Cutting Edge: Stat6-Dependent Substrate Depletion Regulates Nitric Oxide Production

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Nitric oxide production from activated macrophages is essential for the control of a variety of microbial infections (1). However, uncontrolled NO production can also be detrimental, resulting in tissue damage and cell dysfunction or death (2). In macrophages, NO is produced by the conversion of arginine and oxygen to citrulline and NO by the enzyme inducible NO synthase (iNOS) (1). iNOS levels in activated macrophages are regulated through complex transcriptional and posttranscriptional mechanisms (1, 3). These include transcriptional control dependent upon IFN-γ-regulated IFN regulatory factor (IRF) 1 binding to the iNOS promoter synergistically acting with NF-κB (4–6) and regulation of mRNA stability, translation, protein stability, substrate availability, and the activity of NO scavengers (1, 3) or endogenous enzyme inhibitors (7). In contrast to the well-understood pathways of iNOS production, our understanding of how NO levels are reduced, inhibited, or removed are limited.

Three major classes of cytokines have been shown to play roles in negative regulation of NO production from activated macrophages. IL-10 is a relatively weak inhibitor of iNOS levels while TGF-β negatively regulates iNOS levels through multiple mechanisms (8). Studies from mice lacking IL-10 or TGF-β1 have shown that both cytokines play essential, but differing, roles in negative regulation of iNOS (9, 10). The third class includes IL-4 and IL-13, related cytokines that are powerful inhibitors of NO production from activated macrophages (11–14). IL-4 and IL-13 are pleiotropic cytokines that play major roles in the regulation of T and B cell function as well as controlling macrophage activity (15). Because of the essential role of NO in antimicrobial immunity, we were interested in determining the molecular explanation for the inhibitory actions of IL-4/IL-13. Here, we present our conclusions to this question and identify IL-4/IL-13 as down-regulating NO by substrate depletion though Stat6-dependent production of arginase.

Materials and Methods

Mice

Stat6-deficient mice have been described previously (16) and were bred in the St. Jude Animal Resources Center. Control mice (C57BL/6) were derived from littermates of interbred Stat6+/− mice or were purchased from The Jackson Laboratory (no. 100903; Bar Harbor, ME). Mice were age (6–10 wk) and sex matched for experimental use. Suppressor of cytokine signaling-1 (SOCS1)−/−, IFN-γ−/− mice were bred at St. Jude by intercrossing SOCS1−/−, IFN-γ−/− mice. All procedures were performed in accordance with institutional guidelines for the care and handling of experimental animals.

Macrophage isolation and cell culture

Peritoneal-derived macrophages (PDMs) were isolated from the peritoneal cavities of mice injected with 3 ml Brewer’s thioglycolate 3 days before...
harvest. Residual erythrocytes were removed with red cell lysis buffer (Sigma, St. Louis, MO) and plated as described in each figure legend. Bone marrow-derived macrophages (BMDMs) were obtained by flushing bone cavities and isolating macrophages by differentiation in using L-cell-conditioned medium as a source of CSF-1. Cells were plated to the densities described in each figure legend. Adherent macrophages were used 4–6 days after plating. For most experiments, IL-4 and IL-13 were used at a final concentration of 50 ng/ml, and LPS and IFN-γ were used at a final concentrations of 100 and 2 ng/ml, respectively.

**Reagents**

Murine IL-4 was obtained from PeproTech (Rocky Hill, NJ). Murine IL-13 was obtained from R&D Systems (Minneapolis, MN). Recombinant murine IFN-γ was made in Escherichia coli (P. J. Murray, unpublished observations) and contained endotoxin levels <0.06 endotoxin units/ml and was fully functional by titration against a commercially available standard. E. coli LPS was purchased from Sigma (St. Louis, MO). Cytokine stocks were made to 1 µg/ml in complete RPMI 1640 medium. Griess and arginase assay reagents and cycloheximide were all obtained from Sigma. Rabbit polyclonal anti-Stat6 Abs were a gift from Dr. Demin Wang (St. Jude), anti-iNOS Abs were purchased from Biomol (Plymouth Meeting, PA), anti-Grb2 Abs were obtained from Transduction Laboratories (Lexington, KY), anti-phospho-Stat1 Abs were obtained from New England Biolabs (Beverly, MA), and anti-Stat1 Abs (sc-546, E23) and anti-IRF-1 Abs (sc-640, M20) were purchased from Santa Cruz (Santa Cruz, CA). The SOCS1 cDNA probe was a gift from Evan Parganas (St. Jude). A mouse arginase I probe was isolated from IMAGE EST 492949 (GenBank accession number AA097468).

**Enzyme assays**

Griess assays were performed as described elsewhere (17). Arginase assays were performed using the method of Corraliza et al. (18). iNOS enzyme assays were performed as described by Stuehr (19).

**Immunoblotting and immunoprecipitations**

Immunoblotting was performed as described previously (9). Pulse radiolabeling of iNOS was performed as described elsewhere (20) using 0.2 mCi/ml [35S]methionine/cysteine (ICN Pharmaceuticals, Los Angeles, CA). Cell lysates were made in radioimmunoprecipitation assay buffer with the addition of protease inhibitors and immunoprecipitated for 3 h at 4°C. Immunoprecipitates were collected with protein A-Sepharose and separated by electrophoresis on 4–15% gradient gels. Gels were fixed in 10% acetic acid and soaked in 2 M sodium salicylate, dried, and exposed to film for 15 h at −80°C.

**In vitro infections**

PDMs were harvested from C57Bl/6 mice injected 5 days previously with 3% Brewer's thioglycolate. Cells were adjusted to 8 × 10⁶ cells/ml in complete RPMI 1640 and plated at 50 or 250 µl/well in 96- or 24-well plates, respectively. After 4 h, nonadherent cells were washed away with ice-cold PBS and adherent cells allowed to recover in complete medium for 1 h. Cells were incubated with IL-4 (50 ng/ml) for 16 h. LPS and/or IFN-γ was added to some wells and then infected with T. gondii parasites (0.2 parasites/macrophase) for 24 h and then pulsed with [3H]uracil (1 µCi/well), which is preferentially incorporated into parasites. Cultures were treated with or without exogenous arginine and parasite replication was measured by scintillation counting.

**Results and Discussion**

IL-4 and IL-13 share the IL-4Rα chain in their receptor complexes and both cytokines activate Stat6, which mediates a subset of their functions in T and B cells (16, 21–23) and has previously been shown to be required for inhibiting iNOS mRNA production in LPS- and IFN-γ-activated macrophages (24). Our experimental system uses two populations of primary macrophages; thioglycolate-elicited peritoneal inflammatory macrophages (PDMS) and in vitro differentiated BMDMs. To test whether Stat6 was required for IL-4/IL-13 inhibition of NO production, we stimulated macrophages with IL-4 or IL-13 for 16 h and then treated them with LPS/IFN-γ for another 16 h and measured NO with the Griess reagent. B, NO inhibition by IL-4 is dependent on the time of pretreatment. PDMs were plated as described for A and pretreated with IL-4 for the times indicated on the abcissa. Cells were then treated with LPS/IFN-γ for 16 h and nitrites were measured with the Griess reagent. C, NO inhibition by IL-4 is dependent on the number of cells in the culture. PDMs were plated at a final number per well in 24-well plates as shown on the abcissa and pretreated with IL-4 followed by LPS/IFN-γ as described for A. Note that PDMs are postmitotic and thus do not increase in number following plating.
observed at 7.5 x 10^5 cells/well. Similar findings were observed
with BMDMs (data not shown). The inhibition of NO production
by IL-4/IL-13 was not due to the production of a molecule or
substance that interfered with the Griess reaction and was also not
due to the production of a Stat6-regulated soluble factor(s) as
shown by transferring culture supernatants in the presence of neu-
tralizing anti-IL-4 mAbs (data not shown).

At first appraisal, the simplest explanation for the role of IL-4/
IL-13 in blocking NO is the likelihood of iNOS mRNA down-
regulation. Indeed, several reports have implicated inhibition of
IFN-γ signaling, iNOS gene transcription and other mechanisms as
being involved in IL-4 inhibition of NO production (11, 12, 24,
25). To test whether iNOS protein levels were reduced by IL-4/
IL-13, we performed time course experiments to measure the ac-
cumulation of iNOS by immunoblotting. Both IL-4 and IL-13 in-
hibited NO production but iNOS levels were unchanged in either
case (data not shown). IL-4/IL-13 also did not inhibit Stat1 phos-
phorylation or IRF-1 up-regulation when macrophages were stim-
ulated with LPS/IFN-γ, both prerequisites for high-level iNOS
production, ruling out a role for IL-4/IL-13 inhibiting these sig-
naling pathways under these conditions (data not shown). Minor in-
hibition of Stat1 phosphorylation was observed when macrophages
were stimulated with IL-4 and IFN-γ; however, this did not appear to
inhibit IRF-1 or iNOS induction. Interestingly, IL-4 did inhibit the
LPS-mediated induction of iNOS, indicating that IL-4 may activate
alternate pathways that can reduce iNOS protein levels (data not
shown). We also tested whether IL-4 regulated newly synthesized
iNOS protein by pulse labeling and immunoprecipitation analysis.

FIGURE 2. IL-4 controls arginase levels and de-
pletion of iNOS substrate. A, Arginase induction by
IL-4 is Stat6 dependent. BMDMs from wild-type or
Stat6^-/- mice were plated at the densities shown in
the abscissa in 24-well plates and stimulated with
IL-4 for 16 h followed by LPS/IFN-γ stimulation.
Arginase activity in cell lysates was measured using
the method of Corraliza et al. (18). B, Exogenous
arginine addition restores NO production in IL-4-
stimulated macrophages. BMDMs were plated at
2 x 10^6/well in 24-well plates and stimulated as
shown on the abscissa. Exogenous arginine was
added to the medium (2 mM) at the time of LPS/
IFN-γ addition (filled bars) or immediately before
performing the Griess assay 16 h after LPS/IFN-γ
addition. Nitrates were measured in the culture su-
pernatsants and arginase activity was measured in
cell lysates from the same cultures. C, Arginase I
mRNA is regulated indirectly by Stat6. BMDMs
from wild-type or Stat6^-/- mice were plated at 8 x
10^5 in 10-cm dishes and stimulated with IL-4 for 0,
1, 2, or 6 h in the presence or absence of cyclohex-
imide (CHX, 10 μg/ml). Total RNA was prepared
and arginase I and GAPDH (loading control) levels
were measured by Northern blotting.
Under these conditions, we found that iNOS activity was equivalent in enzyme activity using a method established by Stuehr (19). However, this hypothesis has not been directly tested in a situation shown). Finally, since we had observed that IL-4 induces expression of SOCS1, a protein inhibitor of IFN-γ signaling, the role of SOCS1 was also tested. Using Northern blotting to check for SOCS1 expression and SOCS1−/− macrophages, we failed to implicate this molecule in IL-4-mediated inhibition of NO production (data not shown). In summary, the results indicate that IL-4/IL-13 regulates NO production using a molecular mechanism distinct from inhibiting iNOS levels.

Previous studies have shown that arginase levels can be up-regulated in macrophages treated with IL-4 and then LPS/IFN-γ. Cell-free extracts were used to measure iNOS enzyme activity using a method established by Stuehr (19). Under these conditions, we found that iNOS activity was equivalent in lysates that had been previously treated with IL-4 and LPS/IFN-γ or LPS/IFN-γ alone, even though samples that had been pretreated with IL-4 showed complete inhibition of NO production (data not shown). Finally, since we had observed that IL-4 induces expression of SOCS1, a protein inhibitor of IFN-γ signaling, the role of SOCS1 was also tested. Using Northern blotting to check for SOCS1 expression and SOCS1−/− macrophages, we failed to implicate this molecule in IL-4-mediated inhibition of NO production (data not shown). In summary, the results indicate that IL-4/IL-13 regulates NO production using a molecular mechanism distinct from inhibiting iNOS levels.

Again, these results failed to reveal any effect of IL-4 on iNOS synthesis (data not shown). We also tested whether the activity of iNOS enzyme was affected in macrophages treated with IL-4 and then LPS/IFN-γ. Cell-free extracts were used to measure iNOS enzyme activity using a method established by Stuehr (19). Under these conditions, we found that iNOS activity was equivalent in lysates that had been previously treated with IL-4 and LPS/IFN-γ or LPS/IFN-γ alone, even though samples that had been pretreated with IL-4 showed complete inhibition of NO production (data not shown). Finally, since we had observed that IL-4 induces expression of SOCS1, a protein inhibitor of IFN-γ signaling, the role of SOCS1 was also tested. Using Northern blotting to check for SOCS1 expression and SOCS1−/− macrophages, we failed to implicate this molecule in IL-4-mediated inhibition of NO production (data not shown). In summary, the results indicate that IL-4/IL-13 regulates NO production using a molecular mechanism distinct from inhibiting iNOS levels.

Figure 3. l-Arginine restores NO production and inhibits the growth of T. gondii in IFN-γ/LPS-activated macrophages pretreated with IL-4. Thioglycolate-elicited peritoneal macrophages from C57BL/6 mice were plated and activated as described in this figure. After a 16-h incubation, designated cells were stimulated with IFN-γ/LPS, supplemented with l-arginine (2 mM), and all cells were infected with RH tachyzoites (0.2 parasites/cell). After an additional 24-h incubation, supernatants were removed for NO analysis and infected cells were pulsed with [3H]luracil. Growth of T. gondii was measured 24 h later and recorded as incorporated radioactivity in cpm (A). Background incorporation of similarly stimulated, noninfected macrophages was subtracted. Nitrite levels were analyzed as described and are shown in B. Results are representative of four similar experiments.

A

B

Since previous studies have shown that arginase I (liver isoform) is up-regulated by IL-4 (27, 30), we would expect that this would occur in a Stat6-dependent manner. To test this, macrophages from wild-type or Stat6−/− mice were treated with IL-4 with or without cycloheximide, and arginase I mRNA levels were measured by Northern blotting. Arginase I mRNA levels increased over time and in a Stat6-dependent manner, but was also dependent on new protein synthesis, suggesting that Stat6 is unlikely to act directly on the arginase promoter (Fig. 2C). A previous report claimed six potential Stat6 binding sites are present in the rat arginase I promoter (30) but our own analysis of this promoter failed to reveal any sites correlating with the Stat6 consensus binding site (TTCC(A > T,N)GGAA; Ref. 31). There was a single site conserved to a potential Stat6 binding site (TTCNNNNGA; Ref. 32); however, our observations showing that intermediate proteins are required downstream of Stat6 to regulate arginase I expression suggests that this site is unlikely to be physiologically relevant in the regulation of arginase I by IL-4. Finally, the Northern blot data reveal that ~6 h are required to observe significant arginase I mRNA levels, correlating with the time-dependent requirement for IL-4 pretreatment of macrophages (Fig. 1).

Since arginase and iNOS compete for arginine, we predicted that the depletion of substrate would play a significant role in macrophage-mediated immune mechanisms where NO is important. Therefore, we tested this concept in an in vitro T. gondii killing assay (Fig. 3). In this assay, macrophage anti-toxoplasma activity is dependent upon NO generation (33). IL-4 pretreatment significantly inhibited LPS/IFN-γ killing of T. gondii, while no parasite replication was detected when macrophages were treated with LPS/IFN-γ. Strikingly, addition of exogenous arginine restored complete killing of T. gondii in the IL-4-treated macrophages, suggesting that as long as substrate is available to iNOS the enzyme will actively generate NO (Fig. 3).

The results of this study have implications for our understanding of NO in macrophage-mediated immune responses. We can deduce that macrophages exposed to IL-4/IL-13 will elevate the level
of arginase I, leading to competition for substrate and inhibition of the amount of NO that can be produced. This scenario is likely to play a role where arginine is limited rather than in well-perfused tissues (29). Examples could include granulomas in mycobacterial and schistosomal infections or autoimmune diseases, repairing tissues, airway hyperinflammation, solid tumors (34), or regions of the brain. In these examples, NO generation could be significantly reduced, even though iNOS levels could be high. Therefore, a consequence of this study is that immunological detection of iNOS as a marker for NO levels could have limited value if the same cells or tissues also express significant levels of arginase. Another prediction of this study is that arginase could be a useful therapeutic agent in cases where both arginase I and iNOS are competing for substrate. This has been observed in an in vivo model of Trypanosoma brucei infection where administration of arginine into the peritoneal cavity partially restored parasite killing, presumably by activated macrophages now capable of generating NO (35). Restoration of arginine may be a simple method of providing macrophages with the ability to overcome substrate starvation induced by Stat6-mediated activation of arginase I.

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References