Differentiation of C2D Macrophage Cells after Adoptive Transfer

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C2D macrophage cells protect immunocompromised mice from experimentally induced pneumonias after intraperitoneal (i.p.) adoptive transfer. These macrophage cells are immature and display minimal activity in vitro. Therefore, we wanted to understand how adoptive transfer affected these cells. We believe that the in vivo environment affects the phenotypic and functional characteristics of macrophages that help maintain the physiological integrity of the host. To test this hypothesis, we characterized the trafficking patterns and cellular changes of the established macrophage C2D cell line after adoptive transfer. We examined phenotypic changes of the C2D macrophage cells in vivo with and without stimulation with gamma interferon (IFN-γ). After i.p. adoptive transfer, C2D macrophage cells trafficked to the lungs, spleen, lymph nodes, and bone marrow of recipient mice. The cells were detected for as long as 2 months, and the cells expressed increased levels of CD11b, c-fms, and F4/80 on their surface, becoming more differentiated macrophages compared to cells maintained in vitro. Upon in vivo stimulation with IFN-γ, c-fms levels decreased while Gr-1 levels increased compared to in vivo, unstimulated, phosphate-buffered saline-injected controls. These responses were independent of the genetic backgrounds of the recipient mice. These data support the hypothesis and indicate that C2D macrophage cells respond to in vivo signals that are absent during in vitro culture.

Severe pulmonary infections and clinical pneumonia are significant causes of morbidity and mortality associated with immunosuppression (4, 13, 23). Seventy-five percent of all immunocompromised individuals, and 90% of those who died, had evidence of opportunistic pulmonary infection (58, 71).

Antibiotic resistance complicates treatment of the immunocompromised host. Bacteria may be resistant at the start of prophylaxis or can acquire resistance during therapy. In 1994, Doern et al. observed that 24% of Streptococcus pneumoniae isolates in the United States had developed antibiotic resistance (18). Pseudomonas aeruginosa produces biofilms that contribute to antibiotic resistance (20). Methicillin-resistant Staphylococcus aureus (53) and penicillin-resistant pneumococci (25) also pose a threat to patient health. The former organisms account for 15% of bacterial infections in the United States, and nosocomial pneumonias caused by Staphylococcus epidermis and Staphylococcus haemolyticus have drastically increased in recent years as a result of respirator use in both young and elderly patients (21). These pneumonias were often accompanied by bloodstream infections that were lethal in 54 to 59% of reported cases. Heart transplant and surgical patients have similar outcomes (63). Therefore, alternative preventative and treatment methods for bacterial infections associated with immunosuppression are needed.

The use of ex vivo-modified cellular vehicles to express genes to make proteins that are missing in the human body repertoire provides a potential treatment for a wide range of immunodeficiencies (8). However, the isolation of specific cell types that are capable of achieving the reliable, high-level, tissue-specific, and long-term expression of desired genes has proven elusive. Difficulties in isolation, culture, transduction, and gene stability have limited the application of some candidate cell types (74).

Macrophage cell lines offer a novel approach to cell therapy and can be efficiently transfected using either viral or nonviral methods (8). In addition, macrophages migrate to specific tissues in response to chemokine and cytokine stimuli, which make them attractive candidates for cell therapy. However, little is known about the effects of the in vivo environment on the longevity and cellular function of reintroduced cell lines. We previously described the C2D macrophage cell line that maintained some macrophage properties, including the expression of Toll-like receptor 4 (TLR4). Nevertheless, these macrophage cells secreted only a limited number of cytokines, such as interleukin-6 (IL-6), after stimulation in vitro with lipopolysaccharide (3). Interestingly, the adoptive transfer of C2D macrophage cells protected recipient immunocompromised mice from developing pneumonia after experimental challenge with Pasteurella pneumotropica (33). The mechanism for the successful adoptive transfer and protection of mice with C2D macrophage cells was unclear. Adoptive transfer of C2D macrophage cells resulted in an earlier transcription of tumor necrosis factor alpha (TNF-α) in the lungs of B10 and B10 × C2D mice than in control animals (33). However, C2D macrophage cells did not make TNF-α when stimulated in vitro (3). Therefore, it is possible that C2D macrophage cells changed after adoptive transfer. The in vivo microenvironment can affect phenotypic and functional characteristics of macrophages (44, 49, 51). To test this hypothesis, we adoptively transferred C2D macrophage cells, assessed where they trafficked, and characterized them after reisolation from the peritoneal cavity. We demonstrate that C2D macrophage cells respond to the in vivo environment differently from the in vitro

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environment. Adoptive transfer induced C2D macrophage cells to differentiate into mature macrophages and show increased expression levels of c-fms, F4/80, and CD11b. Cell trafficking patterns and additional cellular changes in response to the in vivo environment were characterized.

MATERIALS AND METHODS

Mouse strains. C57BL/6j (B6 MHC-II+/+ TβRb+/+) mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and used as wild-type controls. C57BL/10ScN (B10 MHC-II+/+ TβRb+/+) mice were obtained from the animal resource facility at the NIH. C2D (B6.129-abbb118NST2F0 MHC-II+/+ TLR4+/+) mice are on a B6 background but lack functional histocompatibility class II (MHC-II) genes due to a natural deletion of the Iε gene and a targeted deletion of the Iαε gene (31). B6, B10, and C2D mice were used between 6 and 10 weeks of age. All mice listed served as recipients in adoptive transfer experiments. These mouse strains are syngeneic on the C57BL (H-2b) background and will accept graft transfers of C2D cells. All mice were bred in the rodent facility of the Division of Biology at Kansas State University. All animal experiments were approved by the Institutional Animal Care and Use Committee.

C2D macrophage cell line. The C2D macrophage cell line was created by our group (3). These cells were derived from C2D murine bone marrow and selected in the presence of macrophage colony-stimulating factor (M-CSF). These cells have the MHC-II−/− and TβRb-/- genotype and are histocompatible with mice of the H-2I-E haplotype. C2D cells were grown in Dulbecco's modified Eagle's medium with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% Opti-Mem in 150-mm tissue culture plates.

Adoptive transfer of labeled cells. (i) C2D mouse whole bone marrow. Long bones were recovered from 6-week-old C2D mice, and the marrow cavity was flushed with sterile heparin (Eikins-Sinn, Inc., Cherry Hill, NJ) in phosphate-buffered saline (PBS) (h/PBS) (100 U/ml). Cells were processed as described below. Cells were centrifuged, washed twice to remove serum components, and resuspended in warm (37°C), sterile h/PBS (50 U/ml). Mice were injected with 1 ml of intraperitoneally (i.p.) (2 x 10⁶ cells per ml).

(ii) C2D macrophage cells. Cells were dispensed with 0.25% trypsin with 0.02% EDTA (TEDETA) and resuspended in DMEM-4. Cells were centrifuged at 350 x g for 7 min, resuspended in warmed (37°C), sterile PBS at a concentration of 1.5 x 10⁶ cells/ml, and stained with carboxyfluorescein diacetate-succinimidyl ester (CFDA SE) (catalog no. C1157; Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Briefly, cells were incubated with 22 μM of the probe solution at 37°C for 15 min and then centrifuged at 350 x g for 7 min, resuspended in fresh, prewarmed PBS, and incubated at 37°C for an additional 20 min. After the second incubation, cells were washed two times with DMEM-4 and resuspended at a concentration of 2 x 10⁶ cells/ml in PBS (50 U/ml). One milliliter of either CFDA SE-labeled or unlabeled C2D macrophage cells was injected i.p. per mouse. CFDA SE-labeled C2D macrophage cells were collected from the peritoneal lavage of 1/10 C2D mice by in vitro cell transfer. Unlabeled cells were detected in various organs using reverse transcriptase PCR (RT-PCR), from day 7 posttransfer to day 62 posttransfer, as described below. Unlabeled C2D macrophage cells were also adoptively transferred through the lateral tail vein (intravenously [i.v.]) by injection of 2 x 10⁷ cells per mouse in 200 μl of h/PBS (50 U/ml).

Fluorescence-activated cell sorter analysis. CFDA SE-labeled C2D macrophages were recovered from B6, B10, and C2D mice by peritoneal lavage 1 to 3 days posttransfer with 24 ml of ice-cold sterile PBS after anesthetizing the mice with halothane (Halocarbon, River Edge, NJ) and euthanizing them by cervical dislocation. Cells were first incubated with 0.5 μg of anti-mouse CD11b/CD32 (eBioscience, San Diego, CA) in 50 μl of Hanks' buffered salt solution, and then blocked with 10 μl of rat IgG2a with biotinylated rat IgG2a as the isotype control, 0.46 μg of rat anti-F4/80-APC IgG2a with rat IgG2a-APC as the isotype control, 0.1 μg of rat anti-CD11b-PE IgG2b with rat IgG2b-PE as the isotype control, and 0.04 μg of rat anti-Gr-1-PE IgG2b with rat anti-IgG2b-PE as the isotype control (all from eBioscience) (isotype controls were used at the same concentrations as specific antibodies). In addition, some cells were stained with 9.2 μg of rat anti-Gr-1-PE IgG2a (ATCC TIB166) conjugated with Alexa Fluor 647, and controls were stained with 9.2 μg of rat IgG2a (eBioscience) conjugated with Alexa Fluor 647 (Molecular Probes, Eugene, OR). We used 5.4 μg of rat anti-ER-MP58 IgM purchased from Biomedicals AG and used 0.14 μg of anti-rat IgG-PE (eBioscience) as the secondary antibody. Biotinylated rat anti-Ly-6C IgM was purchased from BD Pharmingen, cells were stained with 1.0 μg, and controls were stained with biotinylated rat IgM. Biotinylated antibodies were detected with 0.46 μg of streptavidin-APC (eBioscience).

Samples were analyzed using a FACSCalibur analytical flow cytometer (Becton Dickinson, San Jose, CA). A total of 10,000 to 20,000 events were measured for each sample. Data analysis was performed with the personal computer-compatible WinList software (Verity Software House, Topsham, ME). Cell sorting was performed with a FACS Vantage SE cell sorter (Becton Dickinson) using specimen optimization and calibration techniques according to the manufacturer's recommendations. Cells were sorted at a rate of 12,000 cells per s, and approximately 1 million cells were collected per group to minimize cell stress and damage. Cells were sorted based on CFDA SE expression, with the lowest 10% of positive cells not selected.

RT-PCR. Total RNA was extracted from cells (where indicated) using Tri reagent according to the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH). RNA concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE) and were diluted to an optimal concentration of 100 ng per μl, and a total of 500 ng per sample was used. cDNA was amplified using specific primers. B10 and B10 x C2D mice were assessed for the expression of TLR4-positive cells after reconstitution using RT-PCR of TLR4 mRNA. These mice have a deletion in the Tlr4 gene that does not allow the successful transcription of mRNA (5, 55). The detection of a 476-bp cDNA fragment was interpreted as the presence of viable TLR4-transcribing cells in TLR4-deleted mice. For this assessment, primers 5’-GTAATGTAAGACTACAAATTCCT-3’ (sense) and 5’-CATGTTAGAACTCTATATCAA-3’ (antisense) (GenBank accession number AF110133) were used. To detect Tnf-α, a 436-bp cDNA product was amplified using primers 5’-GGGATGAGGATGTTCATCCAATG-3’ (sense) and 5’-TCACAGACGGACTGTTCTCAGG-3’ (antisense) (accession number M53296). To detect β-actin, a 600-bp cDNA product was amplified using primers 5’-ATGGATGACGATATCGCT-3’ (antisense) and 5’-ATAGGAGTACGCRGCA-3’ (antisense) (accession number 00NM-007393.1). The RT-PCR conditions were previously described (33, 73). The presence of mRNA was distinguished from contaminating genomic DNA by PCR controls lacking reverse transcriptase. Densitometric comparisons between cytokines and β-actin DNA were performed on ethidium bromide-stained 2% agarose gels using AlphaImager software (Alpha Innotech Corporation, San Leandro, CA).

TRAFFICKING. CFDA SE-labeled C2D macrophage cells were adoptively transferred to either B6, B10, or C2D mice as described above. At 3 days posttransfer, mice were euthanized as described above, and the spleen, pancreatic lymph nodes, and surrounding lymph tissue, liver, and intestine were removed. Spleens were...
and pancreatic lymph nodes were placed in 10% formalin–PBS and assessed by confocal microscopy. Formalin-fixed mouse pancreatic lymph node, perinodal adipose, and spleen tissues were cut into 50-μm-thick slices using a TC-2 tissue sectioner (Sorvall Instruments). Tissue slices were mounted onto glass slides, and differential contrast interference (DIC) images of tissue and CFDA SE-labeled C2D macrophage cells were observed on a model LSM 5 Pa Zeiss laser scanning confocal microscope. Tissues were visualized with 20×0.5 and 40×0.75 Plan Neofluar objectives with DIC. CFDA SE-labeled C2D macrophage cells were visualized using the 488-nm line of an argon ion gas laser (excitation of CFDA SE), an FT 488 primary dichroic beam splitter, an FT 545 secondary dichroic beam slitter, a 505-nm to 530-nm-bandpass filter, a photomultiplier tube, and LSM5 Pa software, version 3.2 SP2. The number of CFDA SE-labeled macrophages per square micrometer of area of pancreatic lymph nodes, white adipose, and spleen tissues was determined using ImagJ v1.37 (NIH). The median lobe of liver was collected and frozen at −80°C in Tissue Tek (Sakura, Torrance, CA). Eight-micrometer-thick slices were then cut using a cryostat, placed onto glass slides, and kept at −80°C until visualization. Tissue was visualized with 20×0.5 and 40×0.75 objectives with DIC using a Nikon Eclipse 80i microscope (Nikon, Melville, NY) equipped with an EXFO Photonics X-Cite 120 fluorescence illumination system (EXFO, Quebec, Canada), a CoolSNAP cf camera (Photometrics, Tucson, AZ), and MetaVue (version 6.3r6) imaging software (Molecular Devices Corp., Downington, PA).

C2D macrophage cell or bone marrow cell differentiation in vitro. To obtain primary bone marrow, B6 mice were sacrificed as described above, and the femora and tibiae of both legs were removed. Bones were washed in 70% ethanol, and the tips of the bones were removed. Using a 26-gauge needle, the bone marrow was washed from the medullary cavity with sterile, cold PBS. Red blood cells were lysed by the bone marrow by exposure to ammonium chloride-potassium lysing buffer for 5 min on ice. After 5 min, the solution was returned to isotonicity by diluting lysing buffer in two times the volume of DMEM-4 and was centrifuged at 350 g for 10 min. The pellet was resuspended in DMEM-4. The cells were then counted and plated in either Biomedia (DMEM-4 plus 10% LM929 supernatant, 150 ng/100 ml M-CSF [R&D Systems, Minneapolis, MN], and 0.2 μg/ml gentamicin) or DMEM-4–cytokine (DMEM-4 plus 500 U/ml IL-4 [R&D Systems], 800 U/ml granulocyte-macrophage colony-stimulating factor [GM-CSF] [R&D Systems], and 0.2 μg/ml gentamicin) (43) at 2 × 10⁶ cells per 100-mm plate. Confluent C2D macrophage cells were dispered with TEDTA and resuspended in DMEM-4. Cells were then counted and plated in either Biomedia or DMEM-4–cytokine at 2 × 10⁵ cells per 100-mm plate. Two days later, medium was removed, and fresh medium was added to both bone marrow cells and C2D macrophage cells. At day 5 postplating, old medium was again removed, and fresh Biomedia or fresh DMEM-4–cytokine plus an additional 100 U/ml TNF-α (R&D systems) were added to both cell types. At day 8 postplating, adherent cells were removed using 0.02% EDTA and resuspended in DMEM-4. Cells were then labeled with antibodies as described above and analyzed by flow cytometry.

Statistics. Statistical values were determined using both the Student's t test (two-tailed, paired) and the Wilcoxon signed-rank test. A P value of <0.05 was considered to be significant if shown by both tests, unless otherwise indicated. Data are presented as means ± standard errors of the means (SEM). Differences between treatment groups were determined using the indicated tests done with the StatMost statistical package (Data XIOM, Los Angeles, CA).

**RESULTS**

Phenotype of C2D macrophage cells in vivo versus that in vitro. C2D macrophage cells, which show little functional activity in vitro, help protect immunocompromised mice from infection (33). Therefore, we hypothesized that once in vivo, C2D macrophage cells adapt and alter their phenotype. The

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**FIG. 1.** Phenotype of C2D macrophage cells after in vivo or in vitro culture. CFDA SE-labeled C2D macrophage cells were adoptively transferred to the peritoneal cavities of B10, B6, or C2D mice. Three days later, peritoneal cavities were washed, cells were collected, and the expression of various surface markers was analyzed on CFDA SE-positive cells. (A to C) Phenotypes of C2D macrophage cells in vivo in B6 mice (A), in B10 mice (B), and in C2D mice (C). (D) Phenotypic comparison of C2D macrophage cells in various mouse strains. The data are presented as means ± SEM (n = 9; analysis of C2D macrophage cells from nine independent samples [three mice pooled per sample]/mouse strain). An asterisk over a bar indicates a significant difference between treatments (P < 0.05).
microenvironment in which cells reside influences heterogeneity (19, 29, 30, 47, 59). Thus, we also hypothesized that the phenotype would differ slightly depending on the mouse strain to which the C2D macrophage cells were adoptively transferred.

To test these hypotheses, we adoptively transferred (i.p.) CFDA SE-labeled C2D macrophage cells into B10, B6, or C2D mice, and 3 days later, we analyzed their phenotype by flow cytometry. The phenotype of C2D macrophage cells in vivo was significantly different from that of C2D macrophage cells in vitro (Fig. 1A to C); however, the phenotypes were similar regardless of the mouse strain (Fig. 1D). In the in vitro environment, C2D macrophage cells express negligible levels of the macrophage markers c-fms, F4/80, and CD11b (Fig. 1A to C), regardless of whether the cells have been stained with CFDA SE or left untreated. However, they express moderate levels (30 to 40%) of Ly-6C, a surface marker expressed mainly by monocytes and immature macrophages, as well as Mac-2 (55 to 70%), a macrophage marker which successively increases with macrophage maturation. The in vivo environment caused C2D macrophage cells to increase their expression of various surface markers, thus acquiring a mature macrophage phenotype. There was a significant increase in the expression of c-fms, F4/80, and CD11b as well as a slight increase in Mac-2. c-fms identifies cells in the macrophage lineage. F4/80, CD11b, and Mac-2 are first expressed by monocytes or immature macrophages and increase in a differentiation-dependent manner.

There was also a significant increase in the surface expression of Ly-6C, CD11c, and Gr-1 but not to the same extent as that seen for c-fms, F4/80, or CD11b (Fig. 1). The increase in Ly-6C indicates that C2D macrophage cells are immature in the in vitro environment, as the level of Ly-6C does not increase on mature macrophages (36). CD11c, expressed by dendritic cells, and Gr-1, a granulocytic marker, can also be expressed on macrophages (1, 24, 40).

Macrophages are known to play a key role in initiating acute inflammatory responses (27, 41). A T-cell cytokine, IFN-γ, has been shown to have profound effects on macrophage function (66), including the induction of macrophage activation (2, 52, 61), increased microbicidal activity (2, 48), and increased surface expression of MHC-II (41, 66). We therefore hypothesized that upon stimulation with IFN-γ, C2D macrophage cells would further alter their phenotype in vivo but would experience minimal effects in vitro. To determine the effect of IFN-γ on C2D macrophage cells, we injected (i.p.) IFN-γ 1 day post-transfer of CFDA SE-labeled C2D cells to either B10, B6, or C2D mice or added IFN-γ to C2D cells in vitro. We analyzed their phenotype 1 day later. In the in vitro environment, IFN-γ stimulation did not significantly alter the phenotype of C2D macrophage cells (Fig. 2D), while stimulation in vivo altered the surface expression of c-fms and Gr-1 (Fig. 2A to C). There

![Figure 2](http://cvi.asm.org/)

**FIG. 2.** Phenotype of C2D macrophage cells after IFN-γ stimulation in vivo and in vitro. CFDA SE-labeled C2D macrophage cells were adoptively transferred to the peritoneal cavities of B10, B6, or C2D mice. One day later, the cells were stimulated by i.p. injection of IFN-γ. The cells were harvested 18 h later, and their phenotypes were analyzed by flow cytometry. For in vitro samples, C2D macrophage cells were treated with IFN-γ, and 18 h later, their phenotypes were analyzed by flow cytometry. (A to C) Phenotypes of C2D macrophage cells in vivo with and without IFN-γ in B6 mice (A), in B10 mice (B), in C2D mice (C). (D) Phenotype of C2D macrophage cells in vitro with and without IFN-γ. The data are presented as means ± SEM (n = 7; analysis of C2D macrophage cells from seven independent samples [two mice pooled per sample] per mouse strain).
was little change in CD31, Ly-6C, CD11c or F4/80, CD11b, and Mac-2 in vivo (Fig. 2A to C). C2D macrophage cells took on a more granulocytic phenotype as c-fms expression significantly decreased and Gr-1 expression significantly increased. There was also no variation in the phenotype between mouse strains.

**Trafficking of C2D macrophage cells.** C2D macrophage cells adapted to the in vivo environment. Therefore, we hypothesized that C2D macrophage cells would traffic to tissues where macrophages are normally found. The adoptive transfer of C2D macrophage cells was successful using either i.v. or i.p. injection (Fig. 3). The C2D macrophage cells were detected in the lungs, spleen, bone marrow (Fig. 3), and lymph nodes (not shown) by RT-PCR. There was no apparent difference between the transfer methods used. Consequently, we chose the i.p. method of adoptive transfer in our experiments due to the ease in animal and cell handling.

To determine the longevity of adoptively transferred whole bone marrow and C2D macrophage cells in B10 mice, we assessed the trafficking patterns of transferred cells in normal mice. In whole bone marrow transfer recipients, TLR4-positive cells were present in the bone marrow by 7 days posttransfer but were not detected in other organs until 62 days posttransfer (Table 1). In contrast, cells derived from the C2D macrophage cell line were detected by RT-PCR in various organs as early as 7 days following transfer (Table 1). This was consistent with initial transfer experiments (Fig. 3). C2D macrophage cells were also detected up to 62 days posttransfer (Table 1). Histopathological assessments of tissue samples taken from mice 30 days after adoptive transfer from C2D macrophage cell recipients were devoid of neoplasia, tissue damage (necrosis or apoptosis), metastasis, tumorigenesis, or autoimmunity. There was slight to moderate lymphoid hyperplasia in the lymphoid tissues of adoptively transferred compared to control mice (Fig. 4).

To determine if intact cells could be detected after adoptive transfer, we further assessed the trafficking patterns of adoptively transferred C2D macrophage cells using confocal microscopy. We found that they could be detected at 3 days posttransfer in the pancreatic lymph nodes, spleen, and perinodal adipose (Fig. 5). There were no significant mouse strain differences. C2D macrophage cells were also detected in the liver but in significantly lower numbers than in the other organs (Fig. 5).

**Response of C2D macrophage cells to signals given in the in vitro environment.** To determine if we could induce C2D macrophage cell differentiation in vitro, we treated C2D macrophage cells with known differentiation regimens. The significant increase in F4/80 and CD11b expression suggests that C2D macrophage cells became more mature in vivo. The small but significant increase in CD11c expression on C2D macrophage cells in response to the in vivo environment also suggests some differentiation toward a dendritic cell phenotype. Therefore, we assessed expression levels of these markers on C2D macrophage cells after in vitro culture with cytokines to drive them toward those lineages: either M-CSF (macrophage) or GM-CSF plus IL-4 (dendritic cell) (Fig. 6). To confirm that the cytokines were active and given at sufficient levels, we also assessed expression levels of F4/80, CD11b, and CD11c on primary bone marrow cells after similar in vitro cytokine treatments.

The expression of CD11b and CD11c was significantly increased on primary bone marrow cells after 8 days with either cytokine treatment (Fig. 6). Moreover, F4/80 was expressed on primary bone marrow cells at higher levels than CD11c in the presence of M-CSF, and CD11c was expressed more than F4/80 when primary bone marrow cells were incubated with GM-CSF and IL-4. Nevertheless, expression levels were not altered on C2D macrophage cells with either treatment. Thus, C2D macrophage cells require signals above and beyond known differentiation factors in order to alter their phenotype.

**TNF-α transcription in C2D macrophages after adoptive transfer.** C2D macrophage cells altered their surface expression phenotype and trafficked to multiple tissues in vivo. The adoptive transfer of C2D macrophages also changes lung inflammation and host resistance to *P. pneumotropica* (33). We hypothesized that changes in the C2D macrophage phenotype

![FIG. 3. Adoptive transfer of whole bone marrow or the C2D macrophage cell line to B10 mice. Mice received an i.v. or i.p. injection of 2.0 × 10⁷ C2D macrophage cells per mouse. After 7 days, mice were euthanized, and the lungs, spleen, and bone marrow were removed. The presence of TLR4-positive cells was determined using RT-PCR and gel electrophoresis.](http://www.asm.org)
after adoptive transfer were associated with changes in macrophage function. To test this hypothesis, we analyzed TNF-α transcription by C2D macrophage cells in vivo versus that in vitro. CFDA SE-labeled C2D macrophage cells were adoptively transferred (i.p.) to B10, B6, or C2D mice. Three days later, cells were washed from the peritoneal cavity and were sorted on a flow cytometer based on CFDA SE expression. As previously described (3), C2D macrophages do not make TNF-α in vitro, even when stimulated with lipopolysaccharide.

FIG. 4. Histopathological assessments of recipient lymphoid tissue after adoptive transfer of C2D macrophage cells. Recipient mouse spleens and inguinal lymph nodes were examined 30 days after adoptive transfer of C2D macrophage cells. (A) Normal lymph node. (B) C2D macrophage cell recipient lymph node. (C) Normal spleen. (D) C2D macrophage cell recipient spleen. The data are representative of more than six samples per treatment group.

FIG. 5. Number of C2D macrophage cells visualized per μm³ of tissue. Immediately after collection, tissues were fixed (as described in Materials and Methods), and each tissue was sectioned into approximately 26 0.9-μm-thick slices by confocal imaging. To determine the number of cells per sample, the slices were first reconstructed (three dimensionally) using ImageJ, and the cell numbers per μm³ of tissue were then assessed. C2D macrophage cells traffic significantly less to the liver than to the other tissues. The data are presented as means ± SEM (six reconstructions per tissue per mouse strain). P. lymph nodes, pancreatic lymph nodes.

FIG. 6. Effects of cytokines on C2D macrophage cell phenotype in vitro. C2D macrophage cells and primary bone marrow cells (BM) (positive control) were treated with either M-CSF to advance the cells through the macrophage lineage or IL-4 and GM-CSF to enhance a dendritic cell phenotype. The cells were treated with cytokines for 8 days (with 100 U/ml TNF-α for the last 5 days), and the phenotype was then analyzed. Neither cytokine treatment had any effect on the phenotype of C2D macrophage cells in the in vitro environment. The data are presented as means ± SEM (n = 4; analysis of four independently treated plates of C2D macrophage cells or primary bone marrow cells). Statistical values were determined using the Student’s t test (two-tailed and paired) as samples were prepared and collected simultaneously.
and/or IFN-γ (Fig. 7). In contrast, after adoptive transfer and reisolation, there was a significant increase in TNF-α transcription in C2D macrophage cells isolated from B6, B10, and C2D mice. Moreover, TNF-α transcription was accompanied by the secretion of approximately 500 pg TNF-α per 2 × 10^5 C2D macrophage cells, regardless of the mouse strain from which the cells were isolated. Therefore, increased differentiation of the C2D macrophages was associated with increased functional activity.

**DISCUSSION**

Monocytes are selectively recruited to different tissues in response to either infection, injury (9, 27, 65), or tissue-specific recruitment factors (30, 65). Upon entry into a tissue, monocytes differentiate into macrophages, which display a phenotype regulated by the local tissue environment (27, 30, 41, 59, 75). Adoptive transfer of C2D macrophages into mice provided enhanced resistance to infection (33). However, it was not clear what happened to those macrophages in the recipient. In this study, we characterized the response of the macrophage cell line C2D to the in vivo microenvironment of the peritoneal cavity in an effort to understand the underlying changes that lead to the protective response. The peritoneal cavity offered a convenient and sterile site (12, 75) to explore the complexity of the in vivo environment and its effects on the C2D macrophage phenotype. The adoptive transfer of C2D macrophage cells allows long-term survival in vivo in the absence of detrimental pathology (33). This is important for studying the effects of the local, uninflamed microenvironment.

C2D macrophage cells responded to in vivo signals (Fig. 8). When grown in vitro, C2D macrophage cells have an immature phenotype based on their low-level expression of the mature macrophage markers F4/80 and CD11b (38, 39), their mid-level expression of Mac-2 (42), and their low-level production of the macrophage cytokine TNF-α. Within the peritoneal cavity, C2D macrophage cells were induced to differentiate...
from immature macrophages to mature macrophages. They expressed high levels of F4/80, CD11b, Mac-2, and c-fms, (37–39). The increase in Ly-6C after adoptive transfer, which can be induced only on immature cells, also confirms the maturation of the C2D macrophages (36). The expression of F4/80 and CD11b and the up-regulation of TNF-α activity by C2D macrophage cells (Fig. 7) were also consistent with the phenotype of differentiated macrophages (49). In fact, the ability of C2D macrophage cells to make TNF-α in vivo supports the observation of earlier TNF-α production in lungs after P. pneumotropica infection in mice adoptively transferred with C2D macrophage cells, which leads to an altered early inflammatory response (3).

We expected there to be some influence of mouse strain on the phenotype of adoptively transferred C2D macrophage cells. The observation that there was no strain dependence suggests that TLR4 and MHC-II genes (or molecules) are not involved in macrophage differentiation and that nonpolymorphic factors independent of these genes are present in all the mouse strains tested. One factor that may play an important role in C2D macrophage function is integrin and/or other adhesion molecules. Knockout mice for some of these molecules exist (e.g., Mac-1 [9] or P/L/E-selectin [14]). Future studies on how C2D macrophage cells traffic in the in vivo environment in these mice may provide insight as to which factors influence the phenotype of C2D macrophage cells found in the spleen, adipose tissue, and lymph nodes.

Although IFN-γ had little effect on many macrophage phenotypic markers in this study, we did see an increase in Gr-1 and a decrease in c-fms expression. The lack of a change in so many molecules was somewhat surprising. IFN-γ has profound effects on macrophage function and activation (2, 48, 52, 61, 66) and influences monocyte maturation (17, 54, 66). The increase in Gr-1 may reflect activation, as macrophage granularity tends to positively correlate with activation (10, 56). IFN-γ is also required for the generation of inhibitory macrophages capable of suppressing T-cell proliferation, which are identified in part by their expression of Gr-1 (24). The decrease in c-fms expression was somewhat surprising because c-fms expression has been shown to be relatively stable, expressed under both steady-state and inflammatory conditions (12). c-fms mRNA transcripts were also unaffected by IFN-γ treatment (32, 46, 67). A possible explanation for the decrease in c-fms surface expression may be increased internalization after IFN-γ stimulation (17). The surface expression of c-fms has also been noted to decrease (while mRNA levels remain increased) on microglial cells within the lesions of multiple sclerosis patients (70). These lesions are characterized by inflammation and tissue destruction (45, 60). As an alternative measure of activation, we noted an increase in nonspecific Ig binding while doing flow cytometry measurements (data not shown), which is indicative of increased surface expression of Fc receptors caused by IFN-γ (15, 22, 34).

C2D macrophage cells responded to IFN-γ in vivo in a similar fashion in all three mouse strains. There is a tight interaction between the signaling pathways of TLR4 and IFN-γ (11, 16, 62), and it was expected that there would be a significant impact on macrophage phenotype in the absence of TLR4. However, C2D macrophage cells transcribe their own TLR4 if it is needed for activation and thus may respond independently of host expression. The presence of MHC-II may also be irrelevant for activation by IFN-γ. Alternatively, we introduced IFN-γ directly to the C2D macrophage cells within the peritoneal cavity, and therefore, the cells were stimulated directly. It is possible that if stimulated indirectly (i.e.), the agonist would stimulate immunological components of the host and thus may impact the C2D macrophage cells in a more host-dependent manner.

The ability of C2D macrophage cells to traffic and be retained in vivo demonstrates that macrophages adapted to long-term in vitro culture can be reintroduced in vivo, traffic to distant organs, and reside in the host for longer than 2 months without causing pathology. Although other researchers have adoptedly transferred macrophage cell lines to restore host immunity, few studies have attempted to maintain the cells in vivo for extended lengths of time. Wiltrout et al. previously found that the WEHI-3 and RAW264 macrophage cell lines injected i.v. were quickly removed over time (72). Nishihara et al. found that transfected J774A.1 macrophages functioned up to 20 days following intratumor injection (50). We detected cells from the C2D macrophage cell line up to 62 days post-transfer, suggesting that they persist and behave in a manner similar to that of resident macrophages and that they may be maintained indefinitely (we did not assay for the presence of cells after 62 days). This novel difference in longevity may be due to subtle differences in cell line characteristics including differentiation state, surface antigen expression (i.e., the lack of MHC-II), and cytokine production.

The detection of cells derived from the C2D macrophage cell line in vivo using RT-PCR was complemented by the identification of whole cells using confocal microscopy and fluorescence-activated cell sorter analysis. We identified C2D macrophage cells in various tissues including bone marrow, spleen, lungs, pancreatic lymph nodes, and perinodal adipose tissue. Their trafficking is consistent with the localization of macrophages throughout the body (28, 29, 41). Brunstetter et al. previously analyzed the migration of adoptively transferred primary bone marrow cells and determined that it took 8 weeks for the immature cells to appear in the alveolar compartment of normal mice (7). Slightly more mature Ly-6C-positive mononuclear cells traffic to the spleen, lung, and liver by approximately 3 days posttransfer (37), while adoptively transferred peritoneal macrophages migrate to the spleen and lymph nodes by 4 h posttransfer and remain up to 6 days (9, 57). Thus, the differentiated phenotype of C2D macrophage cells facilitates their dispersal to peripheral tissue and could prove useful in further investigating the effects of microenvironmental influences on macrophage heterogeneity in future studies.

It is clear that C2D macrophage cells divided in vivo. Cells labeled with CFDA SE were difficult to detect after 3 to 4 days in vivo, indicating that cell division was diluting out the label. Their detection 2 months after injection by RT-PCR could be due to long-lived cells from the original transplant or cells that have divided locally. If the latter possibility is true, C2D cells divided without being tumorigenic (3), and there was no evidence of neoplasia at necropsy or by histology at 1 or 2 months post-adoptive transfer. Local macrophage cell division is not unprecedented. In a quantitative evaluation of macrophage induction.
kinetics, 30% of host alveolar macrophages arose from a local population of dividing mononuclear phagocytes (6).

Differentiation of bone marrow cells in the in vitro environment using various cytokines has long since been established and used to develop a number of cell lines (35). M-CSF is used to differentiate bone marrow cells into macrophages (40), whereas GM-CSF and IL-4 are used to differentiate bone marrow cells into dendritic cells (69). Bone marrow-derived macrophages have been shown to express Mac-1, Mac-2, and F4/80 (40), while the addition of TNF-α to bone marrow results in a myeloid-dendritic cell phenotype, expressing both CD11b and CD11c (69). The treatment of C2D macrophage cells in vitro with either M-CSF or GM-CSF and IL-4 had little to no effect on their phenotype, unlike the phenotype of treated bone marrow cells. As an alternative method of altering the phenotype of C2D macrophage cells in vitro, we treated them with dexamethasone and insulin (data not shown). The addition of dexamethasone and insulin, which causes the differentiation of 3T3-L1 fibroblasts into adipocytes (26), has been shown by our group (L. Xie and S. K. Chapes, unpublished data) to increase the production of IL-6 by C2D macrophage cells in vitro. However, we saw no alteration of the phenotype of C2D macrophage cells after treatment with dexamethasone and insulin.

In summary, our data demonstrate that the peritoneal cavity role of integrin Mac-1 in accelerated macrophage efflux to the lymphatics. Blood 106:3234–3241.


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