Defective Inflammatory Response in Interleukin 6–deficient Mice

By Elena Fattori,* Manuela Cappelletti,* Patrizia Costa,* Carolina SelliJtto,* Lavinia Cantoni,† Maria Carelli,† Raffaella Faggioni,† Gianila Fantuzzi,† Pietro Ghezzi,† and Valeria Poli*

From *Istituto di Ricerche di Biologia Molecolare IRBM P. Angeletti, 00040 Pomezia (Rome), Italy; and †Istituto di Ricerche Farmacologiche "Mario Negri," 20157 Milano, Italy

Summary

Systemic and localized inflammation elicit a number of host responses which include fever, cachexia, hypoglycemia, and major changes in the concentration of liver plasma proteins. Interleukin 6 (IL-6) is considered an important mediator of the inflammatory response, together with IL-1 and tumor necrosis factor α (TNF-α). The purpose of this study was to unequivocally determine the role of IL-6 in these phenomena making use of IL-6-deficient mice that we have recently generated by gene targeting. We report here that in the absence of IL-6, mice are unable to mount a normal inflammatory response to localized tissue damage generated by turpentine injection. The induction of acute phase proteins is dramatically reduced, mice do not lose body weight and only suffer from mild anorexia and hypoglycemia. In contrast, when systemic inflammation is elicited through the injection of bacterial lipopolysaccharide (LPS), these parameters are altered to the same extent both in wild-type and IL-6–deficient mice, demonstrating that under these conditions IL-6 function is dispensable. Moreover, we show that LPS-treated IL-6–deficient mice produce three times more TNF-α than wild-type controls, suggesting that increased TNF-α production might be one of the compensatory mechanisms through which a normal response to LPS is achieved in the absence of IL-6. We also show that corticosterone is normally induced in IL-6–deficient mice, demonstrating that IL-6 is not required for the activation of the hypothalamic-pituitary-adrenal axis. Our results reinforce the idea that different patterns of cytokines are involved in systemic and localized tissue damage, and identify IL-6 as an essential mediator of the inflammatory response to localized inflammation.

Acute inflammation results in a general reaction of the organism which include anorexia, loss of body weight, hypoglycemia, and changes in the serum levels of several plasma proteins produced by the hepatocytes, the so-called liver acute phase response (APR) (1). Several stimuli of different origin such as bacterial infection, endotoxemia and sterile tissue damage (burns, surgical traumas, ischemic necrosis) can trigger the host inflammatory response (1). These conditions can be reproduced by a number of experimental treatments. In particular, intraperitoneal injection of bacterial endotoxin (LPS), a component of the Gram negative bacterial wall, is commonly used to reproduce systemic inflammation, and subcu-
glycophorin (AGP), and serum amyloid A and P (SAA and SAP), are induced by IL-1 and TNF-α, either alone or in combination with IL-6. In contrast class 2 genes, which include α1-antichymotrypsin, fibrinogen, and α2-macroglobulin (α2M), respond to IL-6 alone. Interestingly, it has been recently shown that a family of cytokines related to IL-6, namely leukemia inhibitory factor (LIF), oncostatin M (OM), IL-11, and ciliary neurotrophic factor (CNTF), are able to induce the same spectrum of acute phase genes as IL-6 in hepatic cells (11 and references therein), suggesting functional redundancy.

Corticosteroid hormones participate in the induction of most APPs, being required for their optimal induction (12). Both LPS and turpentine are able to rapidly induce corticosterone production through the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the induction of ACTH (7, 13, 14). IL-1 is known to be one of the main mediators of this induction, as IL-1 blocking antibodies or IL-1 receptor antagonist can block the ACTH and corticosterone response to LPS (15, 16); IL-6 has been proposed to be able to synergize with IL-1 to generate maximal response (17). Corticosteroids exert in turn an inhibitory effect on the production of inflammatory cytokines, activating thus a negative feedback loop (18-20).

We have recently described the generation of IL-6-deficient mice by gene targeting, and showed that they are viable and fertile (21). Here we show that the complete absence of IL-6 abolishes or dramatically reduces several aspects of the acute host response to turpentine, including the liver acute phase reaction, anorexia, loss of body weight, and hypoglycemia. However, the same responses are only slightly modified after LPS treatment, indicating that in this case functional redundancy between different cytokines can compensate for IL-6 loss of function.

Materials and Methods

Animals and Treatments. 8–10-wk-old IL-6−/− and +/+ mice bred in our barrier facility were used. The generation and genetic background of these mice was described recently (21). Mice were maintained in standard conditions under a 12-h light-dark cycle, provided irradiated food (4RF21; Mucedola SILL; Settimo Milanese, Milan, Italy) and chlorinated water ad libitum. Procedures involving animals and their care were conducted in conformity with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana No. 40, February 18, 1992; National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH publication No. 85-23, 1985). LPS (From Escherichia coli serotype 026:B6) was purchased from Sigma Chemical Co. (St. Louis, MO), resuspended in sterile pyrogen-free saline solution and injected intraperitoneally at a dose of 1 mg/kg body weight. Steam distilled turpentine was injected subcutaneously. A single injection of 100 μl was given for the analysis of mRNAs, CS, TNF-α, and IL-1α. Two injections, one over each hind limb, were given for the analysis of body weight, food intake and hemaglucose. Control mice were injected intraperitoneally or subcutaneously with sterile pyrogen-free saline solution. Blood was collected from the retroorbital cavity.

RNA Expression. At different time intervals after treatment, mice were killed by cervical dislocation and RNA was isolated by the guanidine isothiocyanate method (22) and subjected to Northern blot analysis as previously described (23). 32P-labeled cDNAs for rat α2-M (24), mouse serum amyloid P (25), rat α1-acidic glycoprotein (26), rat haptoglobin (26), rat hemopexin (27), mouse albumin (28), and mouse GAPDH (29) were used as probes. Poly(A)+ mRNA was prepared using oligo d(T) columns (5 Prime–3 Prime, Inc., Boulder, CO) and hybridized with 32P-labeled rat α2M and mouse GAPDH cDNA. The relative abundance of the different mRNAs was measured by densitometric scanning of the autoradiographs.

Serum Protein Profile and Serum SAA Measurement. Sera were subjected to cellulose acetate electrophoresis. Strips were stained with Ponceau red solution and densitometric profiles were determined. SAA was measured in serum samples using an ELISA assay as previously described (30).

TNF, CS, and IL1α Measurement. TNF-α bioactivity in serum samples was measured 1 h 30 min after LPS or turpentine treatment by standard cytotoxicity assay using L929 cells (31). CS was measured in serum 1 h 30 min after LPS or turpentine treatment by a RIA, using an antiserum to corticosterone obtained from Sigma Chemical Co. and following manufacturer’s instructions. [3H]Corticosterone was purchased from Amersham Corp. (Arlington Heights, IL). IL-1α was measured in the serum 2 h after LPS treatment and 18 h after turpentine treatment using a mouse IL-1α ELISA kit purchased from Genzyme Corp. (Cambridge, MA) and following manufacturer’s instructions.

Hematic Glucose. Hematic glucose level was determined using the automatic analyzer Cell-Dyn 800 (Sequoia, Santa Barbara, CA).

Body Weight and Food Intake Measurements. The mice were weighed every 24 h starting from the day and time of treatment (day 0). Food intake was measured for each group at the same time intervals and mean food intake per mouse per day was calculated.

Statistical Analysis. Comparisons of mean values were performed by Student’s t test for independent samples.

Results

The Liver Acute Phase Response to LPS Injection Is Similar in IL-6−/− and +/+ Mice. To evaluate the specific role of IL-6 in the LPS induced liver APR, LPS was injected into IL-6-deficient (IL-6−/−) mice or wild-type (IL-6+/+) age matched controls, the mice were killed at different time intervals, and total liver RNA was extracted. The mRNA levels for several positive APPs, as well as for albumin, a negative acute phase reactant, were evaluated by Northern blot. As shown in Fig. 1, the changes of most mRNAs analyzed were comparable in the mutant and wild-type mice. After normalization with the internal control, the extent of albumin decrease was identical, and only minor differences were noticed in the induction of Hpx, Hp, and AGP. However, SAP mRNA was induced considerably less in the IL-6−/− mice, and no induction of the α2M mRNA was detected. Interestingly, when measured 18 h after LPS treatment, SAP mRNA levels were only two- to threefold lower in the IL-6−/− mice than in the IL-6+/+ controls, but dropped to almost background levels at 24 h, suggesting that the most critical role played by IL-6 in the induction of this gene in response to LPS is probably to sustain its activation rather than to initiate it. These results suggest that, as far as the liver APR is concerned, in systemic inflammation the absence of IL-6
Figure 1. Northern blots showing liver acute phase mRNA in IL-6-/- and IL-6+/+ mice following LPS treatment. Mice were treated with LPS (1 mg/Kg, i.p.), total liver RNA was extracted at 0, 2, 18, and 24 h after the treatment and hybridized with the indicated cDNAs. GAPDH was used as an internal control.

Figure 2. Northern blots showing liver acute phase mRNA in IL-6-/- and IL-6+/+ mice after turpentine treatment. Mice were treated subcutaneously with 100 μl of turpentine. Total liver RNA was extracted at the time intervals indicated and analyzed as described in the legend to Fig. 1.

can mostly be compensated for by other cytokines known to be induced by LPS like IL-1 and TNF-α.

Impaired Liver Acute Phase Response to Turpentine Injection in the IL-6-/- Mice. The reaction to turpentine of both IL-6-/- and IL-6+/+ mice was analyzed essentially as described for LPS. In this case, the difference in the levels of mRNAs encoding APPs after the treatment was dramatic (Fig. 2). Whereas in IL-6+/+ mice the various mRNAs were induced between 9- and 30-fold, the maximal induction in IL-6-/- mice was about fourfold. Furthermore, induction started earlier in the IL-6+/+ than in the IL-6-/- mice, being already detectable 2 h after treatment. Consistent with what was observed upon LPS treatment, the most dramatic difference was noticed for α2M mRNA. Albumin mRNA levels did not decrease at all in the IL-6-/- mice, confirming that IL-6 also plays an important role in the regulation of negative acute phase reactants, at least in this model of inflammation. These data confirm and extend the previous observation that monoclonal anti-IL-6 antibodies can block the serum accumulation of SAP in response to a turpentine abscess (9), and imply that IL-6 plays a unique role in the regulation of the liver APR induced by localized tissue damage.

The mRNA Coding for the Class 2 APP α2M Is Not Induced in the IL-6-/- Mice. To confirm that neither LPS nor turpentine are able to induce the α2M RNA in the IL-6-/- mice, we prepared polyA+ RNA from the samples analyzed above and hybridized it with the α2M probe. As shown in Fig. 3, α2M RNA was never detectable in the liver from the IL-6-/- mice, despite the strong induction occurring in the IL-6+/+ mice. Interestingly, the IL-6 related cytokines LIF, CNTF, OM, and IL-11 have been shown to be able to induce several class 2 acute phase genes by themselves (11). Our results suggest however that under physiological conditions these cytokines are not able to compensate for the absence of IL-6, at least under the experimental conditions described.

Serum Protein Profiles and Serum SAA. Although the regulation of APP production is known to occur mostly at the transcription level (23, 32), posttranscriptional and/or posttranslational effects have been described (33, 34). To evaluate if the alterations in mRNA induction observed are reflected by equivalent changes in circulating plasma proteins, we analyzed the protein profiles of sera from LPS- and turpentine-treated mice (Fig. 4 A). In IL-6+/+ mice both inflammatory stimuli caused a comparable decrease in the albumin peak and increase in the α and β globulin peaks, which include most of the major APPs. As a result, the ratio of albumin
to globulin decreased by ~50%. In contrast, turpentine treatment did not significantly modify the protein profile of the IL-6−/− mice, in agreement with the results obtained by Northern analysis and the albumin/globulin ratio was unchanged, while in response to LPS treatment it decreased almost to the same extent as in the IL-6+/+ mice.

To further extend these results, we measured serum SAA levels 18 and 24 h after LPS or turpentine injection. As shown in Fig. 4 B, SAA was undetectable in the serum of the turpentine treated IL-6−/− mice, while its levels were very high in the serum of IL-6+/+ mice at both time points analyzed. Interestingly, in the case of LPS injection the SAA levels detected after 18 h were significantly lower in the IL-6−/− mice than in the IL-6+/+ controls. This difference was not maintained after 24 h, when SAA concentration had already started to drop. This phenomenon was not predicted from the kinetics of induction observed for the mRNAs. Since SAA is known to be regulated also posttranscriptionally (33), our results seem to suggest a role for IL-6 in the regulation of the liver APR also at this level.

Corticosterone Induction Is Normal in the IL-6−/− Mice. Corticosteroid hormones are important cofactors in the induction of AP genes, and their action is considered of particular importance in the induction of the AGP and α2M genes (35, 36). For these reasons we decided to measure serum corticosterone (CS) both before and after turpentine or LPS treatment. As shown in Fig. 5 (A and B), both basal and induced CS levels were equivalent in IL-6−/− and IL-6+/+ mice, indicating that the impaired induction of acute phase genes described above is not due to a secondary effect through a defective CS induction.

Induction of TNF-α and IL-1 in the IL-6−/− Mice. Both TNF-α and IL-1 are prominent cytokines involved in the regulation of different aspects of the host response to inflammation, and they are thought to exert at least part of their effects through the induction of IL-6 (37). It was therefore important to analyze their levels in the IL-6−/− mice.

Interestingly, TNF-α induction by LPS was found to be approximately threefold higher in IL-6−/− than in IL-6+/+ mice (P <0.05) (Fig. 5 C). It was reported that IL-6 can inhibit TNF-α transcription (38); our observation might therefore be a consequence of the absence of this negative feedback in the IL-6−/− mice. Whatever the mechanism, the increased TNF-α levels might contribute to compensate for the absence of IL-6, at least for those acute phase genes known to be responsive to this cytokine. Confirming what was already reported by others (6), we could not detect any TNF-α activity after turpentine injection at the time points analyzed (data not shown).

In contrast to what was observed for TNF-α, there was no statistically significant difference in the extent of IL-1α induction in the IL-6−/− and IL-6+/+ mice (Fig. 5 D and E). In both cases the absolute levels reached after turpentine injection (Fig. 5 E) were lower than after LPS treatment (Fig. 5 D). The observation that IL-1 is induced normally in mutant mice suggests that also this cytokine may be involved in compensating for IL-6 absence.

Anorexia, Food Intake, and Hypoglycemia in the IL-6−/− Mice. It has been reported that antibodies against IL-6 partially blocked both LPS and turpentine induced weight loss and hypoglycemia (7–9). To test the effects of a complete absence of IL-6 on these clinically important aspects of inflammation, we decided to measure these parameters in IL-6−/− mice.

The measures of body weight, daily food intake and hematic glucose after LPS injection are shown in Fig. 6 (A, B, and
Figure 5. Effects of LPS and turpentine on the induction of serum CS, TNF-α, and IL-1α in IL-6+/+ and IL-6-/- mice. Mice were treated as described in the text with LPS, turpentine, or sterile saline solution as control. TNF-α and IL-1α in the control mice were undetectable and were therefore not reported in the graphic. (A and B) CS levels were measured by a RIA in the serum 1.5 h after treatment with turpentine (A, TURP.) or LPS (B). Black bars and open bars represent IL-6+/+ and IL-6-/- mice, respectively. (A) four mice/group; (B) seven mice/group. Data are shown as mean values ± SD. (C) TNF-α was measured by standard cytotoxicity assay 1.5 h after LPS treatment (seven mice/group); (*) significantly different from IL-6+/+, p < 0.05. (D and E) IL-1α was measured by ELISA 2 h after LPS treatment (D), and 18 h after turpentine treatment (E). Nine mice/group were used. The differences were not statistically significant.

C, respectively). All three parameters decreased equivalently in the IL-6+/+ and IL-6-/- mice, and body weight and food intake returned to normal values 3 d after the treatment. These results seem to indicate that IL-6 action is not required for the generation of these host responses to LPS, confirming that an overall normal inflammation process in response to endotoxin can take place in the absence of IL-6. The response to turpentine was however completely different. During the first 3 d after treatment the body weight did not decrease at all in the IL-6-/- mice, while the IL-6+/+ mice lost a considerable amount of weight (Fig. 7,A). In addition, daily food intake and glucose concentration decreased significantly less in IL-6-/- than in IL-6+/+ mice (Fig. 7, B and C). Surprisingly, whereas after 4 d the IL-6+/+ mice started to regain weight and increase their food intake, both parameters started to drop dramatically in IL-6-/- mice (Fig. 7, A and B). One of them died at day 7, and all mice were killed at day 8 to avoid chronic effects of turpentine toxicity. We do not know the mechanism for the phenomenon described. Likely, the absence of an efficient response to the inflamma-

Figure 6. Changes in body weight, food intake, and hematic glucose after LPS treatment of IL-6-/– and IL-6+/+ mice (six mice/group). Mice were treated as described above. The values of body weight and food intake did not change in the control mice, and were therefore not reported. (A) Body weight was measured every 24 h at the same time of the day starting from the day of treatment (day 0) until the mice regained the original weight (3 d); the mean values are indicated. (B) Cumulative food intake was measured for the different groups every 24 h and mean values for individuals were derived. (C) Hematic glucose was measured 4 h after treatment with LPS or pyrogen free saline solution. Black bars and open bars represent IL-6+/+ and IL-6-/- mice, respectively. Data are shown as mean values ± SD.
The overall characteristics of the host response to inflammation are remarkably similar, whatever the triggering stimulus may be. However, different kinds of injury involve different cell types, and as a consequence the mediators produced and their site of release are different. A striking example is the finding that endotoxin induces the synthesis of IL-6 by liver Kupffer cells, whereas sterile tissue damage does not (6). This difference is probably due to the fact that LPS rapidly enters the circulation, and can reach and directly stimulate liver nonparenchymal cells. Another important difference is that TNF-α, a prominent cytokine in LPS-generated systemic inflammation, is not produced in response to turpentine induced localized inflammation. Distinguishing between the specific roles the different mediators play in regulating the inflammatory response has not been an easy task so far, due to the complex interactions between cytokines, glucocorticoid hormones, and their signal transmission pathways, and to the lack of suitable genetic models. The interpretation of the results obtained by injecting in vivo recombinant cytokines is therefore not straightforward, and experiments with blocking antibodies cannot be conclusive, both because a complete inactivation is difficult to achieve and to demonstrate, and because monoclonal antibodies in particular against IL-6 have been shown to complex the cytokine and to inactivate it, and because monoclonal antibodies in particular against IL-6 have been shown to complex the cytokine and to inactivate it, and because monoclonal antibodies in particular against IL-6 have been shown to complex the cytokine and to inactivate it, and because monoclonal antibodies in particular against IL-6 have been shown to complex the cytokine and to increase its half-life (8, 9). Mutant mice generated through the targeted mutagenesis of the mouse genome may provide the opportunity required to obviate these problems by studying the inflammatory reaction in the complete absence of a given cytokine.

We have used IL-6-deficient mice generated by gene targeting to explore the specific functions of IL-6 in determining the host response of two fundamentally different types of injury, systemic inflammation, reproduced by means of LPS injection, and localized inflammation, mimicked by sterile turpentine abscesses. While this manuscript was in preparation, Kopf et al. (39) reported similar studies on independently derived IL-6-deficient mice. They found that the AP reactants Hp, AGP, and SAA are not induced by sterile tissue damage or by infection with Gram positive bacteria in the mutant mice, whereas the induction of these genes and proteins is almost normal after LPS injection. Our results confirm their findings; however, we could identify at least one specific defect in the response to LPS, that is the lack of induction of the α2M mRNA in the IL-6-/- mice. Moreover, we explored several aspects of the host reactions to localized inflammation in addition to the liver APR, and showed that IL-6 is an essential component in determining clinically relevant systemic responses such as anorexia, loss of body weight, and hypoglycemia. In contrast, only minor differences were detected in the same responses to LPS, demonstrating that IL-6 is in this case dispensable.

The finding that IL-6 is not required to generate in inflammatory response to LPS was somewhat surprising, as IL-6 is produced in large quantities upon LPS injection (9). Presumably its activity can be substituted for by other cytokines, in particular IL-1 and TNF-α. It is interesting to note that LPS induced threefold higher TNF-α levels in IL-6-/- mice than in the IL-6+/- controls; compensation through an increased TNF-α production might therefore account, at least partially, for the overall normal inflammatory reaction to LPS we observed in the IL-6-/- mice. This idea is further corroborated by the fact that turpentine abscesses, which do not elicit a normal response in the absence of IL-6, failed to induce TNF-α production.

We have used IL-6-deficient mice generated by gene targeting to explore the specific functions of IL-6 in determining the host response of two fundamentally different types of injury, systemic inflammation, reproduced by means of LPS injection, and localized inflammation, mimicked by sterile turpentine abscesses. While this manuscript was in preparation, Kopf et al. (39) reported similar studies on independently derived IL-6-deficient mice. They found that the AP reactants Hp, AGP, and SAA are not induced by sterile tissue damage or by infection with Gram positive bacteria in the mutant mice, whereas the induction of these genes and proteins is almost normal after LPS injection. Our results confirm their findings; however, we could identify at least one specific defect in the response to LPS, that is the lack of induction of the α2M mRNA in the IL-6-/- mice. Moreover, we explored several aspects of the host reactions to localized inflammation in addition to the liver APR, and showed that IL-6 is an essential component in determining clinically relevant systemic responses such as anorexia, loss of body weight, and hypoglycemia. In contrast, only minor differences were detected in the same responses to LPS, demonstrating that IL-6 is in this case dispensable.

The finding that IL-6 is not required to generate in inflammatory response to LPS was somewhat surprising, as IL-6 is produced in large quantities upon LPS injection (9). Presumably its activity can be substituted for by other cytokines, in particular IL-1 and TNF-α. It is interesting to note that LPS induced threefold higher TNF-α levels in IL-6-/- mice than in the IL-6+/- controls; compensation through an increased TNF-α production might therefore account, at least partially, for the overall normal inflammatory reaction to LPS we observed in the IL-6-/- mice. This idea is further corroborated by the fact that turpentine abscesses, which do not elicit a normal response in the absence of IL-6, failed to induce TNF-α production. Moreover, the most prominent
Although IL-6 has been shown to synergize with IL-1 in the induction of the HPA axis (17), we found that CS induction is not defective in the IL-6−/− mice (Fig. 5, a and b). We have shown that CNTF is equivalent to IL-6 in eliciting CS induction when co-injected with IL-1 (Pantuzzi, G., manuscript submitted for publication). We propose therefore that CNTF and/or some other cytokine belonging to the IL-6 family are physiologically involved in the stimulation of the HPA axis, thus rendering IL-6 function dispensable.

IL-6 has been proposed as playing a role in the pathogenesis of several diseases, including rheumatoid arthritis, multiple myeloma (reviewed in reference 43), and osteoporosis (21, 44). We have shown that IL-6 is required for several aspects of the inflammatory response to sterile tissue damage, which can be commonly caused by surgery or burns. Although chronic or excessive liver APR, anorexia, and weight loss can contribute to morbidity, these body responses are thought to be part of the host defense reactions to acute inflammation. APPs include several protease inhibitors and heme-binding proteins, which can limit the damage at the site of injury and preserve the iron reserves of the organism, and mobilization of body fat and lean tissue may provide the substrates and energy required for tissue repair and recovery. IL-6, being an essential mediator of these responses, might therefore exert a protective effect during acute inflammation.

We wish to thank C. Stewart for help with embryo re derivation; F. Tenuta for the serum profiles; H. Baumann, U. Muller-Eberhard, and S. Maeda for the kind gift of probes; M. Aquilina and S. Germoni for animal care; G. Ciliberto, J. Clench, R. Cortese, J. Jirikny, and C. Toniatti for critically reading the manuscript; and Y. M. Cully and M. Emili for graphical work.

Address correspondence to Valeria Poli, Istituto di Ricerche di Biologia Molecolare IRBM P. Angeletti, Via Pontina Km. 30.600, 00040 Pomezia (Roma), Italy.

Received for publication 25 March 1994 and in revised form 23 May 1994.

References

11. Banmann, H., S.F. Ziegler, B. Mosley, K.K. Morella, S. Pa-


