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M-1/M-2 Macrophages and the Th1/Th2 Paradigm[1]

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Evidence is provided that macrophages can make M-1 or M-2 responses. The concept of M-1/M-2 fomented from observations that macrophages from prototypical Th1 strains (C57BL/6, B10D2) are more easily activated to produce NO with either IFN-γ or LPS than macrophages from Th2 strains (BALB/c, DBA/2). In marked contrast, LPS stimulates Th2, but not Th1, macrophages to increase arginine metabolism to ornithine. Thus, M-1/M-2 does not simply describe activated or unactivated macrophages, but cells expressing distinct metabolic programs. Because NO inhibits cell division, while ornithine can stimulate cell division (via polyamines), these results also indicate that M-1 and M-2 responses can influence inflammatory reactions in opposite ways. Macrophage TGF-β1, which inhibits inducible NO synthase and stimulates arginase, appears to play an important role in regulating the balance between M-1 and M-2. M-1/M-2 phenotypes are independent of T or B lymphocytes because C57BL/6 and BALB/c NUDE or SCID macrophages also exhibit M-1/M-2. Indeed, M-1/M-2 proclivities are magnified in NUDE and SCID mice. Finally, C57BL/6 SCID macrophages cause CB6F$_1$ lymphocytes to increase IFN-γ production, while BALB/c SCID macrophages increase TGF-β production. Together, the results indicate that M-1- or M-2-dominant macrophage responses can influence whether Th1/Th2 or other types of inflammatory responses occur. *The Journal of Immunology,* **2000, 164: 6166–6173.**

Macrophages are essential for host defense (1–7). In primitive organisms, macrophages are the host defense, in that they are responsible for everything from recognition to engulfment to destruction of threats (8). During phylogeny, lymphocytes take on progressively more of these macrophage duties using their superior recognition structures. The shift in the importance from innate to adaptive immune responses in evolution (9) is illustrated by the fact that lymphocyte-deficient rodents or humans succumb rapidly to infection. In line with the increasing lymphocentricity of immune systems, a paradigm has developed over the last decade or so in which T or other types of lymphocytes control the type of immune responses generated by the profile of cytokines they secrete (10–12). In particular, immune responses highlighted by T lymphocyte production of IFN-γ, which causes macrophage activation, are called Th1. Th2 immune responses are associated with IL-4, IL-5, and IL-10, which, in contrast to IFN-γ, inhibit macrophage activation and instead stimulate Ab production. *Leishmania major* is the prototypical model of Th1 and Th2 responses. Resistant C57BL/6 T lymphocytes produce IFN-γ that activates macrophages to produce NO and kill the parasite, while susceptible BALB/c T lymphocytes instead produce more IL-4 that suppresses macrophages (13, 14). In addition, IL-4 and other Th2 cytokines have been reported to increase macrophage arginine metabolism via argininase, which produces ornithine and urea (15). T lymphocytes of other strains of mice have also been reported to have a tendency to produce Th1 (B10D2) or Th2 (DBA/2) cytokine profiles (16).

Whereas the aforementioned results clearly indicate that T lymphocytes from different strains of mice have a tendency to produce cytokines that activate or inhibit macrophages, other reports have shown that macrophages from the Th1 strains are more easily activated than those from Th2 strains (17–21). Thus, the ability to generate a Th1- or Th2-type response does not wholly depend on T lymphocytes. That macrophages themselves may determine immunologic outcomes is suggested by results such as those showing that *Leishmania* infection of macrophages can increase their ability to stimulate a Th2 response instead of a Th1 response (22). In a related vein, it has been reported recently that dendritic cells have the potential to shepherd T lymphocytes into Th1- or Th2-dominant phenotypes (23–26). That macrophage and dendritic cells can both influence lymphocyte responses is in keeping with the knowledge that macrophages are a precursor of dendritic cells (27).

In the course of our ongoing investigations into factors that regulate macrophage arginine metabolism, we found evidence that Th1 and Th2 macrophages not only differ in their ability to be activated in the classical sense, but made qualitatively different responses to the same stimuli. For example, we confirmed that macrophages from Th1-like strains are more easily activated to produce NO than macrophages from Th2-like strains. More importantly, however, we also discovered that in response to certain stimuli (LPS), Th2 macrophages not only do not produce NO, but instead increase arginine metabolism to ornithine; Th1 macrophages do not. Because NO inhibits cell replication (7, 28, 29), while ornithine (as a precursor of polyamines) can stimulate replication, these results suggested that macrophages from Th1 and Th2 mice can influence immune reactions in opposite ways. The results to follow provide evidence to support this postulate and will show that macrophages from Th1 and Th2 strains differentially influence whether Th1, Th2, or other immune response occurs. We propose that these different macrophage responses be termed M-1 and M-2.

Materials and Methods

Animals

C57BL/6, B10D2, DBA/2, BALB/c, B6D2F$_1$, CB6F$_1$, C57BL/6J-*Hfh11*^{nu}, BALB/cByJ-*Hfh11*^{nu}, C57BL/6J-*Prkdc*^{scid}/SzJ, and BALB/cByJ-*Prkdc*^{scid}/J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice used for experiments were between 9 and 14 wk old.

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They were routinely tested for common murine pathogens by the University of Minnesota Research Animal Resources (Minneapolis, MN). Mice were euthanized with carbon dioxide.

Spleen cell culture

Spleens from five mice were pooled and cultured for 3 days in the presence of 5 μg/ml Con A, as previously described (30). The medium employed is described below. Supernatants were collected and assayed for IFN-γ and IL-4.

Resident peritoneal macrophage culture

Resident peritoneal macrophages (PEC)[3] were cultured as in preceding studies (30, 31). Briefly, PEC from five or more mice were harvested by three injections of 5 ml PBS containing antibiotics. PEC were resuspended in RPMI 1640 supplemented with antibiotics, 1×10^{-2} M morpholinopropane sulfonic acid (buffer), 5×10^{-5} M 2-ME, BSA (2.5 mg/ml), transferrin (10 μg/ml), and insulin (1 μU/ml). Tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD) or Sigma (St. Louis, MO). All experiments were performed in this serum-free medium (SFM), except for the spleen cell culture in Fig. 1. In this experiment, the basal medium was supplemented with 10% FBS (HyClone, Logan, UT), as in preceding studies (30). All medium components contained less than 0.015 ng endotoxin/ml. Cells were plated at 3×10^6 or 1×10^6/ml in 0.1 ml in quadruplicate flat-bottom microtiter wells. Adherent and nonadherent cells were separated by repeated washing after a 2-h incubation at 37°C in an atmosphere of 7% CO_2. PEC were then cultured for 48 h before supernatant collection to measure NO, cytokine production, or amino acid metabolism. IFN-γ was purchased from Life Technologies. LPS 055:B5 was purchased from Difco (Detroit, MI). Unless indicated, macrophages were cultured with 500 pg/ml IFN-γ (10 U/ml) and/or 1 ng/ml LPS. Any experiment comparing strains of mice was head to head, in that it was conducted on the same day with the same medium, reagents, etc.

Nitrite assay

NO production by PEC was measured in supernatants collected after 48 h of culture, as described previously (32). Briefly, 0.05 ml of Griess reagent (prepared with reagents from Sigma) was added to 0.05 ml of supernatant, and absorbance was read at 550 nm using an automated plate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

IFN-γ, IL-4, and TGF-β1 determination

Cytokines in cell culture supernatants were measured using commercially available kits from Genzyme (Cambridge, MA) (Figs. 1 and 3) or R&D Systems (Minneapolis, MN) (Fig. 6). The two companies' kits behaved similarly. We switched companies by necessity; R&D Systems purchased Genzyme's ELISA kit division. The sensitivity limits for the assays were 5 pg/ml (IFN-γ and IL-4) and 7 pg/ml (TGF-β1). Latent plus active TGF-β was measured. Experimental values were determined from standard curves calculated in Excel. The SFM employed contained undetectable quantities of these cytokines.

Amino acid analysis

Amino acids were measured as in previous publications (31). Briefly, samples for amino acid analysis were deproteinized with a solution containing sulfosalicylic (Seraprep; Pickering Labs, Mountain View, CA) and 250 μM norleucine internal standard. Denatured protein is removed by centrifugation at 14,000 $\times$ *g* for 5 min. The pH of the supernatant was adjusted to 2.2 by the addition of a lithium-based buffer solution. Amino acids were measured using a Dionex BioLC Amino Acid Analyzer System (Dionex, Sunnyvale, CA) with lithium eluents. Samples with amino acid concentrations greater then 5 μM can be measured with this procedure. Variation between replicate amino acid runs routinely averaged less than 5%.

Anti-TGF-β1 Ab

Polyclonal neutralizing Ab against TGF-β1 (catalog AB-101-NA) and control chicken Ig were purchased from R&D Systems and were used at a concentration of 50 μg/ml.

Macrophage phagocytosis

After 48 h of culture, 0.05 ml of Fluoresbrite Plain YG 2 μm microspheres (Polysciences, Warrington, PA) at a concentration of 3×10^8 beads/ml were added per well (~50 beads/cell). A test plate (37°C) and a control plate (4°C) were incubated for 2 h. At that time, the wells were washed three times each with 0.2 ml of PBS to remove beads not actively phagocytosed. A final 0.1 ml of PBS was added and the plate was read at 480 nm on a Perkin-Elmer Luminescence Spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, U.K.). Fluorescence in replicate control wells was subtracted from the test wells to calculate the phagocytic index.

[3] Abbreviations used in this paper: PEC, resident peritoneal macrophages; iNOS, inducible NO synthase; SFM, serum-free medium.

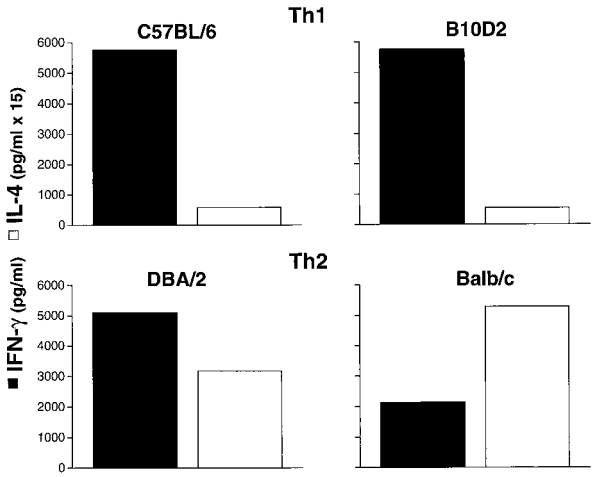

FIGURE 1. Spleen cytokine profiles of the Th1- and Th2-type mice used in this study. Spleen cells (3×10^5) were cultured in 5 μg/ml Con A for 3 days, and supernatants were collected for IFN-γ or IL-4 measurement. Other concentrations of Con A gave the same pattern.

Macrophage/lymphocyte coculture

PEC from C57BL/6 or BALB/c SCID mice were collected, irradiated (1000 rad), and then cultured at 1×10^5/well in 0.2 ml. After 2 days, 0.1 ml was removed and 3×10^5 (C57BL/6 $\times$ BALB/c)F_1 spleen cells depleted of macrophages by adherence were added in 0.05 ml to the macrophage cultures. Con A (0.06 μg/ml) was added in 0.05 ml. After 2 days, 1 μCi [^{3}H]thymidine (6.7 Ci/mmol) was added in 0.025 ml. Cells were harvested 16 h later, and [^{3}H]thymidine uptake was determined.

Data presentation

Data reported are means $\pm$ 1 SD from a representative experiment. The error bars represent the average $\pm$ SD of quadruplicate microtiter wells. If the SD was smaller than the width of the bar, it is not shown. All of the experiments reported in this work were repeated two to three times with the same pattern of results. Student's unpaired *t* test was used to analyze statistical significance. Inverse correlation statistics (Pearson's correlation coefficient (ρ) and *p* value) were obtained from a simple regression analysis (exponential model) using GB-Stat PPC 5.5.

Results and Discussion

Th1/Th2 profiles of selected inbred mice

Before undertaking a detailed comparison of macrophages from selected Th1- and Th2-type mouse strains, it was considered important to first characterize their lymphocyte cytokine propensities. It can be seen first in Fig. 1 that Con A stimulation of spleen cells from C57BL/6 mice results in a Th1 response characterized by high IFN-γ/low IL-4 production, while BALB/c spleen cells exhibit a Th2 response with low IFN-γ/high IL-4 production. B10D2 spleen cells also exhibit the Th1 profile of high IFN-γ/low IL-4 production. DBA/2 spleen cells exhibit something of an intermediate phenotype, producing a significant quantity of both IFN-γ and IL-4. B10D2 and DBA/2 were chosen for analysis because they share major histocompatibility type H-2^d with BALB/c mice and they are two other strains that have been reported to exhibit Th1 and Th2 T lymphocyte tendencies, respectively (16). Because the polyclonal activator Con A was used as the stimulant, the results also demonstrate that the tendencies of mice to produce

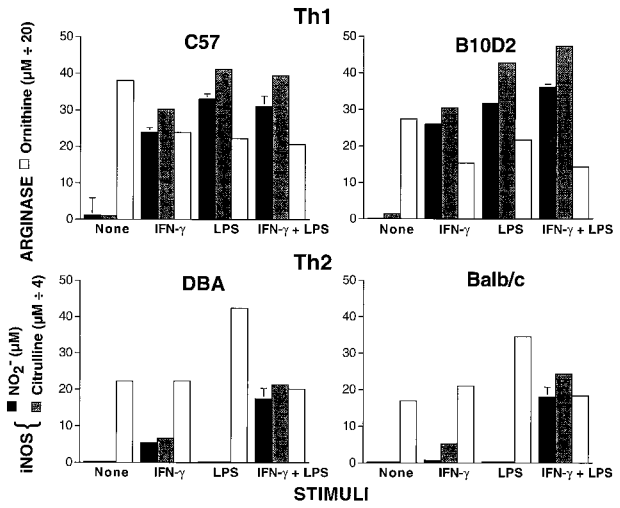

FIGURE 2. Th1 macrophages preferentially produce NO from arginine; Th2 macrophages produce ornithine. PEC from Th1 or Th2 strains of mice were cultured for 48 h, and supernatants were measured for NO_2^- and amino acids. RPMI 1640 contains 1100 μM arginine. Stimuli, 500 pg/ml IFN-γ and/or 1 ng/ml LPS.

IFN-γ or IL-4 are Ag independent. These results provide a background for the macrophage studies by defining the T lymphocyte cytokine profiles of the strains of mice to be used.

Differential arginine metabolism by Th1 and Th2 macrophages

Results such as those in Fig. 1 suggest that lymphocytes from certain inbred mice have a predilection to produce IFN-γ and/or IL-4. However, if differences in cytokines produced by lymphocytes could wholly explain susceptibility to diseases like *Leishmania major*, then macrophages from C57BL/6 and BALB/c should be similarly responsive to IFN-γ, the primary Th1 cytokine that activates macrophages. Fig. 2 shows that this is not the case. It can be seen, in agreement with some recent reports (17–21), that PEC from two Th1-like strains (C57BL/6 and B10D2) are more easily activated by IFN-γ to produce NO than macrophages from the two Th2-like strains (BALB/c, DBA/2). The concentration of citrulline, the other product of the iNOS pathway, parallels that of NO, as would be expected. It can also be seen that C57BL/6 and B10D2 macrophages respond more readily to LPS than DBA/2 or BALB/c macrophages, in agreement with a recent report (21). The simplest explanation for these data is that Th1 macrophages become activated and Th2 do not.

However, in addition to their role in host defense, macrophages are known to perform critical functions in Ag presentation (33, 34), tissue repair (35), angiogenesis (36), and other constructive processes. One would predict that macrophages performing these functions are not activated in the classical sense, which involves destructive processes (3). Evidence to support the concept of macrophage activation for either defense or nondefense purposes includes observations from this laboratory (37) that during wound healing, macrophages produce little NO/citrulline, but instead metabolize arginine primarily to ornithine/urea via arginase. This pattern of arginine metabolism makes sense for constructive processes such as wound healing because excess NO would inhibit cell replication/healing, while ornithine could promote cell replication/healing because it is a precursor for polyamines and collagen (7, 28, 29). That ornithine plays a role in healing is supported by findings that inhibition of ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis, inhibits healing, while provision of polyamines promotes healing (38, 39). Also, ornithine has been reported to be a proline precursor in healing wounds (40).

Thus, the pathway macrophages utilize to metabolize arginine could alter the outcome of inflammation in opposite ways. Therefore, it was also of interest to compare not only the iNOS, but also the arginase pathway in Th1/Th2 macrophages. Results in Fig. 2 show that macrophages from Th1 and Th2 mice can activate opposing pathways of arginine metabolism in response to the same stimuli. Specifically, the results in Fig. 2 demonstrate that in this experiment, Th2 macrophages not only do not produce NO in response to LPS, but instead increase their production of ornithine/urea. Specifically, there is a 91% increase for DBA/2 and a 103% increase for BALB/c in the extracellular ornithine concentration. In contrast, Th1 macrophages, if anything, slightly decreased ornithine production in response to LPS. Thus, these results directly demonstrate that under the same circumstances, Th1 and Th2 macrophages can be activated to express qualitatively different metabolic programs, which could affect inflammatory outcomes in opposite ways. In vivo evidence of such differential changes in macrophage arginine metabolism includes the findings that macrophages up-regulate NO production during tumor rejection, and up-regulate ornithine production during progressive tumor growth (41). From the results in this section, we propose that macrophages predominately expressing the iNOS or arginase pathway be termed M-1 or M-2, respectively.

Another difference observed between the way that M-1 or M-2 macrophages respond is that LPS strongly synergizes with IFN-γ in DBA/2 and BALB/c macrophages, but much less so with C57BL/6 or B10D2. How can these results be explained in terms of the two-signal concept of macrophage activation (42, 43)? Part of the explanation seems to be that some of the original experiments were performed with BALB/c mice, in which strong synergy between IFN-γ and LPS is observed. Also, most investigators use medium supplemented with 10% FBS, whereas the experiments reported in this work and in certain other recent reports (44) have used SFM. SFM was selected because results to follow will show that 10% FBS contains enough TGF-β to inhibit macrophage activation. In turn, it may only be necessary to use both IFN-γ and LPS to optimally stimulate macrophages if there is a significant quantity of TGF-β in the medium.

Macrophage TGF-β1 production is inversely proportional to macrophage NO production in M-1 and M-2 strains of mice

Results in the preceding section indicate that macrophages from M-1 and M-2 strains have a propensity to activate the iNOS or arginase pathway, respectively. To probe the molecular basis for these differences, TGF-β1 production by M-1 and M-2 macrophages was measured. TGF-β1 was selected for study because it is known to be a powerful inhibitor of macrophage NO production (45, 46). In addition, TGF-β1 has been reported to increase arginase activity (47). In vivo, the level of TGF-β correlates with susceptibility to *Leishmania* (20, 48, 49). Also, inhibition of TGF-β1 has been shown to heighten resistance to diseases, in which macrophages are the likely effector (50). On the other hand, NO has been reported to inhibit macrophage TGF-β production (44). Thus, although the foregoing results indicate that a chicken or egg question remains regarding who regulates whom, important links exist between TGF-β and NO. In turn, it was considered important to determine whether there is a relationship between NO and TGF-β production in M-1 and M-2 macrophages.

It can be seen first in Fig. 3 that C57BL/6 and B10D2 macrophage again responded more readily to IFN-γ by production of NO. It can be seen that BALB/c macrophages in this experiment produced NO in response to LPS. We do observe some day to day

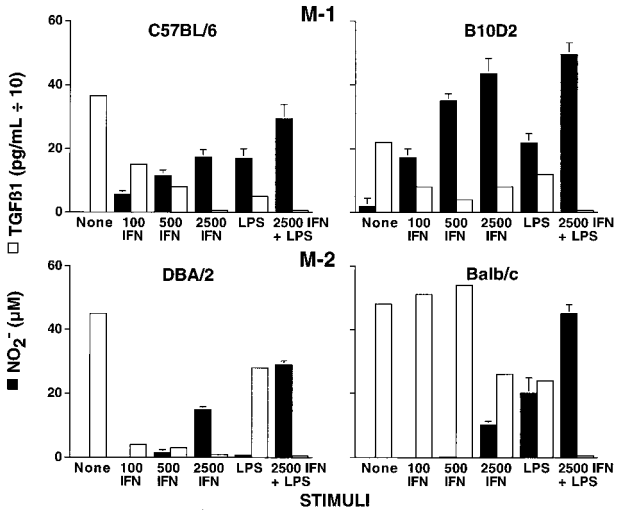

FIGURE 3. Inverse relationship between macrophage TGF-β1 production and macrophage NO production. PEC from M-1 or M-2 mouse strains were cultured for 48 h, and TGF-β1 and NO_2^- were measured in the supernatants.

variation in responsiveness to LPS, but not IFN-γ. The variability occurs despite using medium components from the same lot and with very low endotoxin, and using mice of the same age, etc. On average, however, BALB/c and DBA macrophages both produce significantly less NO than C57BL/6 or B10D2 in response to either IFN-γ or LPS in agreement with the results of others (17–21). Most importantly, BALB/c macrophages again responded in a qualitatively different manner to LPS than C57BL/6, as evidenced by increased ornithine production. Specifically, medium ornithine concentrations (μM) in the experiment in Fig. 3 were: BALB/c, NONE = 564; 500 IFN-γ = 659; LPS = 761; IFN-γ + LPS = 394. C57BL/6, NONE = 320; 500 IFN-γ = 239; LPS = 236; IFN-γ + LPS = 165.

As for the production of TGF-β by M-1 and M-2 macrophages, it can be seen that both produce a significant quantity of TGF-β1 (200–400 pg/ml); no consistent difference between strains has been observed. However, more importantly, it can be seen that the quantity of NO produced by M-1 and M-2 macrophages stimulated with different concentrations of IFN-γ or LPS is inversely proportional to the quantity of TGF-β1 produced. That there is a causal link between NO and TGF-β1 production is suggested by the findings that in all four strains of mice examined, and with essentially all of the different stimuli, a highly significant inverse relationship is observed ($\rho = -0.81$; $p < 0.0001$). That macrophage TGF-β is physiologically important is suggested by the fact that the quantity of TGF-β1 produced (400 pg/ml) is in the range that has been shown to inhibit NO production (45). Although total (latent plus active) TGF-β1 was measured in this study, macrophages are known to activate latent TGF-β1, so the values are in the correct range (51). Together, these results are consistent with the conclusion that M-1- and M-2-dominant responses occur, in part, because of autocrine macrophage regulation by TGF-β1.

Macrophage TGF-β1 is an endogenous inhibitor of NO production

The results in Fig. 3 suggested that TGF-β1 produced by macrophages plays a role in down-regulating NO production, or vice versa. To measure TGF-β1, it was necessary to use SFM because 10% FBS that is used by most investigators provides about 750 pg/ml TGF-β1, and thus would obscure that produced by macrophages. Evidence that the quantity of TGF-β1 contained in medium supplemented with 10% FBS is sufficient to inhibit macrophage NO production is shown in Fig. 4*A*. Macrophages cultured in a medium containing 10% FBS produce less NO than macrophages cultured in SFM only supplemented with BSA, transferrin, and insulin. Also, addition of FBS to SFM inhibits NO production, indicating that inhibitory components of FBS rather than stimulatory components in SFM are responsible. That the inhibitory component in FBS is TGF-β1 is further suggested by the finding that the addition of 750 pg/ml TGF-β1 to SFM (the same concentration provided by 10% FBS) inhibits NO production. Thus, the use of SFM, in addition to allowing TGF-β1 to be measured, also serves

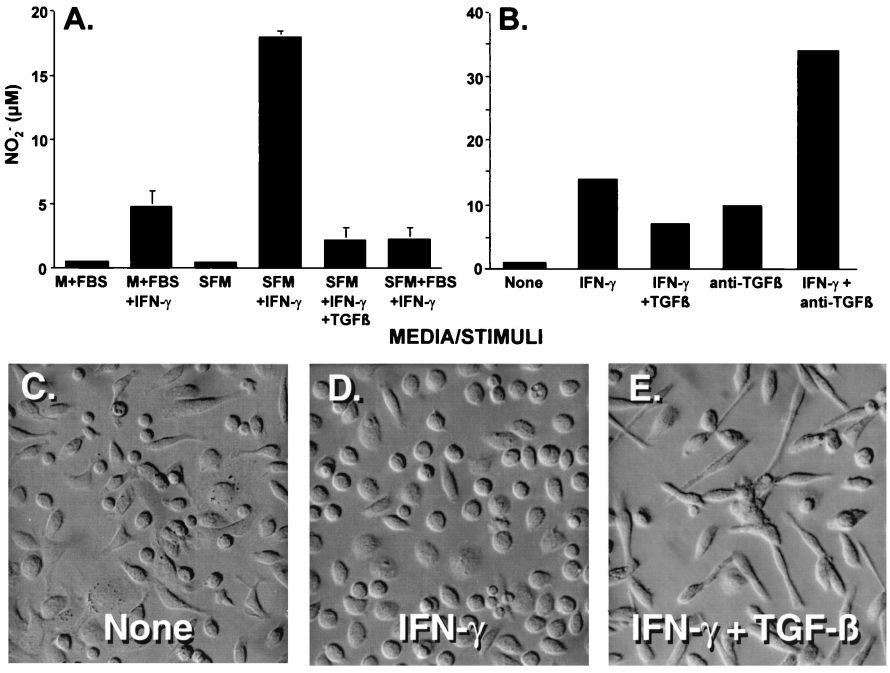

FIGURE 4. Macrophage TGF-β1 is an endogenous inhibitor of NO production. *A*, NO_2^- production medium (M, RPMI 1640, MOPS, 2-ME, antibiotics) with 10% FBS or in SFM or in SFM supplemented with 10% FBS (CM). *B*, NO_2^- production stimulated by anti-TGF-β1 Ab. *C*, *D*, and *E*, PEC after 2 days of culture in SFM (None), IFN-γ, or IFN-γ plus TGF-β1. Resident C57BL/6 $\times$ DBA/2 F_1 macrophages were used in these experiments.

to demonstrate that culturing macrophages in standard FBS-containing medium may be misleading because the TGF-β1 artificially inhibits macrophage activation.

To directly test the hypothesis that TGF-β1 is an endogenous autocrine inhibitor of macrophage NO production, anti-TGF-β1 Ab (50 μg/ml) was added to macrophage cultures and NO production was measured. It can be seen first in Fig. 4*B* that IFN-γ stimulates significant NO production. However, more importantly, addition of anti-TGF-β1 Ab without IFN-γ also increases NO production. That endogenously produced TGF-β1 inhibits macrophage activation is further evidenced by the additional finding that addition of IFN-γ and anti-TGF-β1 results in the highest level of NO production. Control Ab (nonimmune chicken Ig) does not stimulate NO production. Also, anti-TGF-β1 increases NO production in C3H/He macrophages, indicating that endotoxin is not responsible for the activity observed (not shown). Thus, the results in this section indicate that macrophages produce a quantity of TGF-β1 that is sufficient to endogenously down-regulate their activation state.

The impact of TGF-β1 on macrophages is not subtle. Fig. 4, *C*, *D*, and *E*, shows what PEC look like after 2 days of culture in SFM alone (*C*), SFM plus IFN-γ (*D*), or SFM plus IFN-γ and TGF-β1 (*E*). It can be seen that TGF-β1 (1 ng/ml) induces a dramatic change in macrophage morphology, causing them to take on a fibroblast-like appearance. In addition to their reduced NO production, these TGF-β-induced long slender cells have markedly reduced phagocytic capacity. Specifically, macrophages cultured alone or with IFN-γ had a phagocytic index of 3.5 ± 0.4 and 5.5 ± 0.5, respectively, while macrophages cultured in IFN-γ plus TGF-β had an index of only 1.5 ± 0.5 ($p = 0.0034$ and $p < 0.0001$, respectively). In this regard, it has been previously reported that TGF-β did not decrease phagocytic activity (52). The most likely explanation for this discrepancy is that we have observed that 10% FBS (containing TGF-β) as used in other studies itself causes macrophages to assume this fibroblast-like appearance. In turn, addition of exogenous TGF-β may not have further decreased phagocytosis under these conditions. It is not clear at present what functions these fibroblast-like cells induced by TGF-β do perform. One possibility being explored is that they exhibit an enhanced Ag presentation capability because TGF-β has been shown to promote the formation of dendritic cells (53).

As for the molecular basis of how TGF-β1 so dramatically alters macrophage morphology, there are suggestions that its effect may result from changes in arginine metabolism. For example, in a previous publication, we observed that macrophages cultured in a low arginine environment also exhibited this fibroblast-like morphology (54). Also, there have been repeated suggestions that macrophages in culture eventually transform into fibroblasts (55, 56): circumstances again in which the arginine concentration in the medium would have been greatly reduced by the macrophages. Relatedly, TGF-β1 not only down-regulates the iNOS pathway, but also up-regulates the arginase pathway (47, 57). Thus, perhaps the stimulus for the change in macrophage morphology is low NO concentration, either because of a decrease by TGF-β or a low extracellular arginine concentration.

Macrophages from C57BL/6 and BALB/c NUDE or SCID mice display exaggerated M-1 or M-2 phenotypes

Results in the preceding sections indicated that macrophages from Th1 or Th2 mice can respond very differently when confronted with the same stimuli. Although these results suggest that the macrophages themselves are different, other explanations were possible. For example, macrophages could have been bathed in a Th1 or Th2 atmosphere before culture. Also, a significant number of T lymphocytes could have been present in the macrophage-enriched cultures. To rule out these potentialities, we compared NUDE or SCID macrophages with a C57BL/6 or BALB/c background. It can be seen first in Fig. 5 that macrophages from C57BL/6 NUDE mice spontaneously produce significant quantities of NO. Addition of IFN-γ, LPS, or both does not further increase NO production. C57BL/6 SCID macrophages spontaneously produce even more NO, and again, stimuli do not further elevate production. Thus, absence of T/B lymphocytes strongly enhances the M-1 phenotype. Theoretically, NK cells could be involved here. However, if they are, the results still indicate that differences between M-1 and M-2 macrophages are not dependent on Th1/Th2 influence. It can also be seen in Fig. 5 that in contrast to the up-regulation of the iNOS pathway in C57BL/6 mice, the arginase pathway is preferentially expressed in BALB/c NUDE and SCID macrophages. For example, BALB/c NUDE and SCID macrophages produce about twice the ornithine as age-matched C57BL/6 NUDE macrophages measured on the same day. BALB/c SCID macrophages did produce NO in response to IFN-γ, and produced about the same maximum quantity of NO as C57BL/6 SCID macrophages. Although an explanation for these results requires more investigation, one might speculate that an additional NO-inhibiting cytokine is lost in SCID as compared with NUDE BALB/c mice. Together, the results in this section suggest that the qualitative differences in C57BL/6 and BALB/c macrophage arginine metabolism are not dependent on T or B lymphocytes. Indeed, M-1 and M-2 phenotypes seem exaggerated in their absence, indicating that T and B lymphocytes play a modulating role.

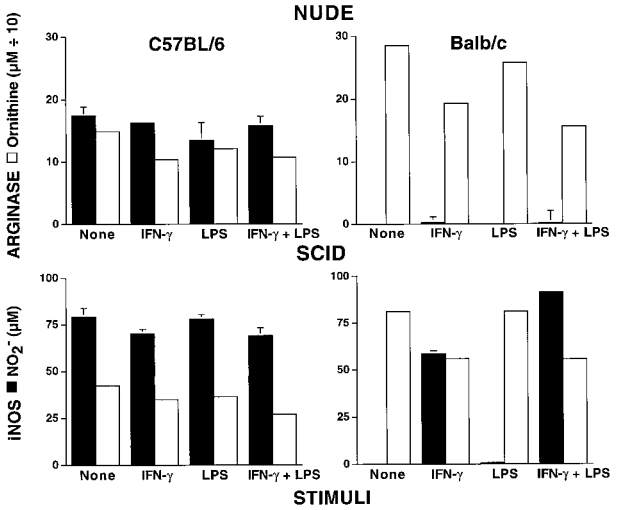

FIGURE 5. Macrophages from NUDE or SCID mice on the C57BL/6 or BALB/c background exhibit exaggerated expression of the iNOS or arginase pathway, respectively. PEC were cultured with 500 pg/ml IFN-γ and/or 1 ng/ml LPS for 48 h, and NO_2^- or ornithine was measured.

M-1 or M-2 macrophages differentially influence lymphocyte responses

Evidence in this communication indicates that macrophages from Th1- and Th2-type mice can respond in qualitatively different ways to stimuli, and therefore could differentially influence what type of lymphocyte responses occur. To test this hypothesis, we again took advantage of SCID mice with an M-1 (C57BL/6) or M-2 (BALB/c) background. Macrophages (1×10^5) from C57BL/6 or BALB/c SCID mice were cultured alone or in the presence of IFN-γ or LPS or both for 2 days. The macrophages

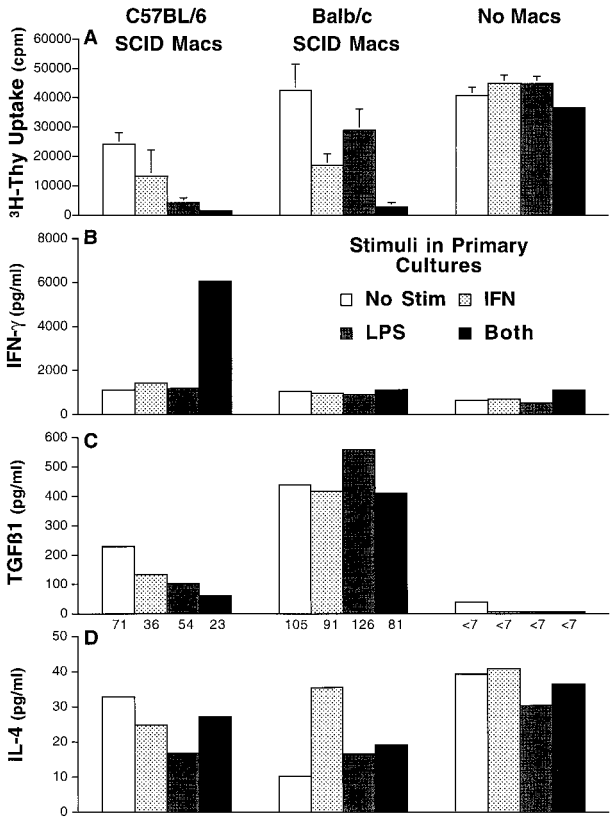

FIGURE 6. M-1 and M-2 macrophages differentially affect lymphocyte responses. Adherent macrophages (1×10^5) in 0.2 ml from C57BL/6 or BALB/c SCID mice were cultured for 2 days, 0.1 ml supernatant was removed, and then C57BL/6 × BALB/c F_1 spleen cells and Con A were added. After an additional 2 days, 0.1 ml of supernatant was removed for cytokine analyses and the cultures were pulsed with [^{3}H]thymidine. *A*, Proliferation; *B*, IFN-γ; *C*, TGF-β1, numbers under histobars are TGF-β1 in macrophage cultures with no $CB6F_1$ lymphocytes added; *D*, IL-4.

were irradiated (1000 rad) before culture to prevent any contribution to proliferation. Macrophage-depleted spleen cells (3×10^5) from (C57BL/6 × BALB/c)F_1 mice ($CB6F_1$) were then added, and the macrophage/lymphocyte cocultures were stimulated with Con A. F_1 lymphocytes were employed so they would not respond to parental Ags. After 2 days, supernatants were removed for cytokine analyses and the cultures were pulsed with [^{3}H]thymidine to measure cell proliferation. It can be seen first in Fig. 6*A* that Con A-induced proliferation of $CB6F_1$ lymphocytes is inhibited to a greater degree by unstimulated C57BL/6 SCID than BALB/c SCID macrophages ($p = 0.03$). Specifically, there is a 59% decrease in proliferation upon coculture with unstimulated C57BL/6 SCID macrophages, whereas proliferation with unstimulated BALB/c SCID macrophages is the same as with $CB6F_1$ lymphocytes cultured without exogenous macrophages. These results are consistent with the findings in Fig. 5 showing that unstimulated C57BL/6 NUDE or SCID macrophages endogenously produce NO, and therefore would be expected to express more suppressor macrophage activity (30) than BALB/c SCID macrophages. It can also be seen in Fig. 6*A* that proliferation is strongly inhibited by either C57BL/6 or BALB/c SCID macrophages if they have been pretreated with IFN-γ and LPS. These findings are also in keeping with results in Fig. 5, which showed that BALB/c macrophages can produce as much NO as C57BL/6 macrophages when stimulated with both IFN-γ and LPS.

In addition to affecting lymphocyte proliferation to different degrees, C57BL/6 and BALB/c SCID macrophages also influence whether Th1- or Th2-associated cytokines are produced by lymphocytes. Specifically, it can be seen in Fig. 6*B* that C57BL/6 SCID macrophages precultured with IFN-γ and LPS cause a marked increase in IFN-γ production by $CB6F_1$ lymphocytes. BALB/c SCID macrophages caused no increase. The vast majority of the IFN-γ measured in the supernatants was produced by $CB6F_1$ lymphocytes (T or NK) because only 500 pg/ml IFN-γ was added to precultured macrophages, and about 6000 pg/ml was produced in the secondary cultures. As for a molecular explanation for the IFN-γ-inducing ability of C57BL/6 SCID macrophages, it has been reported that C57BL/6 macrophages produce more IL-12 than BALB/c macrophages (58). We have also observed that C57BL/6 macrophages produce more IFN-γ-inducing IL-12 than BALB/c macrophages (C. D. Mills, unpublished).

In contrast to IFN-γ, BALB/c SCID macrophages, whether cultured alone or with IFN-γ and LPS, caused $CB6F_1$ lymphocytes to increase production of TGF-β1 more than C57BL/6 SCID macrophages (Fig. 6*C*). Although not strictly a Th2 cytokine, TGF-β does favor the production of Th2 responses by inhibiting Th1-induced macrophage activation. The majority of the TGF-β produced in the cocultures seems to be from $CB6F_1$ lymphocytes (the amount produced in macrophage cultures without lymphocytes is shown under the histobars).

The quantity of the Th2 cytokine IL-4 produced by $CB6F_1$ lymphocytes, whether cocultured with C57BL/6 or BALB/c SCID macrophages, was barely detectable (C57BL/6, 25–30 pg/ml; BALB/c, 10–20 pg/ml). This is much less IL-4 than is even produced by the low IL-4 parent, C57BL/6, as shown in Fig. 1. In this regard, offspring of M-1 and M-2 mice ((C57BL/6 × BALB/c)F_1 or C57BL/6 × DBA/2)F_1) have intermediate phenotypes (not shown). Therefore, the reason for low IL-4 does not appear to be the mouse strain employed, but rather that the serum-free culture conditions do not support IL-4 production like cultures containing 10% FBS, as in Fig. 1. In summary, the results in this section demonstrate that M-1 or M-2 macrophages when confronted with the same stimuli: 1) can affect subsequent lymphocyte proliferation in different ways; 2) influence whether Th1- or Th2-dominant cytokines are produced.

The results in this communication provide evidence that macrophages play a more important role in orchestrating immune responses than is currently appreciated. If one views immune responses in temporal terms, however, it is not really surprising that macrophages play a pivotal role in determining immunological outcomes because they are typically the first cells to receive danger signals (59). Also, T lymphocytes require signals normally provided by macrophages or dendritic cells to respond to Ags (27, 33, 34, 60). In this regard, although the results in this communication are described in terms of M-1 or M-2 macrophage responses, dendritic cells are also likely to be involved in these responses because macrophages are a precursor for dendritic cells (27). Evidence to support this postulate includes results discussed earlier that dendritic cells can stimulate Th1 or Th2 cytokine profiles in vivo (26). Perhaps M-1- or M-2-dominant responses evolve into dendritic cell responses that stimulate Th1 or Th2 responses, respectively. At the same time, because M-1 equates with the production of destructive molecules such as NO, overexpression of this pathway could inhibit lymphocyte responses through a suppressor macrophage effect (30, 61–63), as suggested in Fig. 6.

Our proposed classification of macrophage propensities into M-1 and M-2, while useful for conceptualizing immune responses, certainly could be an oversimplification. In particular, it cannot be concluded from the present results that M-1 or M-2 are clonally

separable cells like Th1 and Th2 clones (13). Instead, there may be a continuum of phenotypes between M-1 and M-2 macrophages. Regardless, the functionally important point is that the net macrophage responses to stimuli between common strains of mice vary in fundamentally different ways, and can be independent of T lymphocyte influence. In turn, the results suggest that macrophages can be an important factor in determining whether Th1/Th2 or other immune responses occur. At the same time, although the present results provide evidence that macrophages can influence lymphocyte responses, there are well-established differences in lymphocyte cytokine profiles. Therefore, the type or intensity of immune response that occurs is likely to be a composite of the propensities of macrophages and lymphocytes acting in concert. As an example, our results show that M-2 macrophages have a propensity to express arginase/ornithine production. Other recent results have shown that the Th2 cytokine IL-4 also increases macrophage arginase/ornithine production (64). Therefore, if macrophages from an individual have an M-2 propensity and his T lymphocytes have a Th2 propensity, then one would predict that there would be synergy in stimulating the arginase/ornithine pathway. Conversely, one could also envision circumstances in which macrophages and lymphocytes have opposite propensities, and thus would antagonize each other. Increasing our understanding of the interplay between macrophages, dendritic cells, and lymphocytes should augment our ability to alter the course of immune responses/inflammation as needed.

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