with phycoerythrin-conjugated rat anti-mouse CD11b (M1/70.15) for 15 min. This was followed by three additional washes before resuspension in 1 ml of staining buffer and analysis by flow cytometry. Macrophages were selected by gating, using forward light scatter and right angle scatter. In addition, staining with M1/70 and F4/80 was used to identify and select macrophages and eliminate medium and large lymphocytes.

Acid elution

To attempt to elute IL-10 that might be bound to a surface receptor, acid elution similar to that described for cell-bound Ig (29) was used. The cells were pelleted by centrifugation and resuspended in acetate buffer, pH 4.4, containing 0.05 M sodium acetate, 0.09 M NaCl, 0.005 M KCl, and 0.05% FCS. The suspension was neutralized after 1 to 15 min by adding PBS containing 0.05% FCS. The suspended cells were immediately underlayed with 1 ml of FCS and centrifuged to pellet the cells. The cells were then washed three times and stained as indicated above. Up to 15 min of acid treatment did not significantly change the cell viability, as determined by forward and right angle scatter, trypan blue exclusion (98% viable untreated vs 93% viable after acid elution), and propidium iodide exclusion (98.4 $\pm$ 0.4% viable untreated vs 97.8 $\pm$ 0.5% viable cells after acid elution). In addition, others have found that acid treatment of lymphocytes for 3 min did not decrease cell viability (29).

IL-10 ELISA

Supernatants from overnight cultures of 2×10^5 cells/ml or from 12j macrophages treated for 3 days were collected, centrifuged at $250 \times g$ to remove cells, and stored at -20°C until assayed. The 12j cell lysate was obtained by incubating 1×10^6 washed cells with 1 ml of PBS containing 0.5% Nonidet P-40 (Sigma Chemical Co.) for 15 min on ice. The lysate was centrifuged for 5 min at 15,000 rpm to remove cellular debris. These lysates were stored at -20°C until assayed. The quantity of IL-10 in the supernatant was determined using an IL-10 ELISA kit (Endogen, Boston, MA). The manufacturer states that the assay is sensitive to 0.14 U/ml, which is equivalent to 35 pg/ml of IL-10, and does not cross-react with IL-2, IL-3, IL-4, granulocyte-macrophage CSF, or TNF-α. The ELISA plate was read at OD$_{450}$ on a Bio-Kinetics Microplate Reader (Bio-Tek Instruments, Winsooki, VT). Replicate measurements were within 10% of each other. Each sample was compared with standard IL-10 diluted in culture supernatant and validated as indicated in the kit.

TNF-α assay

The cellfree supernatants used above were diluted in serial twofold dilutions and incubated with the TNF-α-sensitive cell line, WEHI 164.13 (30). After 16 to 18 h, tetrazolium dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, was added for 4 h. After dissolving the formazan crystals, the OD$_{570}$ was determined using an ELISA reader, and the results were compared with a standard curve of recombinant TNF-α (R&D, Minneapolis, MN) to determine the nanograms of TNF-α per milliliter.

Determination of nitrite concentration

The amount of nitrite produced by the macrophages was measured using the Greiss procedure, as described previously (31). Briefly, 100 μl of the cellfree supernatant prepared above was added to 100 μl Greiss reagent (1% sulfanilamide, 0.1% naphthylethylene diamine, and 2.5% H$_3$PO$_4$ in distilled H$_2$O). After 10-min incubation at room temperature, the OD$_{550}$ was read with an ELISA reader, and the nitrite concentration was determined by comparison with a standard curve of NaNO$_2$ concentrations.

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[4] Abbreviations used in this paper: BSS, balanced salt solution; PEC, peritoneal exudate cells; NMS, normal mouse serum; FcR, Fc receptor.
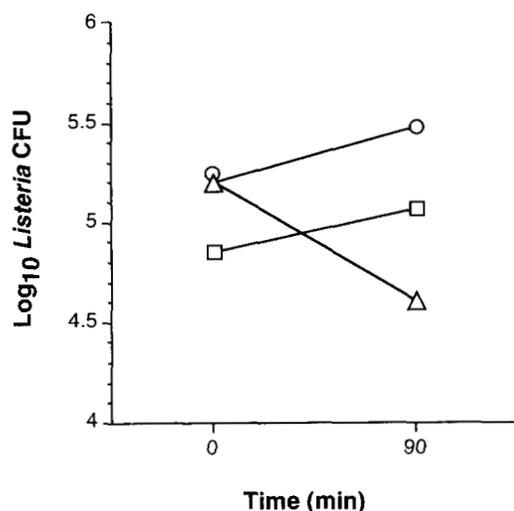
FIGURE 1. Incubation of 12j macrophages with anti-IL-10 mAb induces macrophage bactericidal activity. Untreated (□), anti-IL-10 mAb-treated (△), or isotype control-treated (○) 12j cells were incubated with an equal number of *L. monocytogenes* for a 20-min phagocytosis period before washing three times to remove extracellular bacteria. An aliquot was removed, and the number of bacteria within the cells was determined. Another aliquot of the resuspended cells was removed after 90 min of incubation, and *Listeria* CFUs were determined. This experiment is one of four experiments with similar results.

Statistical analysis

Statistical analysis was performed using the computer software WormStat produced by Small Business Computers of New England (Amherst, NH).

Results

Anti-IL-10 monoclonal antibody induces 12j macrophages to kill L. monocytogenes

It has been reported that macrophages transcribe IL-10 mRNA and that IL-10 can down-regulate many cytokines and activities of macrophages (6–10). Therefore, we hypothesized that IL-10 produced by nonbactericidal 12j macrophages prevents these cells from killing *Listeria*. To test this, 12j macrophages (2×10^5/ml) were cultured with 2 μg/ml of anti-IL-10 mAb for 3 days. On day 3, bactericidal activity and phagocytosis were determined. Figure 1 shows that anti-IL-10-treated 12j macrophages killed *L. monocytogenes*, as indicated by a decrease in the number of *Listeria* CFU after the 90-min culture period. In contrast, the *Listeria* grew within macrophages incubated without mAb or with isotype control Ab (Fig. 1). Kinetics and dose experiments showed that culturing macrophages for 3 days with 2 μg/ml anti-IL-10 mAb induced maximal listericidal activity (data not shown). The possibility of endotoxin contamination contributing to induction of bactericidal activity was ruled out by incubating 12j macrophages with LPS (from 0.1 ng/ml to 1 μg/ml). LPS did not induce 12j macrophages to kill *Listeria* (data not shown). In addition, including polymyxin B, an LPS inhibitor, with the anti-IL-10 treatment did not change the bactericidal activity (data not shown).

It is possible that 12j cells are poorly phagocytic and that anti-IL-10 increases the cell's ability to phagocytose and, consequently, to kill this bacterium. To test this, the phagocytic index of untreated and anti-IL-10-treated 12j cells was determined. Figure 2 shows that phagocytosis of *Listeria* by anti-IL-10-treated 12j cells was not different from that by untreated 12j cells or 12j cells that were treated with isotype control Ab. This indicates that the ability
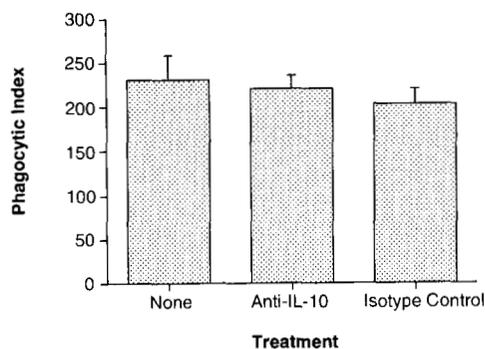

FIGURE 2. Anti-IL-10 mAb treatment does not change phagocytosis by 12j macrophages. Anti-IL-10 mAb-treated, isotype control mAb-treated, or untreated 12j cells were incubated with an equal number of *L. monocytogenes* for a 20-min phagocytosis period and then washed. The phagocytic index (% macrophages containing $\geq$ 1 bacterium) $\times$ (mean bacteria per positive cell) of the treated cells was compared with that of cells that received no treatment. Each point is the mean $\pm$ SEM of five or six experiments.

Table I.   *IL-10 secreted by various cell lines*

Cell Line	IL-10 (pg/ml)[a]
12j	$<35 \pm 0$[b]
12j + *Listeria* T0[c]	40 ± 5
12j + *Listeria* T90[c]	178 ± 24
12j lysate[d]	432 ± 61
CH12.LX[e]	$11,431 \pm 1,525$

[a] Overnight supernatants were collected from cells seeded at 2×10^5 cells/ml and frozen at $-20°$C until assayed as described in *Materials and Methods*. Units/ml were determined by comparison with a standard curve and then converted to pg/ml. Numbers are the mean $\pm$ SEM of 3 to 4 separate samples, each run in duplicate.

[b] Level of detection of the assay is 35 pg/ml. This sample was below the level of detection.

[c] Equal numbers of 12j macrophage hybrid cells and *Listeria* were mixed and incubated for 20 min to allow phagocytosis to occur. The cells were centrifuged and the time 0 (T0) supernatants collected. Extracellular bacteria were removed by washing three times with BSS and the cells incubated for an additional 90 min to allow killing of the intracellular bacteria. After 90 min (T90), the cells were centrifuged and supernatants collected.

[d] The 12j cell lysate was obtained as described in *Materials and Methods* and stored at $-20°$C until assayed.

[e] CH12.LX is a B cell lymphoma previously reported to secrete IL-10 and used as a positive control.

of anti-IL-10 mAb to induce 12j macrophages to kill *Listeria* does not derive from it increasing the phagocytosis of bacteria.

Little IL-10 is secreted by 12j macrophages

Because anti-IL-10 mAb induced 12j macrophages to develop bactericidal activity, we tested whether these cells secreted IL-10. The B cell lymphoma, CH12.LX, was used as a positive control. As shown in Table I, CH12.LX cells secreted 11,431 pg/ml of IL-10, while equal numbers of 12j macrophage hybrids did not secrete detectable amounts of IL-10. This indicates that although treatment with anti-IL-10 Ab induces 12j cells to become listericidal, it does not do so by neutralizing secreted IL-10. Low quantities of IL-10 (432 pg/ml) were found in the 12j whole cell lysate. Very low amounts of IL-10 were secreted following phagocytosis of *Listeria* (40 pg/ml), and IL-10 was increased slightly when 12j cells were incubated with *Listeria* for the 90-min killing period (178 pg/ml).

Secretion of regulatory molecules by 12j macrophages after incubation with anti-IL-10 mAb

The amount of IL-10 secreted by 12j macrophages increased significantly ($p = 0.005$) after incubation with anti-IL-10 mAb for
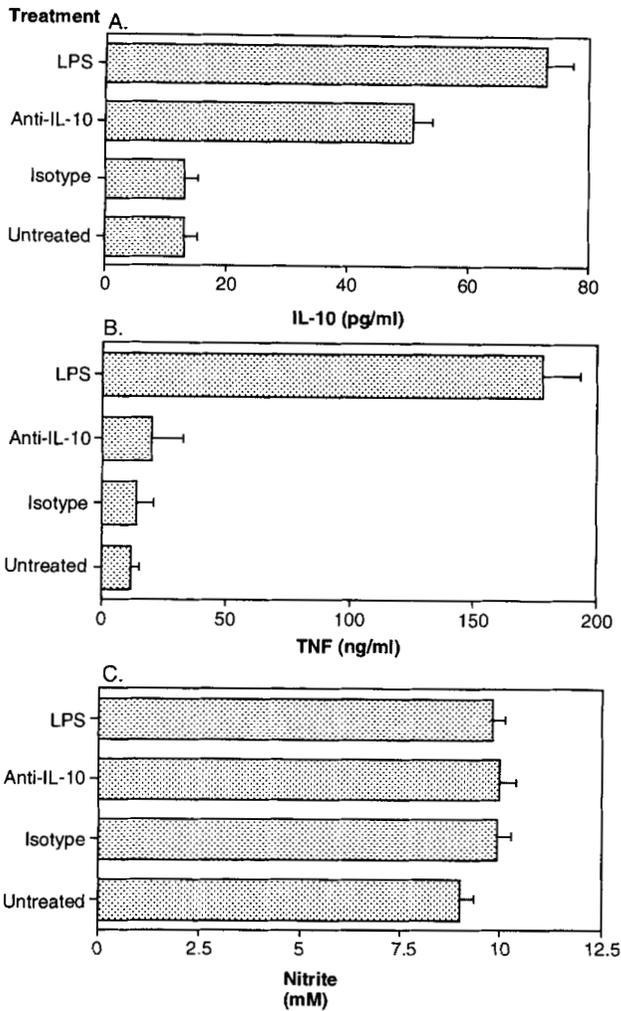
FIGURE 3. Secretion of regulatory molecules by 12j macrophages after 3 days of incubation with anti-IL-10 mAb. Supernatants from anti-IL-10 mAb-treated, isotype control mAb-treated, LPS (1 μg/ml)-treated, or untreated 12j cells were collected, and secreted products were assayed in triplicate as described in *Materials and Methods*. Data represent the mean ± SEM of secreted product in one representative experiment of one to three independent experiments. Significance was determined using WormStat. *A*: LPS vs anti-IL-10, $p = 0.05$; anti-IL-10 vs isotype or medium, $p = 0.005$. *B*: LPS vs anti-IL-10, isotype or untreated, $p = 0.0001$.

FIGURE 4. Flow cytometric analysis of 12j macrophages stained with anti-IL-10 mAb. As described in *Materials and Methods*, 12j macrophages were stained, then analyzed on the FACScan flow cytometer. Cells were not stained (Autofluorescence) or were stained with secondary Ab only (2° Only), isotype control Ab plus secondary Ab (Isotype Control + 2°), or anti-IL-10 mAb plus secondary Ab (Anti-IL-10 + 2°).

brids express IL-10 on their cell surface. High levels of specific binding were detected when 12j cells were stained with anti-IL-10 mAb and a FITC-labeled secondary F(ab′)$_2$ fragment (Fig. 4). Controls, including secondary Ab only and isotype control Ab plus secondary Ab, were negative. Similar results were obtained when F(ab′)$_2$ fragments were used and when Fc receptors (FcR) were blocked using 20% heat-inactivated NMS (data not shown). In contrast, CH12.LX cells were negative when stained with anti-IL-10 mAb (data not shown). These experiments indicate that 12j cells display immunoreactive IL-10 epitopes on the cell surface.

To rule out the possibility that anti-IL-10 mAb binds nonspecifically to the surface of 12j cells, we tested whether adding large amounts of exogenous IL-10 could compete with cell surface IL-10 and prevent staining with anti-IL-10 mAb. When 12j cells were incubated with 100 U/ml IL-10 and 20 μg/ml anti-IL-10 mAb simultaneously, exogenous IL-10 competed for binding of the Ab to the cell surface, as shown by decreased staining of 12j cells (Fig. 5). This indicates that the mAb recognizes IL-10 on the cell surface specifically and not some other cell surface molecule.

The immunoreactive cell surface IL-10 does not appear to be the result of IL-10 binding to an IL-10 receptor

It is also possible that IL-10 is being secreted but immediately binds to an IL-10 receptor that may exist on the macrophage cell surface. Two approaches were used to test this. In one set of experiments we tested whether exogenous IL-10 could bind to 12j macrophages and cause increased surface staining. No change in the relative fluorescence was observed when 12j macrophages were incubated with 100 U/ml IL-10 for either 30 min at 4°C (data not shown) or 15 min at room temperature (Fig. 6). The lack of significant change in mean channel fluorescence suggests that IL-10 is not passively adhering to the cell surface and/or binding to putative IL-10 receptors.

Because these experiments did not rule out the possibility that all IL-10 receptors (if they exist on these cells) are already saturated with endogenously secreted IL-10, a second series of experiments was performed. Kumagai et al. (29) found that incubation of cells in acetate buffer, pH 4 or pH 5, removed Ig from FcR. Preliminary experiments showed that all IL-10 could be eluted from the surface of the mast cell line, MC/9, by incubation for 1 min in acetate buffer, pH 4.4 (data not shown). Moreover, 12j cells

3 days compared with amounts secreted by untreated and isotype control Ab-treated 12j cells (Fig. 3*A*). However, 1 μg/ml LPS stimulation resulted in the 12j macrophages secreting significantly more IL-10 than that seen after anti-IL-10 treatment ($p = 0.05$). In contrast, there was no increase in TNF-α or nitrite production by this macrophage hybrid after 3 days of treatment with anti-IL-10 compared with that after incubation with the isotype control Ab (Fig. 3, *B* and *C*). The lack of IL-10 secretion by unstimulated 12j macrophages is similar to that observed in other reports using primary mouse macrophages (20). In contrast, 12j macrophages are constitutive producers of nitrite (Fig. 3*C*). These data suggest that the cell surface IL-10 may also regulate the secretion of IL-10.

Immunoreactive IL-10 epitopes are displayed on the cell surface of 12j macrophages

Since IL-10 was not secreted by 12j macrophages, we used flow cytometric analysis to determine whether the 12j macrophage hy-
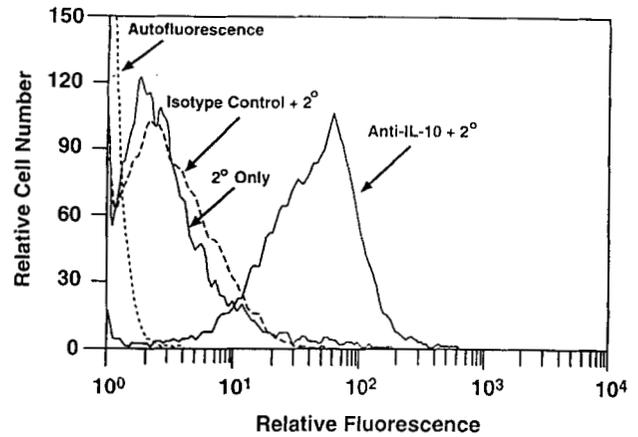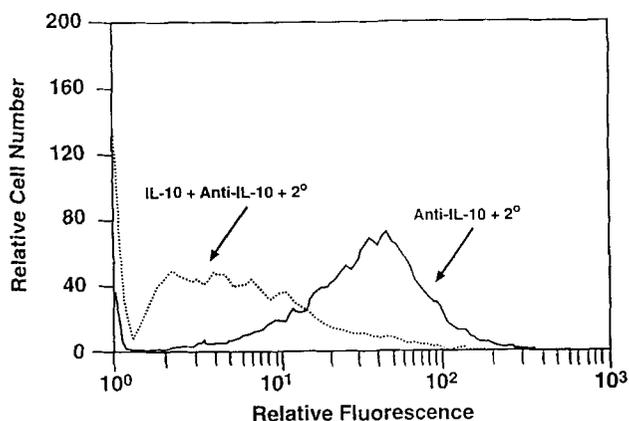
FIGURE 5. IL-10 competitively inhibits 12j macrophage surface staining by anti-IL-10 mAb. 12j macrophages were incubated with 100 U/ml IL-10 and 20 μg/ml anti-IL-10 mAb simultaneously. This was followed by a FITC-labeled secondary Ab (IL-10 + Anti-IL-10 + 2°). Positive control cells were stained with anti-IL-10 mAb followed by secondary Ab (Anti-IL-10 + 2°). The Epics C flow cytometer was used to detect relative fluorescence.
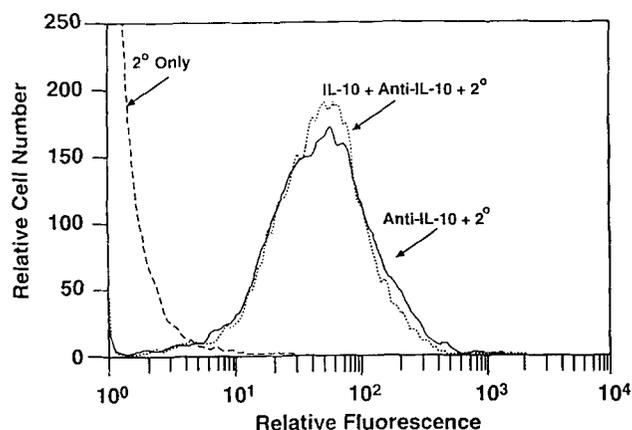

FIGURE 6. Flow cytometric analysis of 12j macrophages preincubated with IL-10 show no change in relative fluorescence after staining with anti-IL-10 mAb. 12j macrophages were incubated with 100 U/ml IL-10 for 15 min at room temperature before staining with anti-IL-10 mAb and secondary Ab (IL-10 + Anti-IL-10 + 2°). Controls cells were stained with secondary Ab only (2° Only) or with anti-IL-10 mAb and secondary Ab (Anti-IL-10 + 2°). All cells were analyzed on a FACScan flow cytometer.

could be incubated in the acid buffer for up to 15 min with minimal cell lysis (>93% viability), as determined by gating, trypan blue exclusion, and propidium iodide exclusion. Figure 7 shows that there was no change in the mean fluorescence of 12j cells stained with anti-IL-10 after 1 min in the acid buffer with (Fig. 7C) or without (Fig. 7B) incubation with exogenous IL-10. This indicates that the staining of 12j cells by anti-IL-10 mAb is probably not due to IL-10 being secreted and immediately binding to IL-10 receptors or other molecules on the cell surface.

Surface IL-10 is present on certain mouse macrophages and may correlate with the absence of bactericidal activity

To determine whether cell surface IL-10 is unique to 12j cells, we asked whether mouse peritoneal macrophages stain with anti-IL-10 mAb. As indicated in Figure 8, the macrophage population (determined as indicated in *Materials and Methods*) contained in
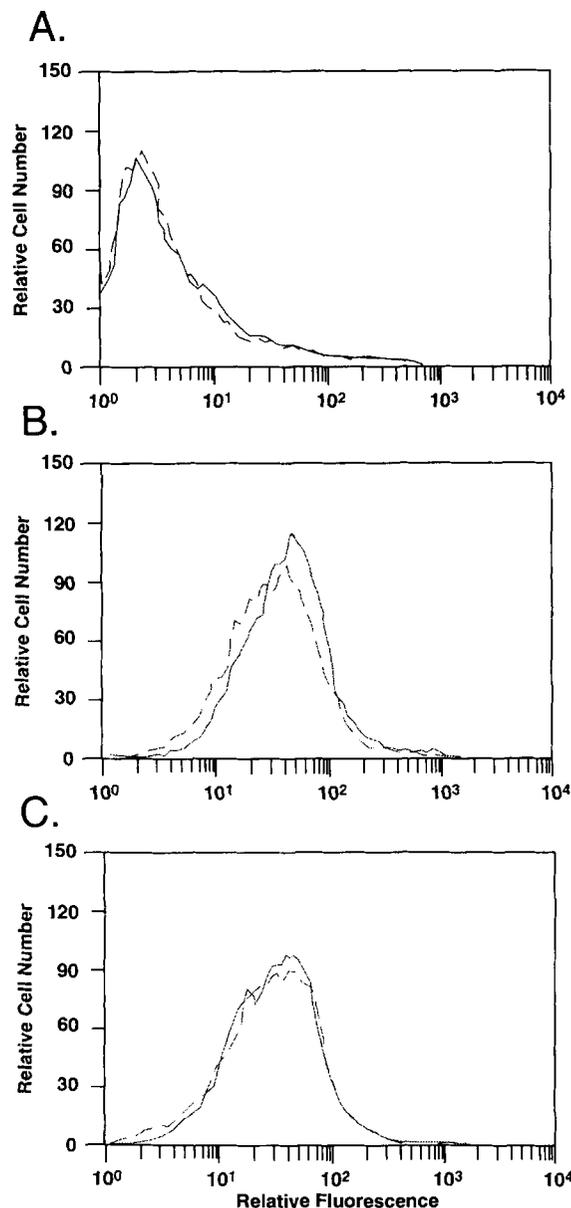

FIGURE 7. Cell surface anti-IL-10 staining does not elute at acidic pH. 12j macrophages were incubated in acetate buffer, pH 4.4 (----), as described in *Materials and Methods*, or in PBS (—), and then all groups were stained for IL-10 with anti-IL-10 mAb and secondary Abs. *A*, Background staining with the isotype control Ab and FITC-labeled secondary Ab. *B*, 12j macrophages were incubated with acid buffer (----) or with PBS (—), and then stained with anti-IL-10 mAb and secondary Ab. *C*, 12j macrophages were incubated with IL-10 before acid elution (----) or PBS incubation (—) and then stained with anti-IL-10 mAb and secondary Ab. All cells were analyzed on a FACScan flow cytometer.

nonlistericidal resident PEC and thioglycolate-elicited PEC showed significantly more fluorescence when stained with anti-IL-10 than with the isotype control. In contrast, listericidal proteose peptone-elicited PEC did not stain significantly with anti-IL-10 mAb (Fig. 8). The mean percentage of IL-10-positive cells within each population varied from mouse to mouse (Table II). The mean percentage of IL-10-positive cells was determined by subtracting the percentage of positive cells stained with isotype control Ab from the percentage of positive cells stained with anti-IL-10. As

In the above experiments, it is possible that the anti-IL-10 mAb and immunoreactive surface IL-10 form immune complexes that stimulate the macrophage to become listericidal. However, Tripp et al. (23) found that immune complexes decreased the listericidal activity of splenic macrophages and induced the production of IL-10. This decrease in killing of *Listeria* was inhibited by anti-IL-10 mAb (23). Finally, Frei et al. (22) showed increased growth of *L. monocytogenes* within IFN-γ-stimulated J774A.1 macrophages treated with IL-10. Unfortunately, the IFN-γ-stimulated macrophages did not kill *Listeria*, but merely slowed its growth from six to four doublings over 8 h (22).

It is also not yet clear which cells produce IL-10 in response to *Listeria* infection in vivo. Flesch and Kaufmann (35) suggest that *Listeria* induces IL-10 production by macrophages. This is based on the observation that IL-10 is produced in response to *Listeria* in RAG-1-deficient mice that have no mature B or T cells; thus, IL-10 production is probably by macrophages. Furthermore, they show that IL-10 is produced by adherent spleen cells, presumably macrophages, after the injection of viable *Listeria*, but very little is produced after the injection of heat-killed *Listeria*. In contrast, Song et al. (34) show that after culture with heat-killed *L. monocytogenes*, splenic B cells have increased IL-10 mRNA, as determined by reverse transcription-PCR analysis. We show here that anti-IL-10 mAbs induce macrophages to secrete increased amounts of IL-10. Therefore, in response to *Listeria* or anti-IL-10 mAb, IL-10 apparently can be secreted by either B cells or macrophages.

We tested whether IL-10 is secreted by the clonally derived, nonbactericidal 12j macrophage and found that when unstimulated, these cells do not secrete IL-10 in quantities detectable by ELISA. We also tested whether treatment with anti-IL-10 mAb could induce these macrophages to acquire bactericidal activity. We found that anti-IL-10 mAb did induce bactericidal activity in 12j cells, as indicated by a decrease in the number of intracellular bacteria within these cells after 90-min co-incubation. Thus, anti-IL-10 mAb induces these macrophages to express bactericidal activity, but not because it neutralizes secreted IL-10. In addition, anti-IL-10 mAb treatment induced low level secretion of IL-10, but this does not inhibit bactericidal activity. The ability of the 12j cells to kill *L. monocytogenes* is not due to anti-IL-10 binding to FcR, since high amounts of NMS, which should saturate FcR, did not decrease the killing (data not shown). Moreover, we have previously shown that bacteria must enter macrophages via CR3 to be killed (38), and bacteria are not killed when entry is mediated by FcR.[5]

Flow cytometric analysis demonstrated IL-10 immunoreactivity on the surface of 12j macrophages. In addition, mouse peritoneal macrophages are heterogeneous for surface IL-10 expression; bactericidal PEC express low levels of surface IL-10, while nonbactericidal PEC express high levels of surface IL-10. We have suggested that the IL-10 is an integral membrane protein and not merely a passively-adsorbed soluble cytokine. However, it is also possible that the macrophage is secreting low quantities of IL-10 that adhere to the cell surface as it is secreted. This appears unlikely for two reasons. First, exogenous IL-10 could not be loaded onto the cell surface, as determined by flow cytometry. This concept is supported by experiments showing that HT-2 T cells, which have IL-10 mRNA and intracellular IL-10, do not stain positive for cell surface IL-10 (39), suggesting that secreted IL-10 may not bind to the cell that secretes it. Second, although the IL-10 receptor has been cloned (40–43) and is expressed in low numbers on the surface of J774.A1 macrophages and the mast cell line, MC/9 (40–

41), it is unknown at this time whether IL-10 receptors exist on the surface of 12j macrophages. However, since exogenous IL-10 did not bind to these cells, it must be assumed that IL-10 receptors on the surface of the 12j cells, if they exist, are full. Even though acid pH removed the staining of IL-10 exogenously added to the mast cell line, MC/9, it did not decrease the staining of 12j cells. This suggests that IL-10 is not bound to surface receptors on 12j macrophages. Additional data indicate that cell surface binding of the anti-IL-10 mAb to 12j macrophages is competitively inhibited by exogenous IL-10, showing that the mAb is specific for IL-10. Therefore, the data presented here show that IL-10 exists as a cell surface molecule on 12j macrophages and on some nonlistericidal mouse macrophages and suggest that it might be an integral membrane protein.

To our knowledge, membrane IL-10 has not been reported on any cell surface. However, the concept of a membrane cytokine is not new. TNF-α and IL-1α have both been reported to be secreted and to have a membrane form that is biologically active (44–47), although the existence of membrane IL-1α is controversial (48). As with membrane TNF-α, the surface IL-10 on 12j macrophages is biologically active, since anti-IL-10 mAb can induce these IL-10-bearing, nonlistericidal macrophages to express listericidal activity and to secrete IL-10. This, too, suggests that the surface IL-10 may be an integral membrane protein that can transduce signals.

The mechanism by which membrane IL-10 inhibits macrophage listericidal activity is not known. Two possibilities come to mind. The first is that membrane IL-10, like soluble IL-10, triggers autocrine signals that down-regulate the ability of macrophages to respond to exogenous biologic mediators. In this unresponsive state, the macrophage would not be bactericidal. However, when surface IL-10 is not expressed, the macrophage may be able to respond to other cytokines and become bactericidal. This may reflect a difference in maturational state, strain of mice, or microenvironmental exposure to other immunomodulators. For instance, immature macrophage precursor hybrids require stimulation by both TNF-α and IFN-γ to become listericidal (31). It is possible that these early macrophages may not express IL-10 and, thus, can respond to these signals, while more mature, membrane IL-10-positive macrophages, such as 12j macrophages or thioglycolate-elicited macrophages, cannot. A second possibility is that macrophage membrane IL-10 acts not on the macrophage that bears it, but, instead, on a near neighbor. In this case, membrane IL-10 could function as does soluble IL-10, inhibiting, in a cognate fashion, the ability of the target macrophage to kill *Listeria*. Both these hypotheses are compatible with the available evidence and are not mutually exclusive.

In conclusion, the results shown here suggest that the reason why 12j macrophage hybrids cannot kill the bacterium, *L. monocytogenes* is because they bear membrane IL-10 and not because they secrete IL-10. Although it has not yet been formally proven, this membrane IL-10 appears to be an integral membrane protein that can transduce signals, since binding of anti-IL-10 induces bactericidal activity.

Acknowledgments

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