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Naive Mouse Macrophages Become Activated following Recognition of L5178Y Lymphoma Cells via Concurrent Ligation of CD40, NKG2D, and CD18 Molecules¹

Ilia N. Buhtoiarov,^{2*§} Alexander L. Rakhmilevich,^{*§} Lewis L. Lanier,[¶] Erik A. Ranheim,^{‡§} and Paul M. Sondel^{2*†§}

Under different circumstances, tumors can inhibit or activate macrophage (M ϕ) effector functions. We studied the mechanisms of tumor-M ϕ interactions leading to M ϕ activation. The results show that L5178Y mouse T cell lymphoma cells can prime naive mouse M ϕ to subsequent LPS stimulation, resulting in increased NO production and antilymphoma effects in vitro. L5178Y cells, but not naive splenocytes, primed M ϕ to ligation of TLR4 but not TLR9. L5178Y-primed M ϕ incubated with LPS showed down-regulation of CD40 and up-regulation of NKG2D expression. Although L5178Y T cell lymphoma cells primed naive mouse M ϕ , several other mouse and human cells lines failed to prime mouse M ϕ . Neither L5178Y-conditioned supernatants nor co-culture of M ϕ and L5178Y cells in Transwells resulted in priming, indicating that direct L5178Y cell-M ϕ contact was needed. Several receptor-ligand pairs are reciprocally expressed on M ϕ and L5178Y cell membranes and can be potentially involved in M ϕ priming. Of these, the CD40-CD154 pair played the most important role, as blocking the interaction of these molecules substantially reduced in vitro M ϕ priming. Furthermore, simultaneous blocking of interactions between CD40-CD154, NKG2D-H60, and CD18-ICAM-1/2 led to complete abrogation of M ϕ -mediated NO secretion and complete inhibition of M ϕ -mediated tumor cell cytostasis. The priming of M ϕ to LPS with L5178Y cells was also observed in vivo. These results suggest that contact with certain tumor cells via CD40, NKG2D, and CD18 molecules on the M ϕ may facilitate M ϕ -mediated antitumor immune surveillance. *The Journal of Immunology*, 2009, 182: 1940–1953.

Activation of cells of the innate immune system, including professional APCs, with vaccine adjuvants strongly influences the efficacy of adaptive immunity induced by vaccination (1). Furthermore, cells of the innate immune system are also the first line of antitumor defense capable of early recognition and killing of autologous cells with aberrant immunophenotypes. NK cells and macrophages (M ϕ)³ are both involved in anti-tumor immune defense (2–4).

An array of stimulatory receptors on naive NK cells allows for recognition of cancer cells with altered surface expression of MHC class I Ags (MHC-I) or MHC-I-like structures, such as H60,

MULT1, and Rae-1 proteins in mice, and MHC-I chain-related proteins (MICA and MICB) and UL16-binding proteins (ULPB) in humans, which results in NK cell activation (5–10). This effect frequently requires mutual coligation of multiple activating receptors and adhesion molecules on NK cells (11, 12) and can further be augmented by stimulation of NK cells via cytokines or TLRs (13). Notably, activation of secretory and cytolytic functions may require different patterns of NK cell costimulation (11, 13). Interactions involved in cancer cell recognition by naive M ϕ are less well characterized. Several studies demonstrated roles for MHC-I and MHC-I-like molecules in regulation of M ϕ activation (10, 14–17). The CD11b-CD18 complex (also known as LFA-1) on M ϕ has also been shown to regulate M ϕ -mediated tumor cell cytotoxicity by facilitating anchoring of the ICAM⁺ tumor cell targets to activated M ϕ (18, 19). M ϕ also express CD40 on their membranes and therefore could be stimulated via CD40 ligation. Indeed, we have recently reported that in vitro and in vivo stimulation of M ϕ with agonistic anti-CD40 mAb (anti-CD40) results in M ϕ activation that could be synergistically amplified via TLR4 by bacterial LPS or via TLR9 by class B CpG-containing oligodeoxynucleotides (20, 21). These anti-CD40-stimulated M ϕ could secrete NO, TNF- α , IL-12, and IFN- γ and mediate antitumor effects in vitro and in vivo against CD40-negative tumors (20, 21).

In several experiments pertaining to our previous studies, we observed that culturing naive M ϕ in vitro with L5178Y T cell lymphoma cells in the presence of low concentrations of LPS resulted in secretion of NO and induced tumor cytostasis. Notably, M ϕ and L5178Y cells cultured separately did not secrete any detectable NO in the presence of LPS. This led to the hypothesis that tumor cells can sensitize naive M ϕ via TLR4. In this study, we investigated the mechanisms of this L5178Y cell-mediated priming of naive M ϕ and found that it required direct L5178Y cell-M ϕ

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³ Abbreviations used in this paper: M ϕ , macrophage; PC, peritoneal cell; PF, paraformaldehyde fixed; TW, Transwell; MHC-I/II, MHC class I/II.

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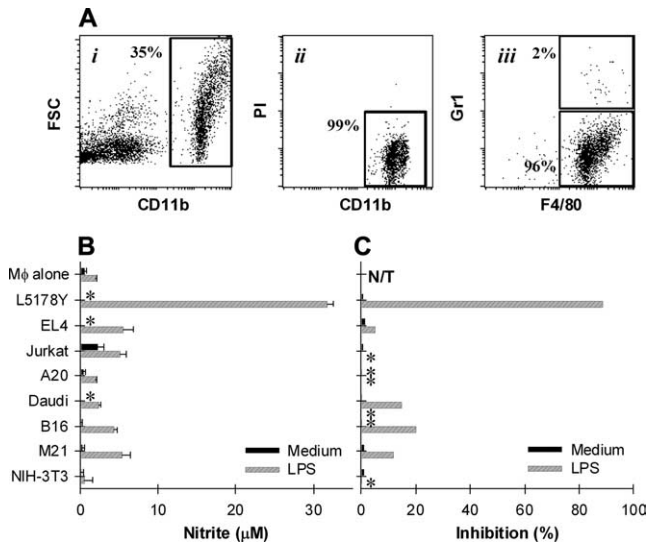


FIGURE 1. L5178Y lymphoma cells prime naive mouse Mφ to LPS, resulting in NO production and antiproliferative effects in vitro. Flow cytometric profile of PC (A). PC obtained from naive C57BL/6 mice contained ~35% of CD11b⁺ cells (Ai). Following 90 min of adhesion, non-adherent PC were removed from cultures by repeated pipetting. The resultant adherent population consisted of 99% CD11b⁺ Mφ (Aii). These adherent cells were comprised of 96% F4/80⁺Gr1⁻ Mφ (Aiii). Naive adherent C57BL/6 Mφ were cultured for 24 h with different mouse and human tumor cell lines in medium with or without LPS. Results are presented as concentrations of NO metabolites (μM) in the supernatants (B) or percentage of inhibition of [³H]TdR incorporation into tumor cells (C). In this experiment, the proliferation of L5178Y cells in medium alone at 24 h was 16.5 ± 0.23 × 10³ counts of [³H]TdR; in other experiments where cultures were assayed at 48 h, counts for L5178Y cells in medium were 70–110 × 10³. *, Negligible value.

interaction and involved simultaneous ligation of CD40, NKG2D, and CD18 on the Mφ surface, with the CD40-CD154 interaction playing the most important role. This cross-talk also led to reciprocal alterations of the immunophenotype of both Mφ and L5178Y cells and resulted in inhibition of L5178Y cell proliferation in vitro. The priming of Mφ with L5178Y cells was also demonstrated in vivo, suggesting that Mφ activation can be a mechanism of immunological lymphoma surveillance.

Materials and Methods

Mice

Six- to 10-wk-old C57BL/6 mice (Harlan Sprague Dawley, The Jackson Laboratory, and Taconic Farms), DBA/2, BALB/c, A/J mice (all from

Table I. Comparison of Mφ from different strains of mice^a

Mouse Strain	Nitrite (μM)		Inhibition (%)	
	Medium	LPS	Medium	LPS
C57BL/6 ^{Tac}	ND	53.1 ± 2.02	6.2	46.8
C57BL/6 ^{Hln}	0.08 ± 0.08	51.9 ± 1.6	11.6	43.6
C57BL/6 ^{Jax}	ND	47.8 ± 3.4	13.2	46.5
DBA/2 ^{Tac}	1.2 ± 0.6	51.2 ± 1.1	9.4	41.4
BALB/c ^{Tac}	ND	ND	8.0	0.3
A/J ^{Tac}	1.8 ± 1.8	ND	10.1	7.3

^a Peritoneal Mφ (3 × 10⁵ cells/0.1 ml), taken from naive mice of different strains obtained from different vendors, were cultured for 24 h with 2.5 × 10⁴/0.1 ml of L5178Y cells in medium with or without 10 ng/ml LPS. After 24 h of coculture, NO concentrations (μM) in the cell culture supernatants, as well as inhibition (percent) of L5178Y cell [³H]TdR incorporation, were evaluated as described in *Materials and Methods*. ND, not detectable: value is below the detection limit.

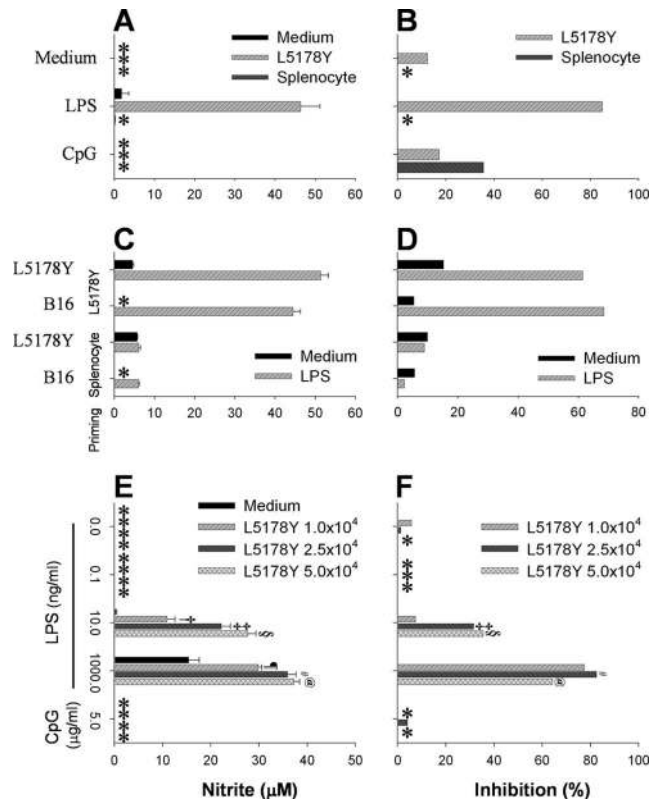


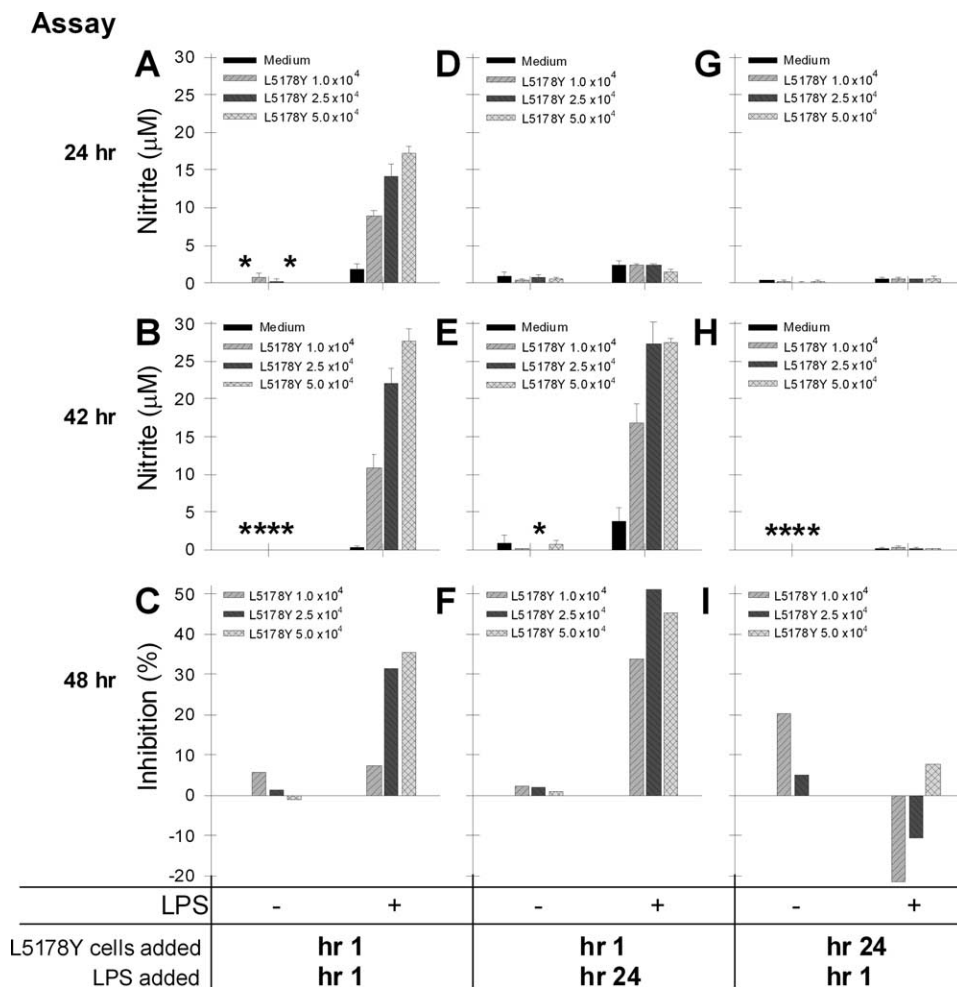
FIGURE 2. L5178Y cells induce dose-dependent priming of naive Mφ to LPS but not CpG. Adherent C57BL/6 Mφ were cultured for 48 h alone or with 2.5 × 10⁴/well of L5178Y lymphoma cells or autologous splenocytes (A and B) or various numbers of L5178Y cells (as shown in the legend box) in a separate experiment (E and F), in medium with or without LPS (10 ng/ml, as well as 0.1 and 1000 ng/ml in E and F) or CpG (5 μg/ml). Alternatively (C and D), C57BL/6 Mφ were cultured with 2.5 × 10⁴/well of L5178Y lymphoma cells or splenocytes from DBA/2 mice for 24 h, followed by thorough removal of L5178Y cells and splenocytes and placement of 2.5 × 10⁴/well of new L5178Y cells or B16 melanoma cells, with or without 10 ng/ml LPS, for another 24 h. At 42 h, cell culture supernatants were harvested for the Griess nitrite test and 1 μCi of [³H]TdR was added for 6 h to the cultures to measure L5178Y cell, B16 cell or splenocyte proliferation. Results are presented as concentrations of NO metabolites (μM) in the supernatants (A and C) or percentage of inhibition of [³H]TdR incorporation into splenocytes (B), B16 cells (D), or L5178Y lymphoma cells (B and D). *, Negligible values. The symbols (in C and D) correspond to the p values for each bar as compared with the previous bar to its left: for C: †, p = 0.0032; ‡, p = 0.0114; §, p = 0.0978; ¶, p = 0.0034; #, p = 0.0325; @, p = 0.5572; for D: †, p = 0.0221; §, p = 0.388; #, p = 0.838; @, p = 0.958; p values for D were calculated by comparing with the mean ± SEM values of original [³H]TdR incorporation counts. The experiments are representative of three separate experiments with similar results.

Taconic Farms), and CD40^{-/-} mice (strain B6.129P2-CD40tm1Kik/J; The Jackson Laboratory) were housed, cared for, and used in accordance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health publication 86-23, National Institutes of Health, Bethesda, MD, 1985).

Tumor cell lines

Mouse L5178Y T cell lymphoma (DBA/2 origin), EL4 T cell lymphoma (C57BL/6), YAC-1 thymoma (A/Sn), A20 B cell lymphoma (BALB/c), B16 melanoma (C57BL/6), and NIH-3T3 fibroblast (BALB/c) cell lines were grown in RPMI 1640 complete cell culture medium supplemented with 10% FBS (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 0.5 μM 2-ME (Invitrogen Life Technologies) at 37°C in a humidified 5% CO₂ atmosphere. Human Jurkat T cell lymphoma, Daudi B cell lymphoma, and M21 melanoma cell lines were grown in RPMI 1640

FIGURE 3. Contact of M ϕ with L5178Y lymphoma cells must precede or occur simultaneously with LPS stimulation to achieve M ϕ activation. Adherent C57BL/6 M ϕ were cultured in vitro for 48 h alone or in the presence of various numbers (as shown in the legend box) of L5178Y lymphoma cells. L5178Y cells were added to M ϕ 1 h after beginning the experiment (A–F) or 24 h after beginning the experiment (G–I). Similarly, LPS was added to M ϕ -L5178Y cell cultures 1 h after beginning the experiment (A–C and G–I) or 24 h after beginning the experiment (D–F). Cell culture supernatants were taken at 24 h (A, D, and G) (just before L5178Y cells (G) or LPS (D) were added to M ϕ cultures) or at 42 h (B, E, and H), before 1 μ Ci of [³H]TdR was added to the cell cultures. Results are presented as concentrations of NO metabolites (μ M) in the supernatants (A and B; D and E; G and H) or percentage of inhibition of [³H]TdR incorporation into L5178Y lymphoma cells (C, F, and I). *, Negligible values. The experiments are representative of three separate experiments with similar results.



complete cell culture medium, as formulated above, but without 2-ME. Mouse fibroblast L cell line (ATCC CRL-2648) and CD40L or control CD32 (FcR)-transfected L cells (CD40L-L and CD32-L, respectively), provided by Dr. E. Ranheim (University of Wisconsin Department of Pathology and Laboratory Medicine) were cultured in RPMI 1640 complete medium with G418 (400 μ g/ml). In separate experiments, we used paraformaldehyde-fixed (PF) L5178Y cells (PF-L5178Y) prepared by exposing L5178Y cells to 2% paraformaldehyde in PBS on ice for 1 h, followed by five separate cell wash cycles with complete cell culture medium. The fixed cells were kept in ice-cold medium for 24 h to allow for complete elimination of any contaminating paraformaldehyde. Cell death was confirmed microscopically by staining with 1% eosin.

Splenocyte preparation

Splenocytes were prepared from whole spleens pooled from three to four C57BL/6 or DBA/2 mice by processing the spleens to a single-cell suspension, followed by lysis of erythrocytes by hypotonic shock.

In vitro M ϕ -mediated tumor cell proliferation inhibition

Peritoneal cells (PC) were obtained from naive mice by peritoneal cavity lavage. Total PC were seeded in 96-microwell flat-bottom cell culture clusters (3×10^6 viable large leukocytes/ml, 0.1 ml/well, unless otherwise indicated). Ninety minutes later, nonadherent cells were removed from the culture by repeated pipetting. This protocol yields a relatively pure population of mature M ϕ , based on 98% expression of F4/80 on CD11b⁺ cells. The resultant adherent M ϕ were thereafter incubated with tumor cells (5×10^5 /ml, 0.05 ml/well, unless otherwise indicated) or splenocytes for 24–48 h in medium with or without 10 ng/ml LPS from *Salmonella enteritidis* (Sigma-Aldrich) or 5 μ g/ml CpG1826 (Coley Pharmaceuticals) in the final volume of 0.2 ml.

In some experiments, M ϕ were activated with recombinant mouse IFN- γ (Roche) or agonistic anti-CD40 mAb (20, 21). The FGK 45.5 hybridoma producing anti-CD40 was a gift from Dr. F. Melchers (Basel In-

stitute for Immunology, Basel, Switzerland). anti-CD40 was obtained from ascites of nude mice injected with the hybridoma cells and enriched for IgG by ammonium sulfate precipitation.

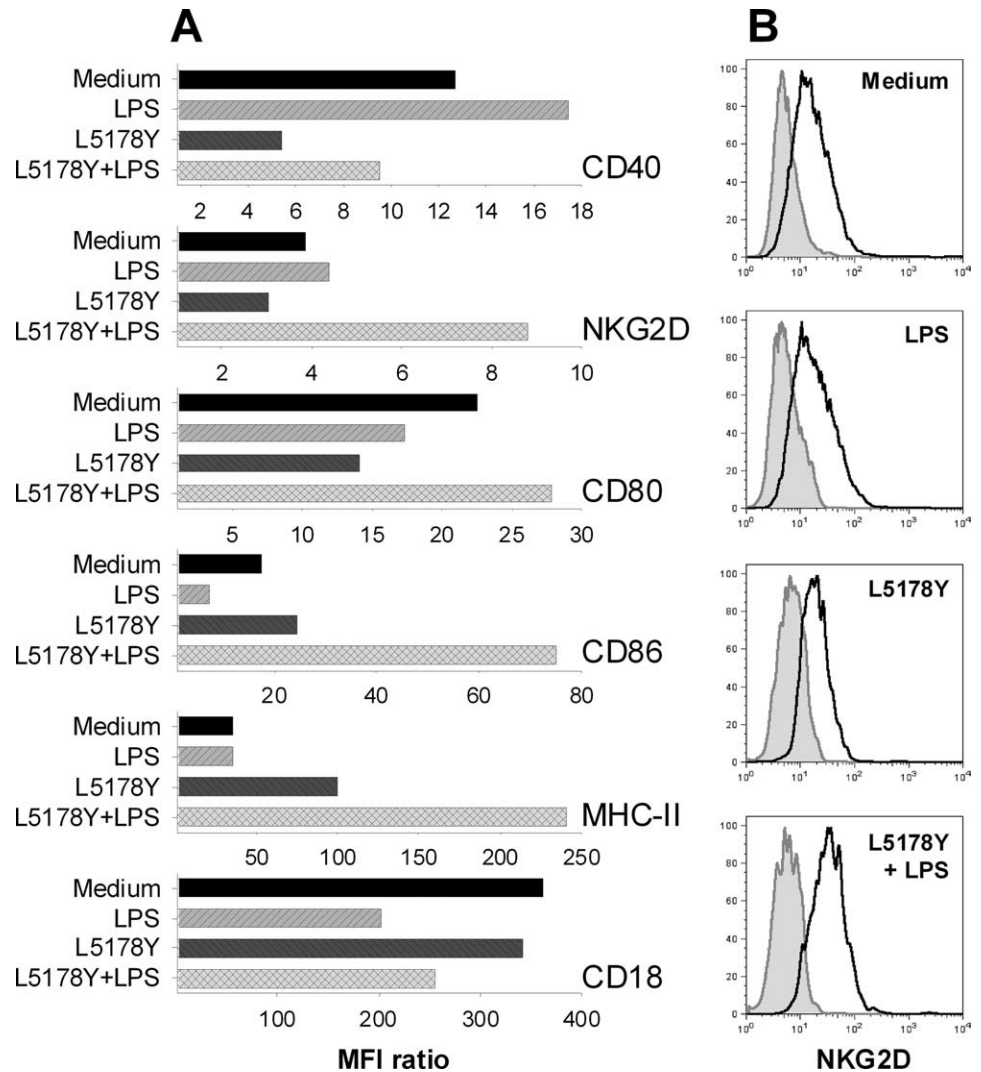
In selected experiments, 10 μ g/ml functional grade blocking mAbs to NKG2D (191004; R&D Systems), CD154 (MR1), CD18 (M18/2), MHC-I (34-1-2S reacts with both H-2K^d and H-2D^d; all from eBioscience), alone or in various combinations, were added into M ϕ cultures at the initiation of the experiment, but 30 min before tumor cells and LPS were added to the cultures. In the setting where anti-CD154 mAb was used, L5178Y cells were additionally precoated with 10 μ g/ml anti-CD154 mAb or control IgG on ice for 30 min and added to M ϕ cultures without additional washing.

To measure cell proliferation, the cells were pulsed with 1 μ Ci/well [³H]thymidine ([³H]TdR) for the last 6 h and retained radioactivity was counted by β scintillation of total cells harvested from the wells onto glass fiber filters (Packard Instrument) using the Packard Matrix 9600 Direct Beta Counter (Packard Instrument). Under these conditions, M ϕ incorporate negligible amounts of [³H]TdR (20), enabling [³H]TdR to reflect the level of proliferation of the tumor cells. Inhibition of proliferation was calculated as $c = [(a - b)/a] \times 100$, where c is the inhibition index; a is the mean value of incorporated [³H]TdR into tumor cells or splenocytes cultured in triplicates in medium without M ϕ in the absence or presence of LPS and CpG; and b is the mean value of incorporated [³H]TdR into tumor cells or splenocytes cultured in triplicates in medium with M ϕ in the absence or presence of LPS and CpG.

Transwell (TW) experiments

In separate experiments, we used the TW system (Costar 3460, 12-macrowell polystyrene plates with 12 mm, 0.4- μ m pore size polyester membrane TW chambers). Total PC at a concentration of 3×10^6 large leukocytes/ml (1.0 ml/well) were plated into wells for 90 min before non-adherent cells were removed, and 1.45 ml of complete cell culture medium was replaced. L5178Y cells (5×10^5 /ml, 0.5 ml/well) were added either

FIGURE 4. Priming of M ϕ by L5178Y cells results in alterations of M ϕ immunophenotype. Naive C57BL/6 M ϕ were cultured for 24 h with or without L5178Y lymphoma cells in medium with or without LPS; M ϕ were then tested by flow cytometry for expression of different surface molecules. Functional grade-purified anti-mouse CD16 + CD32 FcR block from eBioscience (clone 93, rat IgG2a) was used to precoat cells for 30 min on ice followed by washing of the cells. *A*, Results are presented as MFI ratios calculated as described in *Materials and Methods*. *, Negligible values. The experiments are representative of three separate experiments with similar results. *B*, Representative histograms showing NKG2D expression on M ϕ . These correspond to the data shown for NKG2D expression in *A*. Dark gray peak, Isotype-matched IgG control staining. Solid light gray line with gray fill, staining with anti-NKG2D mAb. MFI values for M ϕ stained with isotype-matched control IgG vs anti-NKG2D mAb are 6.67 vs 25.83 (medium), 6.25 vs 27.39 (LPS), 7.44 vs 22.62 (L5178Y), and 6.99 vs 55.02 (L5178Y + LPS).



into the wells or into the TW chamber. Under certain experimental conditions, L5178Y cells were added both in the wells and in the TW chamber. Finally, LPS (0.4 μ g/ml, 0.05 ml/well) was added in the well to achieve the final concentration of 10 ng/ml in 2.0 ml. After 24 h of coculture, the TW chambers were removed from the wells and placed into 15-ml centrifuge conical tubes. To harvest L5178Y cells from the TW chamber, the polyester membrane was delicately perforated by the tip of a 1-ml pipette, and L5178Y cells were washed off by repeated pipetting of cell culture medium through the TW chamber. In the same wells, nonadherent L5178Y cells, cultured together with adherent M ϕ , were harvested from the bottom of the macrowells by gentle pipetting and repeated aspiration of the cell culture supernatants. This technique yielded >95% of total L5178 cells cultured in the well, with <2% of contaminating M ϕ detached from plastic by pipetting. Finally, the harvested L5178Y cells (from the TW chambers or the bottom of the wells) were spun down, the pellet was resuspended in 0.3 ml of the cell culture medium, and the cells were reseeded in 96-microwell round-bottom cell culture clusters in triplicate, 0.1 ml/well. Inhibition of L5178Y cell proliferation was measured with [3 H]TdR as described above.

NO detection

M ϕ were cultured with tumor cells as described above. At different time points, the cell culture supernatants were collected without disturbing the cell monolayers. In experiments involving the TW system, the supernatants were taken from both the well and the TW chamber. Nitrite accumulation in the cell culture supernatants was determined by using the Griess reagent (Sigma-Aldrich) as previously described (20).

Tumor cell medium conditioning

L5178Y cells (5×10^5 cells/ml, 20 ml) were cultured in complete cell culture medium, with or without 10 ng/ml LPS, at 37°C in a humidified 5%

CO $_2$ atmosphere. At 24 h, cell cultures were harvested and cells were pelleted by centrifugation at $453 \times g$ for 20 min. Cell culture supernatants were collected and centrifuged at $3220 \times g$ for 60 min, and the resultant supernatants were subsequently used in *in vitro* experiments.

Tumor cell testing for *Mycoplasma* spp. contamination

L5178Y, YAC-1, and B16 tumor cell lines were tested for contamination with *Mycoplasma* spp. as described elsewhere (22). This method is sensitive for *Mycoplasma hyorhinis*, *Mycoplasma arginini*, *Mycoplasma fermentans*, *Mycoplasma orale*, *Mycoplasma pirum*, *Mycoplasma hominis*, *Mycoplasma salivarium*, and *Mycoplasma laidlawii*. Madin-Darby bovine kidney cells were used as the indicator cell line. To confirm results of the above test, the cells were also cultured for 96 h on the pleuropneumonia-like organism *Mycoplasma* agar and the formed colonies were counted.

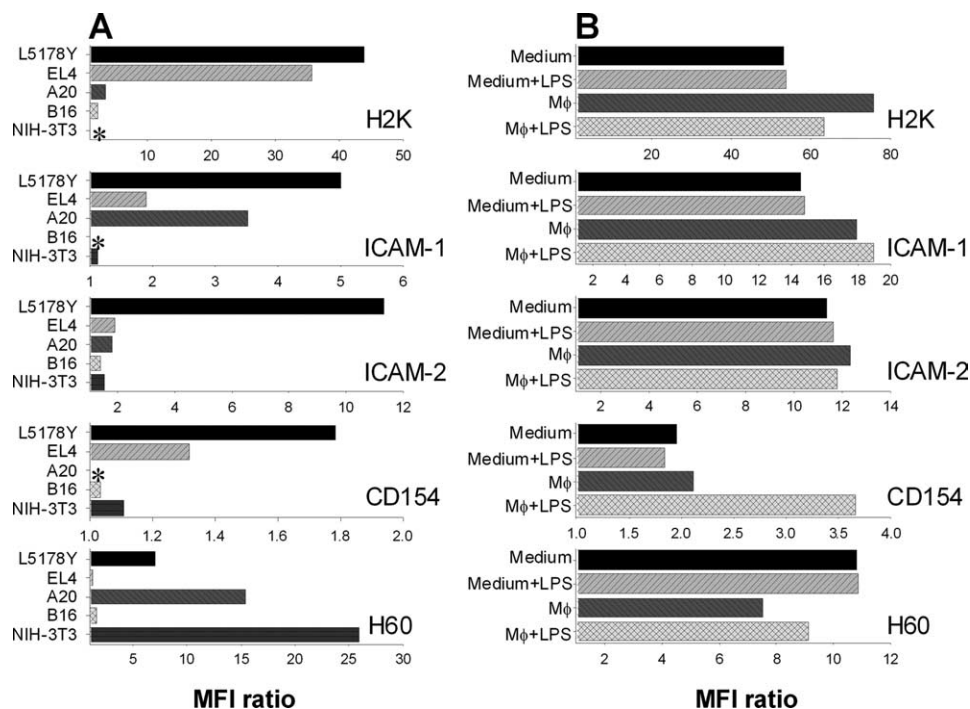
In vivo L5178Y lymphoma-mediated M ϕ priming

DBA/2 mice ($n = 3$) were injected i.p. with 5×10^5 L5178Y cells in 1 ml of PBS. The control group of mice was injected with 1 ml of PBS without tumor cells. Twenty-four hours later, animals were injected i.v. with 20 ng of LPS in 0.2 ml of PBS or with 0.2 ml of PBS (control). At 48 h of the experiment, animals were euthanized and then PC were collected by lavage and seeded in 6-well plates in complete RPMI 1640 medium to allow for purification of M ϕ by adhesion (20).

Flow cytometry analysis

Adhesion-purified peritoneal M ϕ from C57BL/6 mice were harvested as described above and resuspended in ice-cold PBS plus 2% FCS. M ϕ (1×10^5 /sample) were labeled with FITC-conjugated anti-CD11 (M1/70), allophycocyanin-conjugated anti-F4/80 (BM8), and PE-conjugated

FIGURE 5. L5178Y lymphoma cells have unique immunophenotype that can be altered by exposure to M ϕ . A, L5178Y and EL4 T cell lymphoma cell lines, A20 B cell lymphoma cell line, B16 melanoma cell line, and NIH-3T3 fibroblast cell line, cultured in medium alone, were tested by flow cytometry for expression of different surface Ags. Results are presented as MFI ratios calculated as described in *Materials and Methods*. B, L5178Y lymphoma cells were cultured alone or in the presence of naive C57BL/6 M ϕ in medium with or without LPS. After 12 h of coculture, nonadherent cells were harvested and tested for expression of different Ags on F4/80-negative viable L5178Y lymphoma cells by flow cytometry. The data are representative of three separate experiments with similar results.



anti-Gr1 (RB6-8C5), all from eBioscience. Analysis was based on co-expression of F4/80 and Gr1 on CD11b⁺ viable M ϕ . The analysis was performed on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

In separate experiments, adherent M ϕ (3×10^6 cell/well) and L5178Y cells (2.5×10^5 cell/well) were cultured separately or together in medium with or without 10 ng/ml LPS in 12-macrowell polystyrene plates. At 12 h (for analysis of L5178Y cells) or 24 h (for analysis of M ϕ) h, nonadherent L5178Y cells were harvested by repeated gentle pipetting, and adherent M ϕ were harvested by harsh pipetting of the cells following pretreatment with 5% EDTA (Sigma-Aldrich). Cells were pelleted and resuspended in the flow cytometry buffer. All cells were labeled with allophycocyanin-conjugated anti-F4/80 to enable flow cytometric discrimination of F4/80⁻ L5178Y cells from F4/80⁺ M ϕ . In addition, B16 and EL4 tumor cells were labeled with FITC-conjugated anti-H2K^b (AF6-88.5; BD Biosciences), and L5178Y, A20, and NIH-3T3 cells were labeled with FITC-conjugated anti-H2K^d (SF1-1.1; BD Biosciences), FITC-conjugated anti-ICAM-1 (KAT-1; eBioscience), FITC-conjugated anti-ICAM-2 (3C4; BD Biosciences), anti-CD154 (MR1; eBioscience), PE-conjugated anti-H60 (205326, R&D). Separately, M ϕ were labeled with PE-conjugated anti-CD40 (1C10), PE-conjugated anti-NKG2D (CX5), FITC-conjugated anti-CD18 (M18/2), FITC-conjugated anti-MHC class II (M5/114.15.2), FITC-conjugated anti-CD80 (16-10A1), and FITC-conjugated anti-CD86 (GL1), all from eBioscience. Nonspecific staining of M ϕ with anti-NKG2D mAb was prevented by pretreating M ϕ with the functional grade-purified anti-mouse CD16 + CD32 FcR block from eBioscience (clone 93, rat IgG2a) before staining with the anti-NKG2D mAb. Immunophenotyping of L5178Y cells was based on gating on viable F4/80⁻ cells, whereas analysis of M ϕ was based on gating on viable F4/80⁺ cells. The analysis was performed on a FACSCalibur flow cytometer with CellQuest (BD Biosciences) or FlowJo (Tree Star) software. Results presented as the mean fluorescence intensity (MFI) ratio calculated as the value of MFI of staining with specific mAb divided by the value of MFI of staining with isotype-matched control IgG; the MFI ratio equals 1 when an Ag is not expressed. This approach allows for comparison of the same parameter but in different experimental conditions.

In *in vivo* M ϕ -priming experiments, freshly collected PC were resuspended in flow cytometry buffer and stained with anti-F4/80-allophycocyanin and anti-NKG2D-PE or anti-MHC-II-FITC, anti-CD80-FITC, and anti-CD86-FITC. Analysis of the M ϕ phenotype was performed by gating on viable F4/80⁺ cells. In another approach, collected PC were cultured for 9 h in complete medium supplemented with monensin, depleted of nonadherent cells, harvested with 5% EDTA in PBS as described above, and resuspended in flow cytometry buffer followed by staining with anti-F4/80-APC mAb for 40 min on ice. After staining with anti-F4/80-allophycocyanin mAb, cells were washed in ice-cold flow cytometry buffer, fixed, and permeabilized,

as previously described (20, 21), and then stained with anti-IL-4-PE (11B11), anti-IL-10-PE (JES5-16E3), anti-IFN- γ -PE (XMG1.2), anti-TNF-anti-PE (MP6-XT22), or anti-IL-12-PE (C17.8) mAbs. Analysis was performed by gating on F4/80⁺ cells.

In vivo tumoristasis assay

CFSE-labeled L5178Y cells (5×10^5 /ml) were injected *i.p.* into DBA/2 mice ($n = 3$ /group). Twenty-four hours after CFSE⁺L5178Y cell implantation, the mice were treated *i.v.* with 0.2 ml of PBS with or without 20 ng of LPS. After 48 h, mice were euthanized, resident PC plus lymphoma cells were collected from each mouse of the group and pooled, and F4/80⁻CFSE⁺L5178Y were tested by flow cytometry for CFSE fluorescence. In parallel, CFSE⁺ L5178Y cells (5×10^5 /ml, 0.05 ml/well) were cultured *in vitro* in triplicates with naive DBA/2 M ϕ as described above in an *in vitro* M ϕ -mediated tumor cell proliferation inhibition assay. After 48 h, cell cultures were collected by pipetting and pooled into one analysis sample for each experimental group, and F4/80⁻CFSE⁺ L5178Y were tested by flow cytometry for CFSE fluorescence. As a proliferation suppression control, L5178Y cells were γ -irradiated by a ⁶⁰Co γ source.

Statistical analysis

A two-tailed Student's *t* test was used to determine the significance of differences between experimental and relevant control values within one experiment. Statistical analysis of the inhibition of tumor cell proliferation under different experimental conditions was performed on original [³H]TdR incorporation values, which were also used to calculate the percentage of inhibition.

Results

L5178Y lymphoma cells but not other tumor cell lines prime M ϕ to LPS

Based on our initial incidental observation (I.N.B., A.L.R., and H. D. Lum, unpublished data) that L5178Y lymphoma cells primed C57BL/6 M ϕ to LPS, we hypothesized that tumor cells can sensitize naive M ϕ to stimulation via TLR4. Freshly isolated adhesion-purified PC from naive C57BL/6 mice contained 96% of CD11b⁺F4/80⁺Gr1⁻ M ϕ (Fig. 1A). These M ϕ were cultured *in vitro* with mouse L5178Y or EL4 or human Jurkat T cell lymphoma cells, mouse A20 or human Daudi B cell lymphoma cells, mouse B16 or human M21 melanoma cells, or mouse NIH-3T3 fibroblast cells in medium with or without LPS. Following 42 h of

coculture, we tested the cell cultures for NO concentration in the supernatant (Fig. 1B), as well as inhibition of [³H]TdR incorporation into the tumor cells (Fig. 1C). The results of this experiment show that only L5178Y cells, but not any of the other cells tested, were able to prime M ϕ to LPS, as measured by production of NO and antilymphoma effects in vitro. This priming effect of L5178Y cells on M ϕ was similar using PC M ϕ harvested from syngeneic DBA/2 and allogeneic C57BL/6 (but not from BALB/c or A/J) mouse strains obtained from various vendors as summarized in Table I. In contrast, EL4 cells primed neither allogeneic (DBA/2) nor syngeneic (C57BL/6) M ϕ (Fig. 1, B and C, and data not shown).

L5178Y lymphoma cells but not normal splenocytes prime naive M ϕ to LPS but not CpG

We tested whether culturing naive M ϕ with L5178Y lymphoma cells would result in M ϕ priming to CpG as well as LPS, as we have previously reported that both LPS and CpG could trigger CD40 ligation-primed M ϕ (21). M ϕ from C57BL/6 mice were cultured for 48 h with either L5178Y cells or splenocytes from the same donor mice (to further test whether the observed priming effect is specific for L5178Y tumor cells) in culture medium with or without LPS or CpG. As shown in Fig. 2A, naive M ϕ cultured in medium failed to respond to LPS or CpG as measured by NO production. In contrast, M ϕ were found to secrete a substantial amount of NO when cultured with L5178Y cells but not with normal splenocytes and only in response to LPS but not CpG. This pattern of M ϕ secretory activity induced by coculture with L5178Y cells directly correlated with inhibition of L5178Y cell proliferation (Fig. 2B), whereas the antiproliferative effect of M ϕ primed with splenocytes was substantially weaker and not reproducible in every experiment.

To test whether L5178Y cell-mediated priming followed by LPS stimulation would enable M ϕ to be cytotoxic to tumor cells other than L5178Y, we primed C57BL/6 M ϕ with L5178Y cells or splenocytes from DBA/2 mice (so that lymphoma cells and splenocytes would be of the same H-2^d haplotype) for 24 h in complete medium. L5178Y cells or splenocytes were then removed and replaced by freshly prepared L5178Y cells or B16 cells for another 24 h period with or without LPS (Fig. 2, C and D). As in the previous experiment (Fig. 2, A and B), L5178Y cells but not splenocytes from naive donors could prime M ϕ to respond to LPS, resulting in cytotoxicity not only against the priming cell line (L5178Y), but also against the B16 melanoma cell line, which does not possess the priming ability (Fig. 1, B and C).

NO production by M ϕ cultured with L5178Y cells in the presence of LPS correlated with the number of L5178Y cells present in culture (Fig. 2E). The greater the number of L5178Y cells in the culture, the higher the concentration of NO detected in the supernatants when using 10 ng/ml LPS. Increasing the amount of LPS in the cell culture medium to 1000 ng/ml induced NO secretion even in the absence of L5178Y cells; the addition of L5178Y cells did further boost NO secretion, but did not show cell-dose sensitivity in the range tested. Notably, L5178Y cells themselves did not produce any NO in response to LPS or CpG (data not shown), which suggests that this NO was produced by activated M ϕ . The pattern of M ϕ -mediated antiproliferative effects in these same cultures (Fig. 2F) corresponded approximately to NO production (Fig. 2E). These results demonstrate that L5178Y cells, but not normal splenocytes, can augment the sensitivity of naive M ϕ to LPS, but not CpG, that results in *in vitro* NO release and antitumor effects.

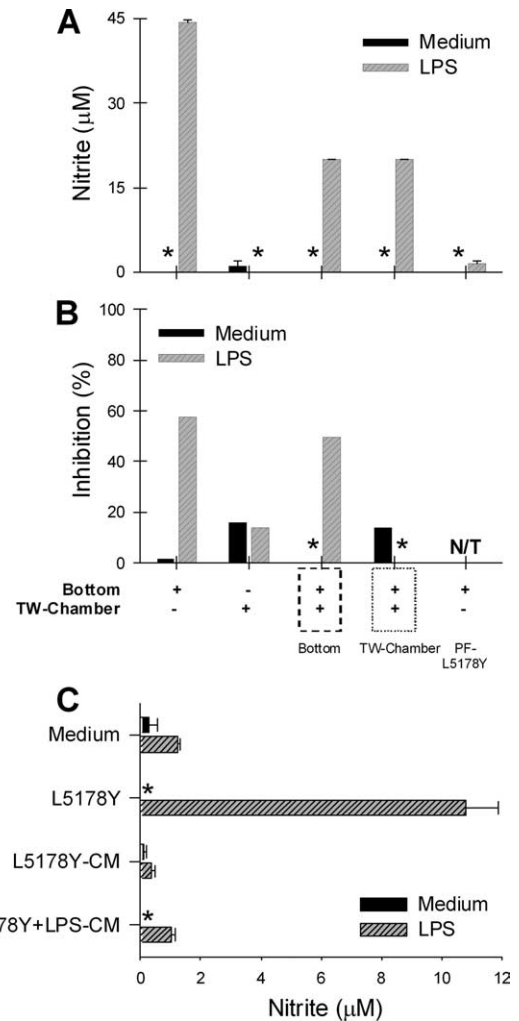


FIGURE 6. Priming of M ϕ by L5178Y lymphoma cells requires direct tumor cell-M ϕ contact. Naive adherent C57BL/6 M ϕ were placed on the bottom of the wells in the TW plates. Viable or PF-L5178Y lymphoma cells were cultured either together with M ϕ (bottom), or placed in the TW chamber in the presence or absence of LPS. In a separate group, M ϕ were placed on the bottom of the wells and L5178Y cells were added both to the bottom of the well and into the TW chamber. In this group, supernatants for NO detection were collected (A) and L5178Y cells (B) were removed for [³H]TdR incorporation evaluation either from the bottom of the wells (dashed rectangle) or from the TW chamber (dotted rectangle). Results are presented as concentrations of NO metabolites (μ M) in the supernatants (A) or percentage of inhibition of [³H]TdR incorporation into tumor cells (B). C, Naive adherent M ϕ were cultured (with or without LPS) either with L5178Y lymphoma cells or in L5178Y cell-conditioned medium or in L5178Y + LPS-conditioned medium (CM). Results are presented as concentrations of NO metabolites (μ M) in the supernatants at 24 h of the experiment. *, Negligible values. N/T, Not tested because the PF-L5178Y cells did not incorporate [³H]TdR. The experiments are representative of three separate experiments with similar results.

Contact with L5178Y cells must precede or occur simultaneously with LPS stimulation in the course of activation of M ϕ

Next, we tested whether priming signals provided by L5178Y cells, and stimulation by LPS, need to occur in a certain sequence to activate cytotoxic M ϕ . Thus, L5178Y cells and LPS were added to M ϕ cultures either simultaneously (Fig. 3, A–C) at the beginning of the experiment, or L5178Y cells were added at the beginning of the experiment and LPS was added at 24 h (Fig. 3, D–F),

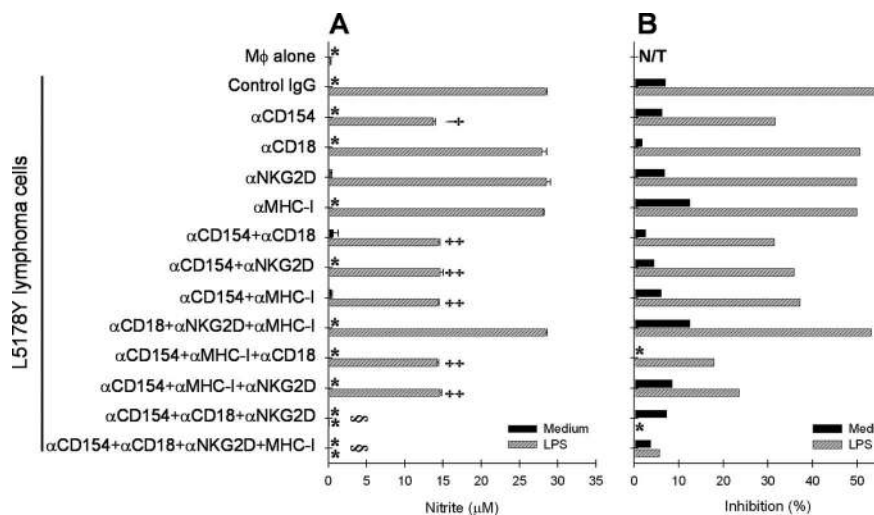


FIGURE 7. L5178Y cell-mediated priming of M ϕ involves engagement of CD40, NKG2D, and CD18 on M ϕ . Naive M ϕ from syngeneic DBA/2 mice were cultured for 24 h alone or with L5178Y cells in medium with or without LPS and in the presence of either control isotype-matched IgG or blocking mAbs against CD154, NKG2D, CD18, and MHC-I used alone or in various combinations. Results are presented as concentrations of NO metabolites (μ M) in the cell culture supernatants (A) or percentage of inhibition of [3 H]TdR incorporation into L5178Y lymphoma cells (B). *, Negligible values. N/T, Not tested. The data are representative of three separate experiments with similar results. †, $p = 0.00064$; ‡, $p > 0.05$; and §, $p = 0.00031$. The † symbols correspond to p values of the anti-CD40 treatment bar compared with the control IgG treatment bar; ‡ symbols correspond to p values of either combined treatment bars compared with the anti-CD40 treatment bar; § symbols correspond to p values of anti-CD40 + anti-CD18 + anti-NKG2D or anti-CD40 + anti-CD18 + anti-NKG2D + anti-MHC-I treatments compared with any other combined treatments.

or LPS was added at the beginning of the experiment and L5178Y cells were added at 24 h in the course of the experiment (Fig. 3, G–I). The overall duration of the experiment was 48 h. Results of this experiment demonstrate that M ϕ exposed simultaneously to both L5178Y cells and LPS were able to secrete progressively higher amounts of NO, as measured at 24 h (Fig. 3A) and 42 h (Fig. 3B) of the experiment, and to inhibit L5178Y cell proliferation at the end of the experiment (Fig. 3C). The magnitude of these effects depended upon the number of L5178Y cells present in the cultures. At the same time, M ϕ cultured with L5178Y cells added at 1 h but without LPS did not secrete any NO or inhibit L5178Y cell proliferation (the two to four columns to the left in Fig. 3, A–C). Similarly, when M ϕ were exposed to L5178Y cells only, i.e., without LPS, for the first 24 h, they secreted only negligible amounts of NO detected at 24 h (Fig. 3D). However, when LPS was added at 24 h to the culture of M ϕ and L5178Y cells, M ϕ rigorously responded to LPS stimulation and secreted a high amount of NO 18 h later (at 42 h of the experiment; Fig. 3E) that was comparable to the amount of NO secreted by M ϕ simultaneously exposed to both L5178Y cells and LPS for 42 h (Fig. 3B). These M ϕ were also able to inhibit L5178Y cell proliferation (Fig. 3F), similar to the results shown in Fig. 2C. In contrast, when M ϕ were first stimulated with LPS for 24 h and then exposed to L5178Y cells for another 24 h, they secreted only negligible amounts of NO at both 24 h (Fig. 3G) and 42 h (Fig. 3H) and were unable to suppress L5178Y cell proliferation (Fig. 3I). These results indicate that the contact of M ϕ with L5178Y cells needs to precede or occur simultaneously with LPS stimulation when these two stimuli are combined to activate M ϕ .

Incubation with L5178Y tumor cells induces alteration of M ϕ immunophenotype

In addition to testing M ϕ abilities to produce NO and suppress tumor cell proliferation, we determined whether M ϕ exposure to L5178Y cells and LPS had an effect on M ϕ immunophenotype. Adherent naive M ϕ were cultured with or without L5178Y cells in the absence or presence of LPS. In our previous

experiments (Fig. 3A), we found that coculture of M ϕ with L5178Y cells and LPS for 24 h led to M ϕ activation and production of a substantial amount of NO. Therefore, after 24 h of coculture, the culture supernatants and the L5178Y cells (growing nonadherently) were removed by repeated gentle pipetting and aspiration. Following this, the adherent M ϕ were additionally washed with warm PBS and harvested by vigorous pipetting with PBS containing EDTA for flow cytometric analysis. Expression of CD40, NKG2D, TLR9, CD18, MHC class II, CD80, and CD86 Ags by M ϕ was evaluated. The results (Fig. 4A) show that culturing M ϕ with L5178Y cells plus LPS suppressed expression of CD40 (7–25% in three separate experiments) and CD18 (17–29%), but induced substantial up-regulation (44–127%) of expression of NKG2D (Fig. 4, A and B), CD80 (16–23%), CD86 (101–328%), and MHC-II (201–585%) on M ϕ . The magnitude of NKG2D expression by M ϕ has remained somewhat controversial, probably because of the low amounts of NKG2D expressed on the cell surface of macrophages. Fig. 4B displays representative histograms of NKG2D expression on naive and manipulated M ϕ . anti-NKG2D mAb stained M ϕ , but not irrelevant cells such as B16 melanoma (MFI values for B16 cells stained with isotype control IgG vs anti-NKG2D mAb were 5.27 vs 5.95, respectively (data not shown)).

Alterations of immunophenotype of L5178Y lymphoma cells exposed to M ϕ

Because L5178Y tumor cells in the presence of LPS altered the phenotype of M ϕ (Fig. 4), we hypothesized that exposure to activated M ϕ may result in phenotypic changes of tumor cells. First, L5178Y, EL4, A20, B16, and NIH-3T3 mouse cell lines (cultured in medium alone) were tested by flow cytometry for expression of MHC-I (H2K), ICAM-1, ICAM-2, CD154, and H60 Ags (Fig. 5A), which were previously shown to be involved in a cross-talk with immune effectors. The results of this experiment suggest that no two cell lines were similar in the pattern and magnitude of expression of the tested surface Ags. Only L5178Y cells were

found to express detectable levels of all of the Ags at the time of analysis. Hence, the phenotype of L5178Y cells was established as MHC-I⁺ICAM-1⁺ICAM-2⁺CD154⁺H60⁺.

We tested whether exposure of L5178Y cells to M ϕ (with or without LPS) would result in alterations of this immunophenotype. L5178Y cells were cultured either alone, as shown in Fig. 4A, or with adherent M ϕ in medium with or without LPS. We did this analysis at 12 h rather than at 24 h (as done for M ϕ) to avoid manipulations with dead or dying L5178Y cells undergoing apoptosis. Following 12 h of coculture, L5178Y cells were harvested and the immunophenotype was evaluated (Fig. 5B). The expression of MHC-I (H2^k) and ICAM-1 was minimally increased following coculture with M ϕ , and the expression of H60 was slightly down-regulated with or without LPS. No regulatory effect on expression of ICAM-2 was documented. Interestingly, the expression of CD154 nearly doubled, provided that L5178Y cells were cultured with M ϕ and LPS. Notably, culture with LPS alone induced no alterations of L5178Y cell immunophenotype. Hence, cross-talk between M ϕ and L5178Y cells results in reciprocal alterations in the immunophenotype of both M ϕ (Fig. 4) and L5178Y cells (Fig. 5B), and priming of M ϕ to LPS via TLR4, that ultimately leads to activation of anti-tumor M ϕ (Figs. 1, B and C, and Fig. 2).

The cross-talk between M ϕ and L5178Y cells requires direct cell-cell contact

We determined whether direct M ϕ -tumor cell contact is required for the effects observed in the previous experiments. We used the TW in vitro system that allows for exchange of soluble secretory products, but not direct cell membrane contacts between M ϕ and L5178Y cells cultured in the same well. M ϕ were adhered to the bottom of the well and L5178Y cells were placed either together with M ϕ at the bottom of the well or in the TW chamber. In some wells, L5178Y cells were placed both on the bottom of the well and in the TW chamber. It was found that M ϕ could secrete substantial amounts of NO (Fig. 6A) and mediate in vitro antilymphoma effects (Fig. 6B) only when both M ϕ and L5178Y cells were cocultured in close proximity, but not when they were separated by the semipermeable membrane. When L5178Y cells were present both on the bottom of the well and in the TW chamber, the LPS-activated antiproliferative effect was mediated only toward those L5178Y cells that were cultured in proximity to M ϕ (Fig. 6B), despite apparent equilibrium of the NO (and presumably other cytotoxic factors secreted by activated M ϕ) in both compartments of the TW system (Fig. 6A). Only viable but not paraformaldehyde-fixed L5178Y cells triggered M ϕ activation, suggesting that either secretory activity or membrane fluidity and surface ligand rearrangements of L5178Y cells were involved in the cross-talk with M ϕ .

To clarify a possible role of L5178Y cell-secreted factors in the M ϕ -L5178Y cross-talk, we used the L5178Y cell-conditioned supernatants from these lymphoma cell cultures grown for 24 h in medium with or without LPS. As shown in Fig. 6C, only L5178Y cells but not L5178Y cell-derived secretory products resulted in M ϕ activation and NO production. Hence, these findings demonstrate that direct and active cross-talk between naive M ϕ and viable L5178Y cells is required for polarization of naive M ϕ into immune effectors with cytotoxic properties.

Priming of M ϕ by L5178Y cells involves concurrent ligation of CD40, CD18, and NKG2D

Multiple receptor-ligand interactions were previously shown to be involved in recognition of tumor cells by various immune effectors. As shown in Fig. 5A, L5178Y cells express CD154, ICAM-1, ICAM-2, and H60, which are known to be the functional ligands

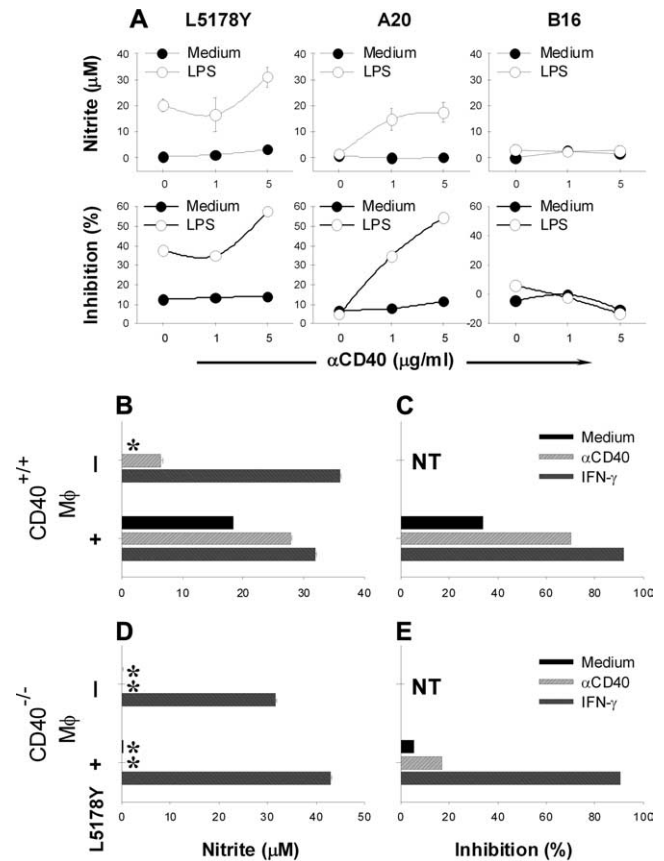


FIGURE 8. Role of CD40-CD40L interaction in priming of M ϕ by tumor cells. A, Peritoneal M ϕ from naive C57BL/6 mice were cultured for 24 h with L5178Y, A20, and B16 tumor cells in medium with or without 10 ng/ml LPS and agonistic α CD40 mAb used at concentrations shown in the x-axis. Tumor cells and anti-CD40 mAb were added to M ϕ cultures 6 h before LPS to enable M ϕ priming. At 24 h, M ϕ activation was evaluated by measuring the cell culture supernatant NO metabolite content (upper panel) and inhibition of [³H]TdR incorporation into tumor cells (lower panel). The amount of NO secreted by M ϕ stimulated with LPS and anti-CD40 mAb but without tumor cells was negligible and is omitted. B–E, Peritoneal M ϕ from CD40^{+/+} (B and C) and CD40^{-/-} (D and E) C57BL/6 mice were cultured for 24 h with or without L5178Y cells in medium with LPS, anti-CD40 mAb, and mouse IFN- γ . Tumor cells, anti-CD40 mAb, and IFN- γ were added to M ϕ cultures 6 h before LPS to enable M ϕ priming. At 24 h of coculture, M ϕ activation was evaluated by measuring supernatant NO metabolite content (B and D) and inhibition of [³H]TdR incorporation into the tumor cells (C and E). *, Value is below the detection limit. N/T, Not tested. M ϕ cultured with L5178Y cells without LPS, with or without anti-CD40 mAb or IFN- γ , secreted negligible amounts of NO and demonstrated no antitumor effect (data not shown).

to CD40, CD18, and NKG2D receptors, respectively, expressed by M ϕ (Fig. 4). Therefore, we tested whether these receptor-ligand pairs are involved in the M ϕ -L5178Y cell cross-talk. To exclude the potential contribution of MHC-I mismatch between M ϕ and L5178Y cells, we used naive M ϕ from DBA/2 mice, which were previously found to be sensitive to the priming effect by L5178Y cells (Table I). To investigate the roles of CD40-CD154, NKG2D-H60, and CD18-ICAM-1/2 interactions in the M ϕ -L5178Y cell cross-talk, we cultured M ϕ and L5178Y cells for 24 h in medium with or without LPS and in the presence of blocking mAbs to the receptors or ligands. These blocking mAbs were used alone or in different combinations. Isotype-matched IgGs were used as control. When a combination of blocking mAbs was tested, anti-MHC-I (specific for both H-2K^d and H-2D^d) was used both as a control for potential Ab-dependent cellular cytotoxicity via FcRs

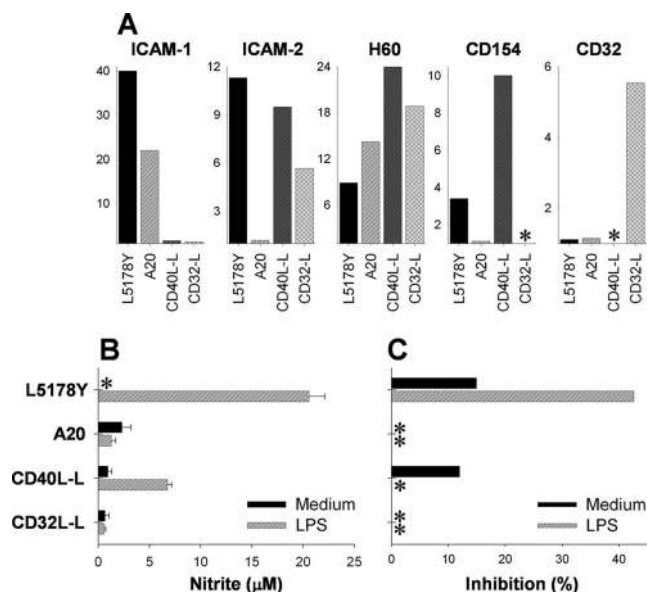


FIGURE 9. CD154⁺ICAM-1/2⁺H60⁺ L5178Y lymphoma cells but not CD154⁺ICAM-2⁺H60⁺ L fibroblasts prime M ϕ . *A*, Lymphoid L5178Y and A20 and nonlymphoid fibroblast CD40L-L and CD32-L cells lines were tested by flow cytometry for expression of ICAM-1, ICAM-2, H60, CD154, and CD32 surface Ags. Results are presented as MFI ratio values as described in *Materials and Methods*. *B* and *C*, Naive adherent C57BL/6 M ϕ were cultured for 24 h with L5178Y, A20, CD40L-L, and CD32-L cell lines in medium with or without LPS, and M ϕ activation was measured. Results are presented as concentrations of NO metabolites (μ M) in the supernatants (*B*) or percentage of inhibition of [³H]TdR incorporation into L5178Y, A20, CD40L-L, and CD32-L cells (*C*). *, Negligible value.

on M ϕ and as a negative control, as it would bind to both the M ϕ and the L5178Y cells, but not directly interact with the specific receptor-ligand pairs that are recognized by the blocking mAbs. When a single blocking mAb was used (anti-CD154), it was found that interference with CD40-CD154 interactions reduced NO production (Fig. 7*A*) and in vitro antiproliferative effects by \sim 50% (Fig. 7*B*). In contrast, blocking single interactions via CD18-ICAM-1/2 (anti-CD18) or NKG2D-H60 (anti-NKG2D) had no distinct inhibitory effect on M ϕ priming. These mAbs did not activate M ϕ , thereby ruling out that their cross-linking by FcRs had a significant agonistic effect. The three mAb combinations of anti-CD154 with either anti-CD18 or with anti-NKG2D and anti-MHC-I mAbs did not add to the effect of anti-CD154 mAb alone. However, simultaneous blocking of CD154, CD18, and NKG2D Ags on M ϕ (using the three mAb combination of anti-CD154, anti-CD18, and anti-NKG2D) led to complete abrogation of M ϕ priming and antilymphoma effects in vitro (Fig. 7). This experiment was repeated three times with similar results.

CD40-CD40L interaction is important for M ϕ priming by tumor cells

In the next series of experiments, the role of CD40-CD40L interaction in M ϕ priming by tumor cells was further addressed. We cultured ICAM-1⁺ICAM-2⁺H60⁺CD154⁺ L5178Y cells, ICAM-1⁺ICAM-2⁻H60⁺CD154⁻ A20 cells, or ICAM-1⁻ICAM-2⁻H60⁻CD154⁻ B16 cells with naive M ϕ in medium with or without LPS in the presence of agonistic anti-CD40 (Fig. 8). In this experiment, we chose to use low (1 and 5 μ g/ml) concentrations of anti-CD40, which we previously found to induce no or very little M ϕ activation in vitro (20). Fig. 8*A* shows that L5178Y cells, but not A20 and B16 cells, could prime M ϕ in the presence of LPS but

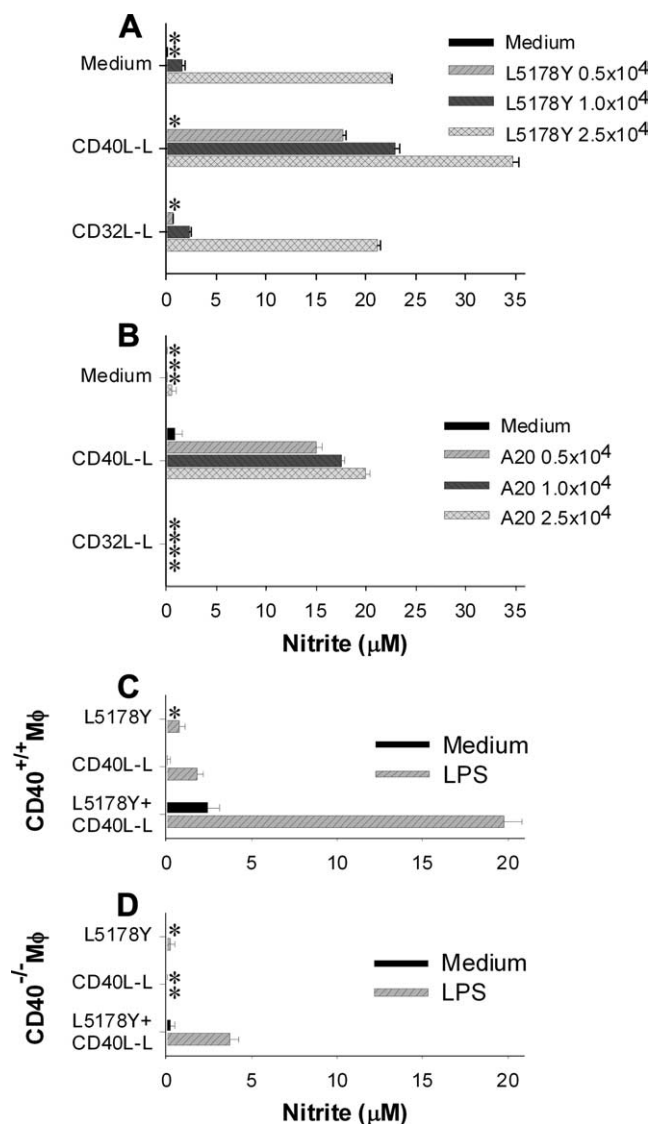


FIGURE 10. Synergistic effect of CD40L-L fibroblasts and L5178Y or A20 lymphoma cells on M ϕ priming. *A* and *B*, Peritoneal M ϕ (\sim 3 \times 10⁵ cells/well) from naive C57BL/6 mice were cultured for 24 h in medium or in the presence of CD40L-L or CD32L-L cells (2.5 \times 10⁴ cells/well) with or without LPS in medium. At 1 h of the experiment, different numbers of L5178Y (*A*) or A20 (*B*) cells were added to some M ϕ -L cell cultures. At 24 h, M ϕ activation was evaluated by measuring concentrations of NO metabolites in cell culture supernatants. *C* and *D*, Peritoneal M ϕ (\sim 3 \times 10⁵ cells/well) from CD40^{+/+} (*C*) and CD40^{-/-} (*D*) C57BL/6 mice were cultured for 24 h in medium, with or without LPS and in the presence of L5178Y cells or CD40L-L cells (1 \times 10⁴ cells/well), added to M ϕ separately or mixed together. At 24 h, M ϕ activation was evaluated by measuring concentrations of NO metabolites in cell culture supernatants.

in the absence of exogenous anti-CD40. Adding anti-CD40 at 1 μ g/ml to these cultures caused M ϕ activation in the presence of A20 cells but not B16 cells, nor did it further enhance the priming induced by L5178Y cells. Adding anti-CD40 at 5 μ g/ml did not induce priming of M ϕ by B16 cells, but increased the M ϕ -priming ability of both L5178Y and A20 cells, as shown by augmented NO production (in the presence of L5178Y cells) and tumorstasis (of both L5178Y and A20 cells). Hence, an additional signal provided by exogenous anti-CD40 was sufficient to enable M ϕ priming by CD154⁻ICAM-1⁺ICAM-2⁻H60⁺ A20 cells, but was not enough to enable M ϕ priming by CD154⁻ICAM-1⁻ICAM-2⁻H60⁻ B16

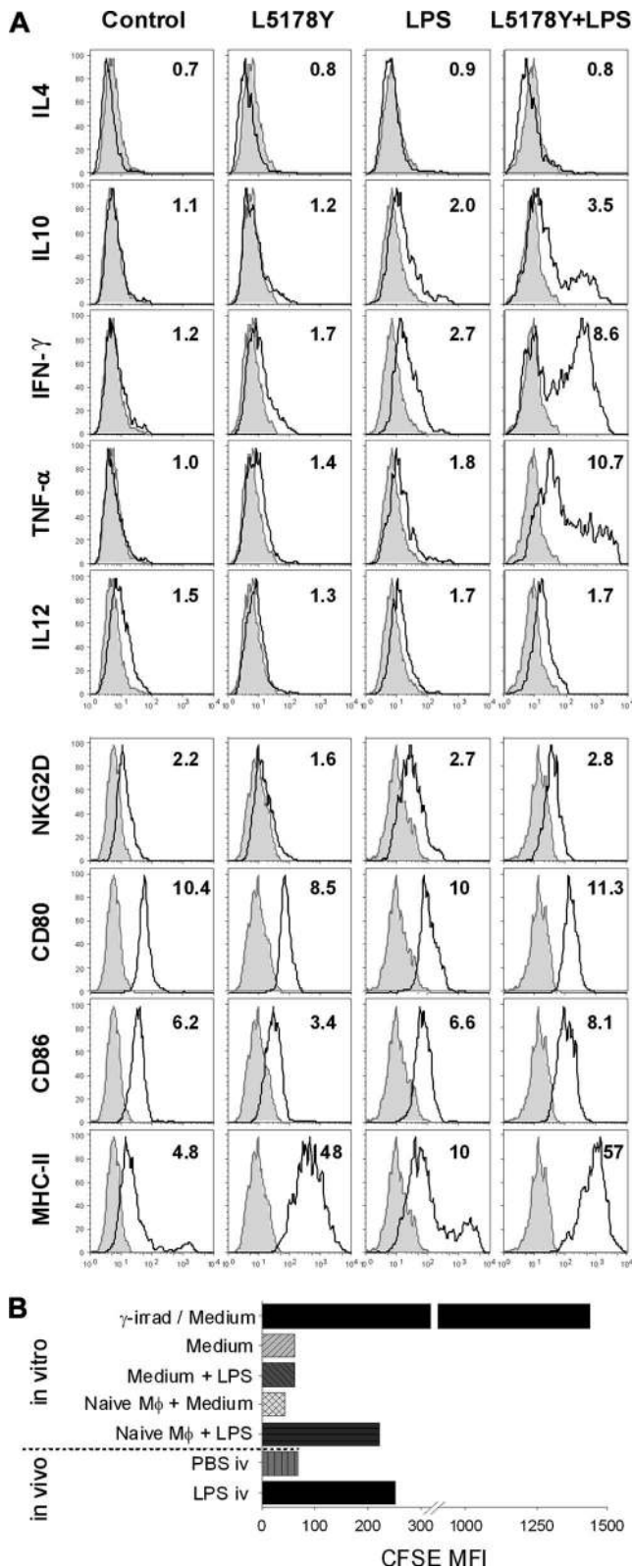


FIGURE 11. L5178Y cells and LPS synergize in activation of M ϕ in vivo. **A**, DBA/2 mice ($n = 3$ /group) were injected i.p. with L5178Y cells (5×10^5 /ml) or PBS (control). Twenty-four hours later, the mice were treated i.v. with 0.2 ml of PBS with or without 20 ng LPS. At 48 h of the experiment, mice were euthanized, resident PC were collected from each mouse of the experimental group and pooled, and F4/80⁺ M ϕ were tested by flow cytometry for production of IL-4, IL-10, IFN- γ , TNF- α , and IL-12 or expression of NKG2D, CD80, CD86, and MHC-II. On histograms: gray filled peaks, staining with isotype-matched control IgG; open black peak, staining with specific mAb. Numbers are MFI ratios calculated as

cells, consistent with the hypothesis that ligation of CD40, CD18, and NKG2D receptors is required for priming of M ϕ . Exogenous anti-CD40 also augmented M ϕ priming by CD154⁺ICAM-1⁺ICAM-2⁺H60⁺ L5178Y cells, but only at the higher dose.

The importance of CD40-CD40L interaction for M ϕ priming was confirmed using M ϕ from CD40^{-/-} mice (Fig. 8, B-E). The priming effect of L5178Y cells and anti-CD40, used alone or in combination, on CD40^{-/-} M ϕ was either abrogated (Fig. 8D) or substantially reduced (Fig. 8E). However, CD40^{-/-} M ϕ were readily primed by IFN- γ , which served as a positive control demonstrating the ability of these M ϕ to become activated via other mechanisms.

To further clarify the role of CD40-CD40L interaction in priming of M ϕ by tumor cells, we used mouse L fibroblasts transfected with CD40L or CD32 (transfection control). In preliminary experiments, CD40L-L cells were confirmed to stimulate proliferation of mouse splenic CD19⁺ B cells (data not shown). These CD40L-L and CD32-L lines also expressed ICAM-2 and H60, thereby showing phenotypic similarity with L5178Y cells (Fig. 9A). However, in contrast to L5178Y cells, CD40L-L cells could not effectively prime M ϕ to subsequent stimulation with LPS, which was shown by a limited NO production (Fig. 9B) and absence of cytostasis (Fig. 9C).

However, when CD40L-L cells were mixed together with a small number of viable L5178Y cells ($0.5-1 \times 10^4$ cells/well) that would be otherwise insufficient to prime M ϕ (Figs. 1-3), M ϕ became activated and were found to secrete NO (Fig. 10A). This synergistic priming effect was observed only when L5178Y cells were combined with CD40L-L but not CD32-L cells, suggesting that the interaction of CD40L on tumor cells with CD40 on M ϕ is required for the priming. Interestingly, A20 cells were also able to provide costimulation to enable CD40L-L cells, but not CD32-L cells, to prime M ϕ (Fig. 10B). These results suggest that costimulation by L5178Y and A20 cells does not require CD154 expression in *cis*, as A20 cells are CD154 negative (Fig. 9A), but might depend on the expression of ICAM-1 or soluble factors secreted by lymphoma cells.

To confirm the importance of CD40-CD40L interaction for efficient priming of M ϕ in this test model, we stimulated M ϕ from CD40^{+/+} and CD40^{-/-} mice with CD40L-L and L5178Y cells (1×10^4 cells/well) alone or in combination. Similarly to the results shown in Fig. 8, B-E, the priming of M ϕ with a combination of these tumor cells was substantially reduced in CD40^{-/-} mice compared with CD40^{+/+} mice, as shown by decreased NO production (Fig. 10C). Along with the findings presented above, these results demonstrate that the M ϕ priming we observed with the tumor cells tested here is a consequence of tumor cell expression of CD40L, ICAM-1, and H60.

described in *Materials and Methods*. **B**, Proliferation of CFSE-labeled L5178Y cells is suppressed in DBA/2 mice after systemic administration of a low amount of LPS. CFSE-labeled L5178Y cells (5×10^5 /ml) were injected i.p. into DBA/2 mice ($n = 3$ /group). Twenty-four hours after tumor cell implantation, the mice were treated i.v. with 0.2 ml of PBS with or without 20 ng of LPS. After 48 h, mice were euthanized, resident PC + tumor cells were collected from each mouse of the group and pooled, and F4/80⁻CFSE⁺ L5178Y were tested by flow cytometry for CFSE fluorescence. In parallel, CFSE-labeled L5178Y cells (5×10^5 /ml, 0.05 ml/well) were cultured in vitro in triplicates with naive DBA/2 M ϕ as described in Fig. 3, D-F. After 48 h, cell cultures were collected by pipetting and pooled into one analysis sample for each experimental group, and F4/80⁻CFSE⁺ L5178Y were tested by flow cytometry for CFSE fluorescence. As a proliferation suppression control, L5178Y cells were γ -irradiated. Results are presented as CFSE MFI.

L5178Y cells prime mouse naive M ϕ in vivo

In the next series of experiments, we tested whether the priming of M ϕ with L5178Y lymphoma cells observed in vitro can also take place in vivo. L5178Y cells (5×10^5) were implanted i.p. into naive DBA/2 mice, followed by i.v. injection of 20 ng of LPS 24 h later. This dose was used to achieve an endotoxin concentration in vivo similar to that used in our in vitro experiments (10 ng/ml): the mice used in the experiments, weighing ~ 30 g each, have a blood volume of ~ 2 ml. Three other groups of mice received L5178Y cells alone, LPS alone, or PBS (control). After 48 h, PC were harvested and M ϕ were tested for production of IL-4, IL-10, IFN- γ , TNF- α , and IL-12p40, as well as alterations of expression of NKG2D, CD80, CD86, and MHC-II surface Ags (Fig. 11A).

Injection of L5178Y cells alone slightly augmented production of IFN- γ (42%) and TNF- α (40%), as well as significantly up-regulated MHC-II (900%) by M ϕ . LPS alone augmented secretion of IFN- γ (125%), TNF- α (80%), IL-10 (100%), and MHC-II (108%) and slightly up-regulated NKG2D (23%). However, in animals that received both L5178Y implantation and LPS, M ϕ were found to secrete significant amounts of IL-10 (218%), IFN- γ (617%), and TNF- α (970%) and up-regulated CD86 (30.6%) and MHC-II (1088%), whereas expression of CD80 (8.7%) and secretion of IL-4 (14%) and IL-12 (13%) was almost unaltered. Although the level of NKG2D expression on mouse M ϕ from the L5178Y plus LPS group was 27% higher than on M ϕ from control pretreatment and L5178Y-only recipient groups (MFI ratio: 2.8 vs 2.2 vs 1.6), it was not different (3.7% increase) from the levels of expression on M ϕ from the LPS-only experimental group (MFI ratio: 2.8 vs 2.7).

In parallel (Fig. 11B), we tested the in vivo antitumor effects of M ϕ primed to LPS by L5178Y cells and found that proliferation of CFSE-labeled L5178Y cells injected i.p. was suppressed in DBA/2 mice after i.v. administration of a low amount of LPS. Thus, CFSE⁺ L5178Y cells unrestrictedly proliferated in control, PBS-treated animals, and by the end of the experiment retained only 4.5% of CFSE fluorescence as compared with CFSE⁺ L5178Y cells that were kept in medium in vitro and were γ -irradiated before incubation to permanently suppress their proliferation. At the same time, CFSE⁺ L5178Y from animals that were challenged with LPS 24 h after CFSE⁺ L5178Y implantation were found to retain $\sim 20\%$ of the amount of CFSE as compared with control, γ -irradiated cells. Similar results were seen with CFSE⁺ L5178Y cells cultured with naive M ϕ in vitro. Together, these results show that L5178Y cells can prime naive M ϕ in vivo to subsequent stimulation with LPS, which results in M ϕ activation, as revealed by phenotypic alterations, change of cytokine expression, and antitumor activity.

Discussion

In this study, we demonstrate that M ϕ can recognize L5178Y tumor cells. Previous studies indicated that isolated ligation of CD40, NKG2D, and CD18 can be involved in activation of M ϕ (17, 20, 23). Our current study dissects a functional hierarchy of these interactions in a polyvalent cross-talk between tumor cells and M ϕ . Even though CD154 is expressed in relatively low amounts on L5178Y cells (Fig. 5A), its role in priming of M ϕ via CD40 was found to have a much greater stimulatory impact on M ϕ than M ϕ cross-linking via CD18 by ICAM-1 and ICAM-2, which are highly expressed on L5178Y cells. Similarly, whereas isolated blockage of NKG2D-H60 interactions had no noticeable effect on M ϕ in in vitro secretory and antitumor activities, it was required, in addition to blockage of CD40-CD154 and CD18-ICAM-1/2, for abrogation of M ϕ activation by L5178Y cells. Thus, the M ϕ -tu-

mor cell cross-talk that ultimately leads to induction of antitumor M ϕ involves concurrent engagement of multiple receptor-ligand pairs.

It also appears that tumor cells should simultaneously express several stimulatory ligands to induce M ϕ activation. In this regard, normal syngeneic mouse splenocytes (essentially comprised of CD3⁺ T cells ($\sim 35\%$), B220⁺ B cells ($\sim 40\%$), CD11b⁺ mononuclear cells ($\sim 10\%$), and CD49b⁺ NK cells (5–7%)) did not prime M ϕ , in contrast to priming by L5178Y cells. Whereas most of the splenocytes expressed ICAM-1 and ICAM-2 (in a very heterogeneous fashion), only a small fraction (mostly CD4⁺T cells, $<15\%$ of total naive splenocytes) expressed CD154 (and only at very low levels), and no splenocytes expressed ligands of NKG2D (data not shown). Activation of splenocytes with LPS and CpG to produce IL-12 and IFN- γ (24–27) might facilitate M ϕ activation and induce moderate levels of antitumor effects in vitro without involvement of the CD40/NKG2D/CD18-dependent mechanism; however, in our in vitro experiments, splenocytes did not trigger NO production by M ϕ . Indeed, culturing M ϕ with 1×10^4 L5178Y cells led to only marginal activation of M ϕ (Figs. 1, B–E, and 2), whereas culturing M ϕ with $2.5\text{--}5 \times 10^4$ L5178Y cells led to robust activation of M ϕ .

Several reports have previously demonstrated that the *M. arginini*-infected L5178Y and YAC-1 cell lines could activate thioglycolate-elicited peritoneal M ϕ in the presence of LPS or exogenous IFN- γ to mediate in vitro antitumor effects via NO- and TNF- α -dependent mechanisms (28, 29). Results of those studies differed in the sensitivity of the model to the suppressive effects of PGE₂, but both agreed that the priming effects could be transferred by the tumor cell-conditioned supernatants or mediated by paraformaldehyde-fixed tumor cells. In our study, we tested the L5178Y cell line for *Mycoplasma* spp. contamination and found no evidence for infection. The absence of *Mycoplasma* spp. in L5178Y cells was also confirmed at a DNA level by using fluorescent probes (data not shown). Most importantly, our results, unlike those published by Young et al. (28) and Ribeiro-Dias et al. (29), suggest that priming of M ϕ required direct cell contact with viable L5178Y cells; M ϕ could not be primed by either L5178Y cell-conditioned supernatants (Fig. 5C) or fixed L5178Y cells. Furthermore, separation of M ϕ and viable L5178Y cells by the TW also abrogated priming of M ϕ . Whereas we cannot exclude a contribution of soluble, LPS-induced tumor cell-derived factors, such as IL-1 (30), our results suggest that membrane-bound, but not soluble factors are important for induction of cytotoxic M ϕ in our in vitro model. The inability of fixed L5178Y cells to prime M ϕ might also be due to potential toxic effects of fixed L5178Y cells on M ϕ (such as leaching of paraformaldehyde). However, our preliminary data argue against this, because coculture of fixed L5178Y cells with viable L5178Y cells did not affect proliferation of viable L5178Y cells as measured by [³H]TdR incorporation (data not shown). The complete absence of M ϕ priming by fixed L5178Y cells in our in vitro experiments could be explained by denaturation of CD154 subunits by paraformaldehyde or by the inability of fixed L5178Y cells to up-regulate CD154 expression upon contact with M ϕ in the presence of LPS, which might be important for compensation of CD40 down-regulation on L5178Y-primed M ϕ . Lastly, loss of L5178Y cell membrane fluidity and inability of PF-L5178Y cells to mobilize CD154-, H60- or ICAM-1- and ICAM-2-containing membrane rafts to the site of direct contact with M ϕ might also lead to this loss of M ϕ -priming properties, provided that the cells express no other priming substances such as those associated with *Mycoplasma* spp. contamination (28, 29).

Despite high concentrations of NO produced by the L5178Y cell plus LPS-stimulated M ϕ , L5178Y cell proliferation in the TW chamber was not suppressed when cocultured with L5178Y cell plus LPS-stimulated M ϕ in the bottom of the TW chamber system. In contrast, direct contact with M ϕ resulted in substantial antitumor effects, suggesting membrane-mediated death signaling. In this regard, we found only very limited expression of membrane-bound TNF- α , FasL, and TRAIL on the surface of M ϕ after their exposure to L5178Y cells and LPS (data not shown). The potential roles of other factors in L5178Y plus LPS-stimulated M ϕ -mediated cytotoxicity remain to be clarified.

Flow cytometric analysis of the immunophenotype of M ϕ and L5178Y cells revealed that exposure of these cells to each other results in changes in expression of some of the Ags forming certain receptor-ligand pairs. Hence, a relative decrease (7–25%) of CD40 expression on M ϕ coincided with up-regulation (31–88%) of CD154 on L5178Y cells after these cells were cultured in the presence of LPS. Similarly, up-regulation (44–127%) of NKG2D on M ϕ coincided with a slight decrease (11–16%) in expression of H60 on L5178Y cells. The observed alterations of the CD40-CD154 pair expression could favor recognition of L5178Y cells via CD154. The decreased expression of H60 on L5178Y cells following contact with M ϕ is in agreement with the observation by Bui et al. (31) that type I IFN down-regulated H60 in some tumors and could be associated with mechanisms of immune evasion (32); yet, we observed up-regulation of NKG2D expression on L5178Y lymphoma-primed M ϕ in the presence of LPS (Fig. 4). Unlike the increased MHC-I we have noted on tumor cells escaping from NK cell effects (33), there was no substantive change in MHC-I expression on L5178Y cells cultured with M ϕ (Fig. 5B). Treatment of L5178Y cells with anti-MHC-I mAb did not alter secretory and antiproliferative functions of M ϕ (Fig. 7). In agreement with this, we have recently demonstrated that anti-CD40-induced M ϕ -mediated *in vivo* antitumor effects can be effectively induced against both highly and weakly immunogenic tumors, independent of MHC-I expression (34).

Although the expression of NKG2D and its potential role in M ϕ activation and M ϕ -mediated killing has been controversial (17, 35), our data document up-regulation of cell surface NKG2D on M ϕ when cultured along with L5178Y cells and LPS. Furthermore, we demonstrate the abrogation of M ϕ activation in these cultures when M ϕ -based CD40, CD18, and NKG2D molecules are simultaneously prevented from recognizing their respective ligands. These data are consistent with the demonstration that mouse M ϕ activated by LPS and IFN- γ up-regulate NKG2D and these activated M ϕ kill tumor cells that express adenovirus E1A in an NKG2D-dependent manner (J. Routes, manuscript in preparation).

In a variety of immunological reactions, M ϕ can serve as APCs providing stimuli for T cells by MHC-II-associated exogenous Ags as well as via a number of costimulatory molecules. However, engagement of certain cell surface molecules on M ϕ causes reciprocal signaling, resulting in M ϕ activation. Several M ϕ membrane-associated surface molecules, including CD40, MHC-II, and CD18, could be involved in this reciprocal stimulation associated with modulation of signaling pathways, production of cytokines, and changes of immunophenotype. Frequently, ligation of more than one type of molecule on the surface of M ϕ is required for induction of cytokines. For example, it was shown that interaction of M ϕ with T cells via CD40-CD154 leads to accumulation of IL-12p40 in M ϕ ; at the same time, additional contacts via the MHC-II-TCR pair was needed to trigger M ϕ to produce IL-12p35, thus enabling pro-

duction of functional IL-12p70 (36–38). Our results demonstrate that L5178Y lymphoma cells express high amounts of H60 as well as ICAM-1 and ICAM-2. Thus, it is possible that H60 and ICAM-1/2 molecules could synergize with CD154 in M ϕ activation. The potential mechanisms of these additional interactions between M ϕ and L5178Y cells remain to be clarified, but could range from a simple anchoring of the ICAM⁺ tumor cells to the surface of CD18⁺ M ϕ (18) (to facilitate cross-talk via CD40-CD154 and NKG2D-H60 pairs) to an active recruitment of a Toll/IL-1R family-like cascade to modulate TLR signaling (39). The latter effect on adapter proteins of TLR signaling pathways might partially explain selective sensitivity of L5178Y-primed M ϕ to LPS but not CpG, although the most likely explanation is that peritoneal mouse M ϕ readily express TLR4 (40), whereas TLR9 expression is negligible without proper stimulation (21).

Alternatively, the ability to prime M ϕ may be restricted to the cells of a certain embryonic origin. Thus, L5178Y cells and A20 cells of lymphoid origin could prime M ϕ (for A20 cells, presence of the exogenous α CD40 (Fig. 8A) or other CD40L-expressing cells (Fig. 10B) was required), whereas B16 melanoma cells (neural crest origin) and mouse fibroblast L cells (mesenchymal origin) did not have this ability even in the presence of exogenous anti-CD40 (B16; Fig. 8A) or after transfection with functional CD40L (CD40L-L; Fig. 9). L5178Y and A20 cells have also been able to facilitate priming by other CD154⁺ cells (CD40L-L), but this costimulatory ability has been lost when cells were paraformaldehyde fixed (data not shown). Importantly, this effect was independent from the CD154-expressing status of these lymphoma cells, as A20 cells are CD154 negative (Fig. 5A). Hence, it remains possible that some soluble factors or other surface costimulatory molecules expressed by lymphoid cells in the process of initial cross-talk between tumor cells and M ϕ , are involved in the process of M ϕ priming.

The conclusions made from *in vitro* experiments were consistent with data obtained in our *in vivo* tumor model. Intra-peritoneal implantation of L5178Y cells resulted in alterations of the phenotype of resident M ϕ (up-regulation of MHC-II and down-regulation of CD80 and CD86), as well as expression of IFN- γ and TNF- α , but not IL-4 or IL-10 (Fig. 11). It is unclear whether these immunological changes are solely due to interaction of peritoneal M ϕ with L5178Y cells or whether this is a result of the cross-talk between M ϕ and tumor cells in the presence of very low, “physiological” concentrations of circulating endotoxin. However, when small doses of LPS were given to the tumor cell recipients, M ϕ expressed much higher levels of MHC-II, CD80, CD86, IFN- γ , and TNF- α than after the treatment with L5178Y cells or LPS alone. Surprisingly, there was a significant increase of production of IL-10, which is known to play an important role in regulation of M ϕ activation. The apparent discrepancy in the magnitude of M ϕ phenotypic changes seen in *in vitro* vs *in vivo* models may reflect differences in these experimental conditions. Thus, in *in vitro* experiments (Fig. 4), M ϕ and L5178Y cells were in constant contact for the entire duration of the experiment, and the volume of distribution of M ϕ -derived and potentially L5178Y cell-derived soluble factors was confined to 0.2 ml. On the other hand, in the *in vivo* experiment, interactions between M ϕ and L5178Y cells could be less durable and the volume of distribution of soluble factors was much larger and potentially affected by physiologic metabolic processes. Even if the overall phenotypic changes are less pronounced *in vivo*, they are consistent with the *in vitro* data that demonstrate the principle that active M ϕ -L5178Y cell

cross-talk results in phenotypic and functional alterations of M ϕ .

Efficient tumor recognition at the early stage of tumor progression might be a critical component of cancer immune surveillance. The state of “dormant” cancer exemplifies immune-mediated tumor growth restriction in the absence of active exogenous immunostimulatory interventions. L5178Y lymphoma in DBA/2 mice has been used for decades to study this biological phenomenon (41–44). Both CTLs and M ϕ are involved in maintaining L5178Y lymphoma in the dormant nonprogressive stage of disease (42–44). In the present study, we suggest an additional mechanism of this immunological lymphoma surveillance: L5178Y cell-induced priming of M ϕ to endotoxin, which is naturally present in biological fluids in minute concentrations (45), might be involved in M ϕ activation and result in antilymphoma effects in vivo. A number of reports demonstrate that human T cell lymphomas, as well as other cancers, can express CD154 (46–51). Whether human M ϕ can recognize autologous cancer cells via similar mechanisms, shown here for L5178Y cells, remains to be determined.

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Disclosures

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