

Gallium Arsenide Selectively Up-Regulates Inflammatory Cytokine Expression at Exposure Site

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Received July 30, 2003; accepted September 8, 2003

ABSTRACT

Gallium arsenide (GaAs), a technologically and economically important semiconductor, is widely utilized in both military and commercial applications. This chemical is a potential health hazard as a carcinogen and immunotoxicant. We previously reported that macrophages at the exposure site exhibit characteristics of activation. *In vitro* culture of macrophages with GaAs fails to recapitulate the *in vivo* phenotype, suggesting that complete GaAs-mediated activation *in vivo* may require other cells or components found in the body's microenvironment. Our present study examined the role of cytokines upon GaAs-mediated macrophage activation. Intraperitoneal administration of GaAs elicited rapid specific recruitment of blood monocytes to the exposure site. This recruitment occurred concomitant with up-regulation of 17 chemokine and inflammatory cytokine mRNAs, while transcripts of three inhibitory

cytokines diminished. Administration of latex beads caused less cytokine induction than GaAs, indicating that changes in mRNA levels could not be attributed to phagocytosis. Four representative chemokines and cytokines were selected for further analysis. Increased cytokine mRNA expression was paralleled by similar increases in cytokine protein levels, and secreted protein products were detected in peritoneal fluid. Cytokine protein expression was constrained to myeloid cells, and to a lesser extent to B cells. Alterations in patterns of cytokine gene expression elucidate mechanisms for increased cellular activation and antigen processing, and modulation of the inflammatory response. Our findings indicate that *in vivo* GaAs exposure alters cytokine gene expression, which may lead to an inflammatory reaction and contribute to pathological tissue damage.

Gallium arsenide (GaAs) is a group IIIa–Va intermetallic semiconductor whose optical and electronic properties promote its use in numerous technological applications such as room temperature lasers, field effect transistors, and integrated circuitry. Processing of GaAs wafers produces fine particulate crystals, which may present a hazard to industrial workers (Harrison, 1986). Previous toxicological studies in animals and culture caused both the Environmental Protection Agency and World Health Organization to classify GaAs as an immunotoxicant and potential carcinogen (Luster et al., 1992; World Health Organization, 1992). Exposure of mice to GaAs, either through intratracheal instillation or intraperitoneal administration, causes systemic inhibition of immune function. Immunosuppression is associated with defects in antigen processing by splenic macrophages, resulting in faulty activation of helper T cells (Lewis et al., 1998a,b) and dose-dependent reduction of the IgM humoral response (Sikorski et al., 1989, 1991a,b; Lewis et al., 1996). Impaired

antigen processing is attributable to decreased proteolytic activity of cathepsins D, B, and L (Lewis et al., 1998b; Harrison and McCoy, 2001), correlating with decreased interleukin (IL)-2 production by helper T lymphocytes.

In contrast to systemic immune defects, GaAs has the opposite effect at the exposure site. A single intratracheal dose of GaAs or chronic inhalation in rodents results in pulmonary edema, fibrosis, and pneumocyte hyperplasia with accompanying accumulation of macrophages and neutrophils (Webb et al., 1986; National Toxicology Program, 2000; Tanaka et al., 2000). These symptoms are indicative of an inflammatory immune response. Following intraperitoneal exposure, both resident and thioglycollate-induced macrophages exhibit an enhanced ability to activate T lymphocytes in response to protein antigens (Hartmann and McCoy, 1996). This enhancement is mediated both by increased proteolytic activity of thiol cathepsins B and L (Lewis et al., 1998b) and increased surface expression of major histocompatibility complex class II and costimulatory molecules (Hartmann and McCoy, 1996; Caffrey-Nolan and McCoy, 1998). Due to up-regulation of these molecules, GaAs-exposed macrophages might exist in an activated state. *In vitro*

This work was supported by National Institutes of Health Grant ES07199. Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>. DOI: 10.1124/jpet.103.057919.

ABBREVIATIONS: GaAs, gallium arsenide; IL, interleukin; RPL, resident peritoneal leukocytes; ELISA, enzyme-linked immunosorbent assay; PLF, peritoneal lavage fluid; RPA, ribonuclease protection assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; MIF, migration inhibitory factor; TGF, transforming growth factor; IFN, interferon.

GaAs exposure of macrophages augments cathepsin activity, but does not increase expression of class II molecules (Harrison and McCoy, 2003). Although GaAs directly affects peritoneal macrophages, the *in vivo* microenvironment also appears to contribute to a completely activated phenotype.

Currently, little is known about the mechanisms by which GaAs enhances macrophage function by GaAs-exposed peritoneal cells. Cytokines are one means by which cells of the immune system modulate their ability to mount responses to antigen. Importantly, cytokines are relevant to inflammatory responses akin to those induced by GaAs administration. The current study investigated the ability of GaAs to influence cytokine expression of a panel of cytokines at local (peritoneal) and distal (splenic) sites. During a time course, GaAs rapidly augmented steady-state mRNA levels of multiple inflammatory cytokines in leukocytes from the exposure site, whereas transcripts of three inhibitory cytokines were decreased. Increased protein quantity of four cytokines was present in the peritoneal fluid. Mainly, macrophages along with some B cells produced three inflammatory cytokine proteins. These findings indicate that GaAs alters cytokine expression at both the mRNA and protein levels, which could contribute to the chemical's adverse biological effects.

Materials and Methods

Mice. Female C3D2F1/J mice (The Jackson Laboratory, Bar Harbor, ME) were between 8 and 12 weeks of age and housed at four per cage. Mice were fed Teklad LM485 food (Harlan, Indianapolis, IN) and tap water *ad libitum*, and housed under specific pathogen-free conditions. Mice were age-matched within each experiment.

Monoclonal and Polyclonal Antibodies. Purified rabbit anti-mouse IL-1 β and rabbit anti-mouse IL-18 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated monoclonal antibodies directed against Mac-1, B220 and Thy1.2, and phycoerythrin-conjugated anti-Mac-3 and anti-MCP-1 (anti-CCL2) were purchased from BD Biosciences PharMingen (San Diego, CA). Goat anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR). Purified rabbit IgG, fluorescein-conjugated streptavidin, and normal mouse IgG were purchased from Zymed Laboratories (South San Francisco, CA). Culture supernatant containing monoclonal 2.4G2 antibody (anti-Fc γ receptor) was a gift from Dr. Gregory Burton (Brigham Young University, Provo, UT).

Chemical Exposure. Mice were injected intraperitoneally with 200 mg/kg body weight GaAs crystals with a 1.5- μ m mean diameter (Research Triangle Institute, Research Triangle Park, NC) suspended in saline containing 0.05% Tween 80 (Sigma-Aldrich Chemical Co., St. Louis, MO). This dose induces systemic immunosuppression without general toxicity (Sikorski et al., 1989; Lewis et al., 1996). Injections were performed intraperitoneally because this route produces results similar to intratracheal instillation without concomitant pulmonary damage (Hartmann and McCoy, 1996). Control mice were injected with saline containing 0.05% Tween 80 (vehicle) or 200 mg/kg body weight latex beads with a 1.0- μ m mean diameter (Sigma-Aldrich) suspended in the vehicle. Cell suspensions were prepared from four mice per exposure group, and sera were pooled from two mice per group.

Preparation of Cell and Fluid Samples. At 6, 18, and 48 h after exposure blood was collected by cardiac puncture and sera were prepared and stored at -20°C until used. For RNA isolation, resident peritoneal leukocytes (RPL) were harvested by lavage and splenic cell suspensions were prepared as described (Lewis et al., 1998b). Cells were suspended in Trizol reagent according to the manufacturer's directions (Invitrogen, Carlsbad, CA). RNA yield was determined by absorbance at 260 nm, and purity and quality were

confirmed by 260 nm/280 nm and 260 nm/230 nm absorbance ratios. For enzyme-linked immunosorbent assay (ELISA), cell-free peritoneal fluid (PLF) was collected by lavage with 1 ml Hanks' balanced salt solution and stored at -20°C until used.

Immunofluorescence Staining and Flow Cytometric Analysis. Cells were stained as described (Hartmann and McCoy, 1996). Briefly, RPL were incubated with normal mouse IgG and/or monoclonal 2.4G2 antibody to block Fc γ receptors. For cell surface staining, cells were incubated with lineage-specific monoclonal antibodies or irrelevant control antibody for 30 min at 4°C and washed. When using biotinylated antibodies, cells were then incubated with fluorescein-labeled streptavidin and washed. Intracellular cytokine staining was conducted using the Cytofix/Cytoperm system (BD Biosciences PharMingen) following the manufacturer's protocol. Briefly, cells were incubated with GolgiPlug (brefeldin A) and stained with lineage-specific antibodies as above. Cells were fixed and permeabilized in Cytofix/Cytoperm buffer, stained with anticytokine or irrelevant control antibody for 30 min at 4°C , and then washed. Fluorescence intensity was measured by a FACScan (BD Biosciences, San Jose, CA). Each experiment analyzed at least 10,000 cells, gated on forward and side scatter to exclude debris. Data analysis was conducted using Cell Quest Pro software (BD Biosciences).

Ribonuclease Protection Assay (RPA). Total RNA was prepared as described above and analyzed using the RiboQuant Multi-Probe Ribonuclease Protection Assay System (BD Biosciences PharMingen). The MultiProbe template sets, including mCK2b, mCK3b, and mCK5b, assessed a panel of 25 different cytokines as well as two housekeeping genes, L32 and GAPDH. Briefly, 2- μ g total RNA was incubated overnight with radiolabeled probes, subjected to digestion by Ribonuclease A + T1, and protected probes were resolved on an 8 M urea-5% acrylamide sequencing gel. Dried gels were exposed overnight to Kodak X-OMAT blue film (Eastman Kodak, Rochester, NY), or exposed in a Molecular Dynamics PhosphorImager cassette (Amersham Corp., Sunnyvale, CA). Cassette exposures were quantitated on a PhosphorImager 445Si, and data analysis and densitometry were achieved using ImageQuant 5.1 (Amersham Corp.). Band intensities for cytokines were calculated as a proportion of the band density of GAPDH to normalize data among lanes and experiments.

Cell Culture. RPL and splenocytes were cultured overnight at 10^6 cells/ml in Dulbecco's modified Eagle's medium fortified with 10% heat-inactivated fetal bovine serum (BioSource International, Camarillo, CA), 100 U/ml penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich). Cell-free culture supernatants were harvested and stored at -20°C until used.

Cytokine ELISA. Murine tumor necrosis factor (TNF)- α and IL-1 β were measured using the Quantikine kit following the manufacturer's recommended protocol (R&D Systems, Minneapolis, MN). IL-18 was measured using a commercially available kit (MBL, Nagoya, Japan), as was CCL2 (Pierce Endogen, Rockford, IL) according to the manufacturers' directions. Samples were assayed in triplicate. Absorbance was measured on a SpectraMax 250 plate reader running SOFTmax Pro 3.1.2 software (Molecular Devices Corp., Sunnyvale, CA). Cytokine quantities in samples were calculated from standard curves of recombinant cytokines.

Statistical Analysis. Parametric analysis of variance was performed using Student's two-tailed *t* test for unmatched pairs in all experiments. GaAs-exposed samples were compared with the vehicle control, and to the latex bead control when applicable. A value of $p < 0.05$ was considered significant.

Results

Early Changes in Cellular Composition after In Vivo GaAs Exposure. *In vivo* GaAs exposure causes immune enhancement without altering cell populations resident

within the peritoneum (Hartmann and McCoy, 1996; Cafrey-Nolan and McCoy, 1998). However, these observations occurred after a 5-day exposure, allowing for the possibility of early modulation of cell populations. To determine whether early changes in cellular composition are initiated by GaAs exposure mice were administered GaAs intraperitoneally, and at 18 h RPL were characterized by flow cytometric analysis for lineage-specific surface molecules including Mac-1, Mac-3, B220, and Thy1.2. As shown in Fig. 1, alteration of the peritoneal population was evident following GaAs exposure compared with vehicle control. RPL from control mice consisted mainly of Mac-1^{hi}/Mac-3⁺ and B220⁺/Mac-3⁻ cells with a negligible fraction (<5%) of Thy1.2⁺ cells. In contrast, RPL from GaAs-exposed mice were skewed toward a single Mac-1^{mod}/Mac-3^{mod} phenotype consistent with monocytes with relatively few (15%) B220⁺ cells. The proportion of Thy1.2⁺ cells appeared to be slightly reduced by GaAs. Thus, peritoneal subpopulations changed early after GaAs exposure.

Altered Cytokine mRNA Expression after GaAs Exposure. A rapid shift in the cellular profile of RPL may be due to monocyte recruitment to the exposure site, perhaps mediated by proinflammatory cytokines. Multiprobe RPA was used to determine the presence and relative concentration of proinflammatory cytokine mRNA transcripts following GaAs exposure. Latex bead exposure was included as an additional control to determine the effect of nonspecific phagocytosis of particles on cytokine production. Steady-state levels of 25 cytokine transcripts were determined during a time period up to 48 h after exposure. Of these, 21 transcripts in RPL showed altered expression at one or more time points, while the others were unaffected by GaAs exposure during the time course. RPAs were also performed on samples of splenic leukocytes. For all cytokines tested, mRNA expression levels in splenocytes were not significantly different among the exposure groups during the 48-h period

(data not shown). Thus, significant changes in cytokine transcript expression after GaAs exposure were limited to RPL.

As shown in a representative gel in Fig. 2, numerous cytokine transcripts were detected following 18-h GaAs exposure, including CCL5, CCL4, CCL3, CXCL1, and CCL2. In contrast, neither vehicle control nor latex bead-exposed RPL appeared to express high levels of these cytokines. Figure 3 depicts a 48-h time course for mRNA expression of three cytokines. During this period, chemokine CXCL1 transcript levels were rapidly elevated at 6 h, and gradually returned to basal level by 48 h (Fig. 3A). A similar expression pattern was observed for the monocyte chemotactic protein CCL2 (Fig. 3B). Vehicle administration caused a small increase in the 6-h expression of these transcripts compared with later time points. Latex bead exposure caused only a slight, short-lived increase in the expression of CXCL1 at 6 h ($p < 0.05$), the magnitude of which was significantly less than that of GaAs exposure ($p < 0.01$). Monocyte migration inhibitory factor (MIF) mRNA in RPL also displayed a rapid increase (Fig. 3C); however, its expression was maintained above control throughout the 48-h time course.

Expression of cytokine transcripts of IL-1 family members indicated that each was specifically up-regulated following GaAs exposure (Fig. 4). As before, transcripts accumulated rapidly after exposure and returned to control levels within 48 h. IL-1 β displayed the largest increase in relative expression of all cytokines tested, with a maximal level nearly three times that of the housekeeping gene GAPDH. IL-1 transcript expression after vehicle or latex bead exposure was virtually undetectable. In addition, mRNA transcripts for the proinflammatory cytokines, TNF- α and IL-18, displayed expression time courses similar to those observed for the above cytokines (Fig. 5).

Members of the tumor growth factor (TGF)- β family of cytokines are generally considered to have anti-inflammatory activity. In marked contrast to the preceding cytokines,

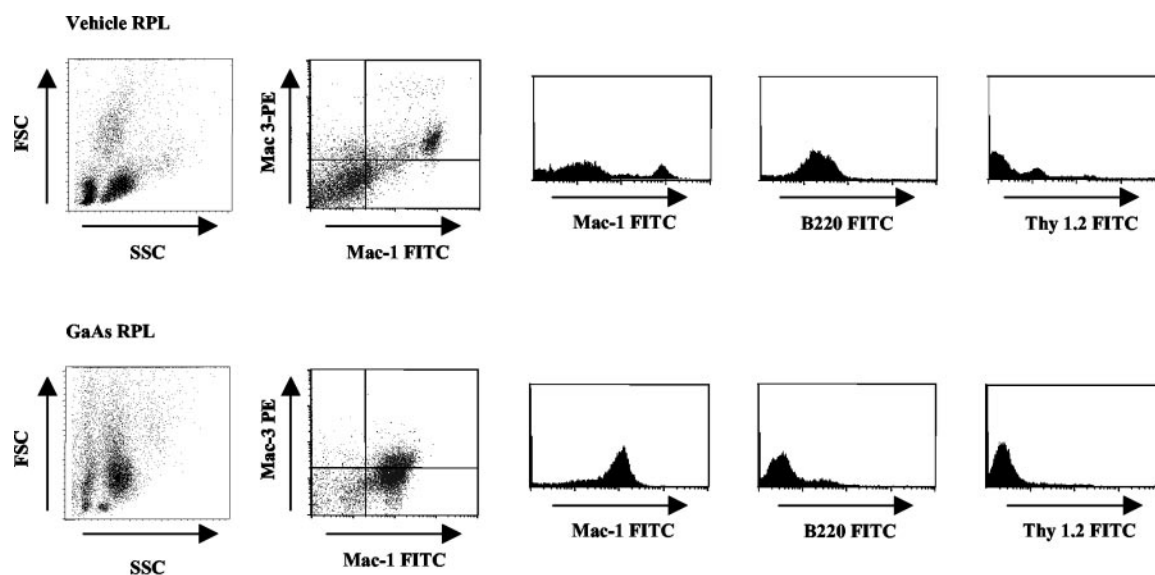


Fig. 1. Resident peritoneal cell types were altered following GaAs exposure. Flow cytometric analysis of peritoneal leukocytes (RPL) from mice exposed intraperitoneally to vehicle (top panels) or GaAs (bottom panels). Cells were harvested 18 h after exposure and stained with fluorochrome-conjugated antibodies specific for Mac-1, Mac-3, B220, and Thy1.2 as described under *Materials and Methods*. Fluorescence intensity was quantified by flow cytometry. Data are from one experiment representative of three repetitions. FSC, forward scatter; SSC, side scatter; PE, phycoerythrin; FITC, fluorescein.

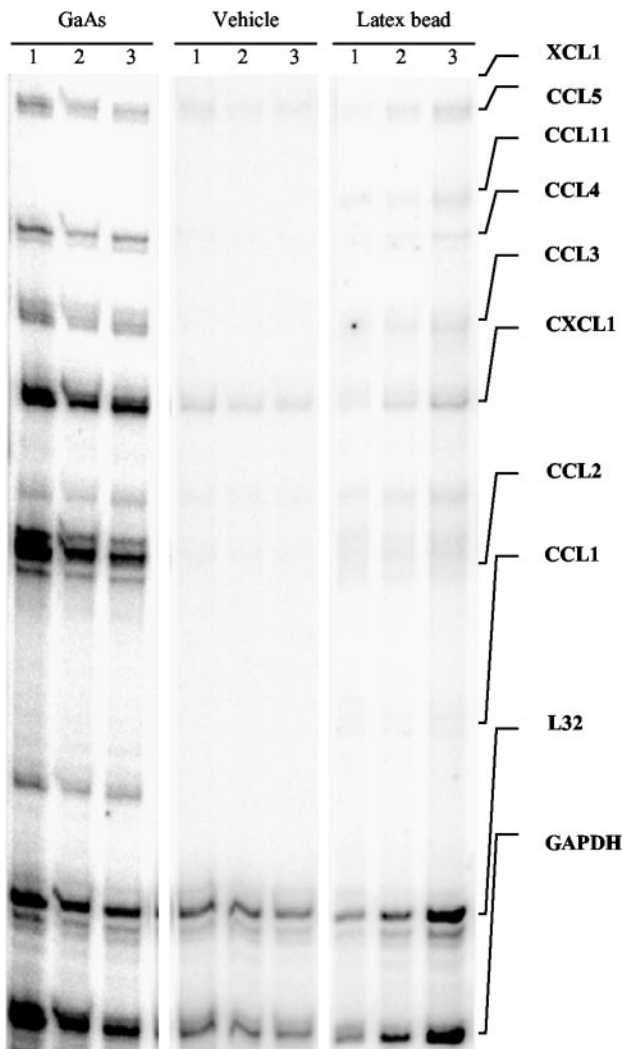


Fig. 2. Up-regulation of chemokine mRNA by GaAs. Total RNA was isolated from GaAs-, latex bead-, and vehicle-exposed peritoneal leukocytes 18 h after exposure. RNA was assayed for the presence of proinflammatory cytokine transcripts by RPA and resolved by gel electrophoresis. Three independently prepared samples are shown for each group.

transcripts for TGF- β 2 and TGF- β 3 were reduced to below detection level by GaAs exposure (Fig. 6, A and B). Reduction was observed at 6 h, and was complete by 18 h. Although a slight induction of TGF- β 1 was apparent in vehicle- and latex bead-exposed cells, its expression remained low in GaAs-exposed RPL throughout the time course (Fig. 6C).

Altered levels of several additional cytokine mRNAs were detected within RPL following GaAs exposure (Table 1). CCL3 and IL-6 mRNA were significantly up-regulated at the 6-h time point, while TNF- β mRNA was slightly reduced. By 18 h, CCL3 and IL-6 transcripts remained elevated (Table 1), whereas TNF- β returned to basal level (data not shown). Furthermore, up-regulation of six more cytokines occurred at 18 h (Table 1), and included the chemokines CCL4, CCL1, and CCL5 and the cytokines interferon (IFN)- γ , IL-6, IL-12 p40, IL-10, and IL-12 p35. Again, the mRNA expression of these nine cytokines was normal at 48 h (data not shown).

Induction of Cytokine Secretion by GaAs. To determine whether increases in cytokine transcripts were reflected at the protein level, four cytokines were selected for

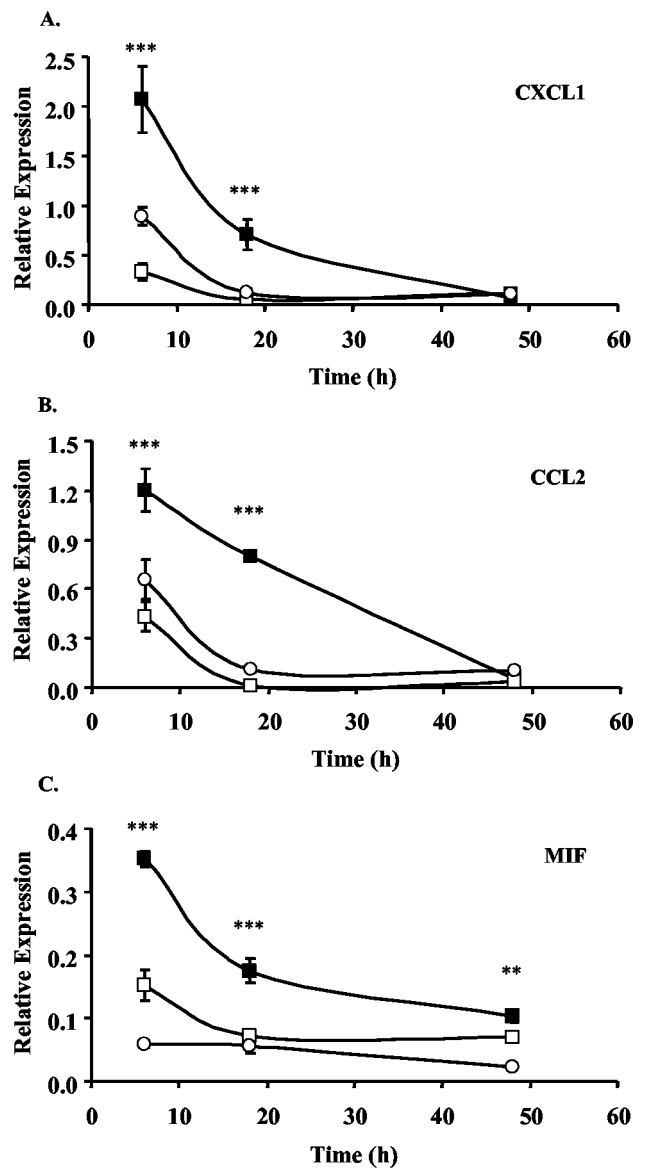


Fig. 3. Time course of increased levels of chemokine mRNA. Sample mRNA were prepared and assayed by RPA as in Fig. 2 legend. Quantitation of relative mRNA levels for CXCL1 (A), CCL2 (B), and MIF (C). GaAs- (■), latex bead- (○), and vehicle-exposed (□) mRNA levels are expressed proportional to the expression of the housekeeping gene GAPDH. Expression levels were assayed in triplicate, and values are mean \pm S.D. Significance between GaAs- and vehicle-exposed mRNA level was determined using Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$.

further analysis at the 18-h time point. These were selected to represent both proinflammatory cytokine and chemokine families. Following exposure, sera and peritoneal lavage fluids were tested for the presence of IL-1 β , IL-18, CCL2, and TNF- α protein by ELISA. Additionally, harvested RPL and splenocytes were cultured in medium without a stimulus overnight, and culture supernatants were assayed. As shown in Fig. 7A, IL-1 β protein was elevated in PLF from GaAs-exposed mice, while no significant change was detected in sera or culture supernatants. IL-18, like IL-1 β , was significantly increased only in PLF from GaAs-exposed mice (Fig. 7B). The monocyte chemokine CCL2 was elevated in both peritoneal fluid and sera from GaAs-exposed mice (Fig. 7C). Secretion of TNF- α was elevated in PLF, but not in serum

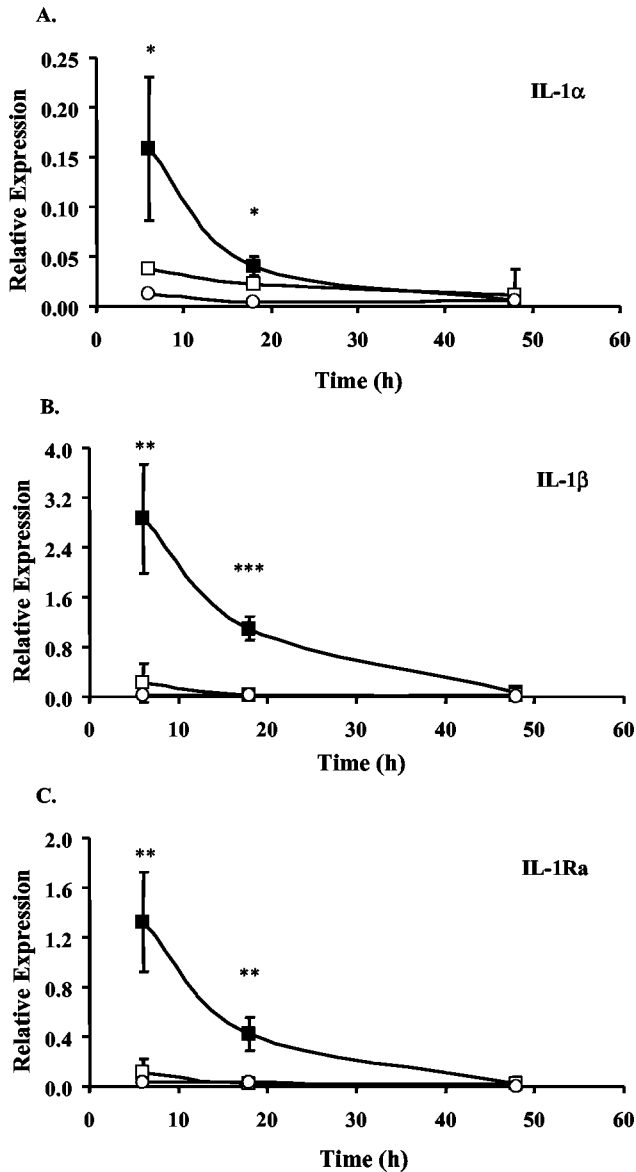


Fig. 4. Cytokines of the IL-1 family are rapidly induced after GaAs exposure. Sample mRNA was prepared as described in Fig. 2 legend and assayed by RPA. IL-1 α (A), IL-1 β (B), and IL-1Ra (C) were normalized relative to GAPDH from GaAs- (■), latex bead- (○), and vehicle-exposed (□) RPL. Expression levels are mean \pm S.D. from three independent samples. Significance between GaAs- and vehicle-exposed mRNA levels was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(Fig. 7D). No differences in cytokine protein levels were detected in culture supernatants generated from RPL or splenocytes. Overall, the increased level of inflammatory cytokine mRNA within the peritoneum was mirrored by similar increases in secreted protein.

Identification of Cells Producing Cytokines. To determine which cells in the peritoneum were producing inflammatory cytokines, intracellular immunofluorescence staining of RPL for CCL2, IL-1 β , and IL-18 was performed. A representative experiment is shown in Fig. 8. Following GaAs exposure, intracellular protein expression of cytokines was detected. Approximately 11% of RPL were CCL2⁺ with the overwhelming majority (>95%) costaining for Mac-1. IL-1 β was expressed in approximately 16% of RPL, of which most

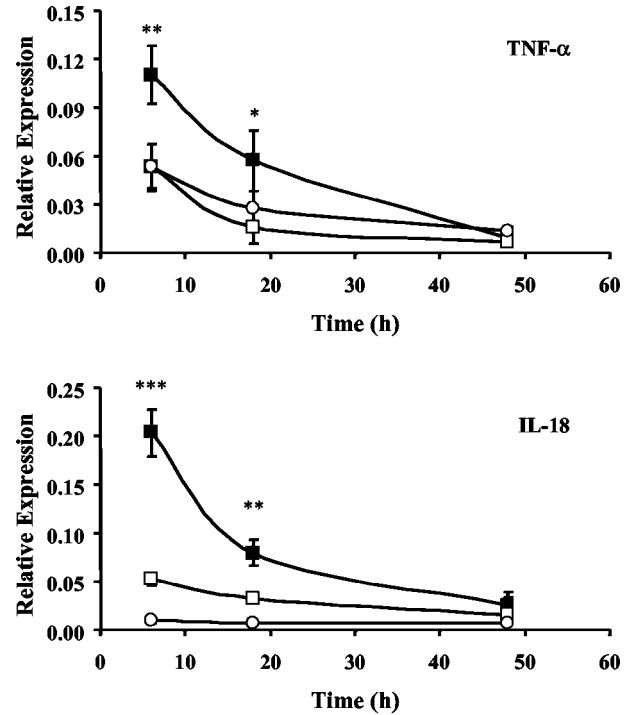


Fig. 5. GaAs induces transcripts for proinflammatory cytokines TNF- α and IL-18. Sample mRNA was prepared from GaAs- (■), latex bead- (○), and vehicle-exposed (□) mice as described in Fig. 2 legend. RNA was assayed by RPA, and mRNA level is expressed proportional to expression of the housekeeping gene GAPDH. Expression levels were assayed in triplicate and are presented as mean \pm S.D. Significant difference between GaAs- and vehicle-exposed mRNA level was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(58%) were Mac-1⁺, and the remainder positively stained for B220. IL-18 was expressed in approximately 6.5% of RPL. Again, most (66%) were Mac-1⁺, and the remainder were B220⁺. No intracellular cytokine staining was detected in Thy1.2⁺ cells. Hence, the main cell type producing the three cytokines examined was Mac-1⁺.

Discussion

Although GaAs induces an activated phenotype in directly exposed macrophages, the mechanism for this activation is unknown. Our current study investigated the role of cytokines as a possible means. Peripheral monocytes, presumably from the blood, were rapidly recruited to the peritoneum following intraperitoneal GaAs exposure. We assessed mRNA expression of 25 cytokines in peritoneal cells by RPA. Concurrently, the mRNA transcripts encoding 17 cytokines implicated in promoting inflammation and leukocyte chemotaxis were detected at significantly higher levels than after vehicle treatment in the peritoneum, while three anti-inflammatory cytokines (TGF- β family members) were diminished to below detection level. Changes in mRNA expression in response to latex beads had a markedly reduced magnitude and duration compared with GaAs, and in many cases were not different from the vehicle control, indicating that the effects of particulate GaAs were specific to the nature of the particle and not simply due to phagocytosis. Generally, cytokine mRNA steady-state levels rapidly increased, and then gradually returned to basal levels by 48 h. In contrast, suppression TGF- β transcript expression remained complete at

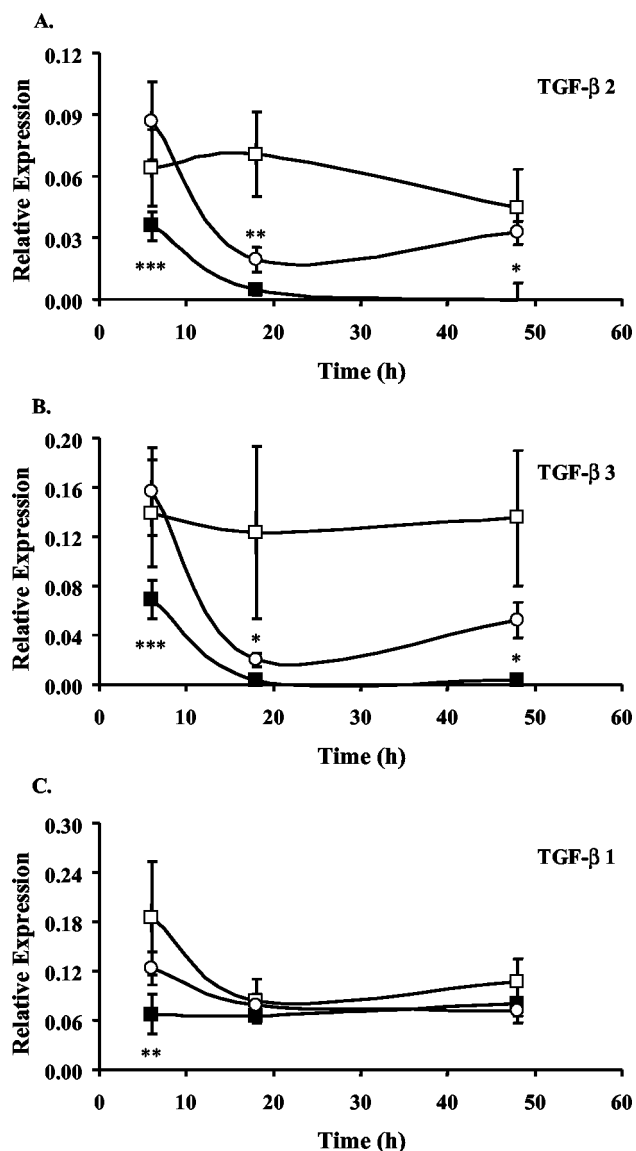


Fig. 6. Anti-inflammatory cytokines of the TGF- β family are decreased after GaAs exposure. RNA was prepared as for Fig. 2 and assayed by RPA for expression of TGF- β 2 (A), TGF- β 3 (B), and TGF- β 1 (C). GaAs exposed (■), latex bead (○), and vehicle (□) mRNA levels are expressed proportional to that of the housekeeping gene GAPDH. Expression levels were assayed in triplicate and are presented as mean \pm S.D. Significance between GaAs- and vehicle-exposed mRNA levels was determined using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

48 h, which could also promote inflammation. Our findings are the first evidence that in vivo GaAs exposure affects cytokine mRNA expression.

For altered steady-state mRNA cytokine levels to initiate an inflammatory response, the gene products must be efficiently translated and secreted at the exposure site. We found that four transcripts, which were examined, were efficiently translated in vivo, yielding increased cytokine protein levels within the peritoneal fluid of GaAs-exposed mice relative to vehicle control. In contrast, splenic cells did not significantly differ in proinflammatory cytokine expression after GaAs exposure, neither at the mRNA nor protein level. Only the chemokine CCL2, whose function is dependent upon dissemination for formation of a chemotactic gradient, was detected in sera. Cytokine expression, as determined by in-

tracellular cytokine staining, was predominantly restricted to Mac-1⁺ cells, although some immunofluorescence staining was detected in B220⁺ cells. Other investigators have reported that B lymphocytes are capable of secreting proinflammatory cytokines, including IL-1 β , IL-18, and TNF- α (Bonney et al., 1989; Nakanishi et al., 2001; Ferreira et al., 2003). Hence, GaAs may induce direct activation of these cells in vivo. Alternatively, cytokine production by peritoneal B cells might be attributed to the secondary activation of these cells by monocyte-derived IL-1 β . Secreted cytokines were mainly detected only within the peritoneal lavage fluid, indicating that the inflammatory response is localized, not systemic. Therefore, we conclude that direct exposure of peritoneal macrophages to GaAs results in production of inflammatory cytokines at the exposure site.

The kinetics pattern of proinflammatory cytokine mRNA expression may indicate that the GaAs-mediated inflammatory reaction has a limited duration. However, GaAs particles persist at the exposure site for extended periods up to 28 days (Webb et al., 1986; Burns et al., 1991). This persistence may trigger a second wave of cytokine production, possibly leading to chronic inflammation. Furthermore, secretion of one cytokine typically causes the subsequent induction of others, which might also contribute to a second round of cytokine production. Thus, analysis of later time points may identify additional cytokines that could influence leukocyte recruitment and activation, and inflammation.

The IL-1 family, including IL-1 α , IL-1 β , and the IL-1 receptor antagonist (IL-1Ra), is implicated in inflammatory reactions in many experimental systems. The massive upregulation of IL-1 β mRNA (>60-fold) and protein (8-fold), which we observed following GaAs exposure, indicates that this cytokine may be a primary driver of the inflammatory response to GaAs. Furthermore, transcripts of five other cytokines that regulate cell migration were expressed at >3-fold higher than the vehicle control (CCL2, CCL3, CCL5, CXCL1, and MIF). The relatively modest increases in the expression of the other proinflammatory cytokines, such as TNF- α and IL-6, suggest these cytokines may play a more limited role. Signaling through the Type I IL-1 receptor activates monocytes and macrophages (Fenton et al., 1987, 1988) and results in the generation of inflammatory lipid mediators, such as prostaglandins or leukotrienes (Gronich et al., 1994). Such a mechanism is consistent with our previous findings of an activated state of GaAs-exposed macrophages (Hartmann and McCoy, 1996; Caffrey-Nolan and McCoy, 1998).

Monocytes are strongly attracted to CCL2 present in inflamed tissues (Kuziel et al., 1997; Siebert et al., 2000). CCL2 was found in sera from GaAs-exposed mice, where it potentially produces a concentration gradient for directed migration of monocytes into peritoneum. After exiting the blood, monocytes mature into macrophages. Macrophages can enhance and sustain inflammation via cytokine secretion (Warren et al., 1988; Bogdan and Nathan, 1993), or differentiate into dendritic cells that migrate to secondary lymphoid organs (Kabel et al., 1989; Zhou and Tedder, 1996). We hypothesize that recruited monocytes, which are evident within the peritoneum at 18 h exposure, phagocytose GaAs particles, become activated, and acquire the macrophage phenotype that is observed 5 days after exposure.

Exposure of macrophages to GaAs in culture stimulates

TABLE 1

Impact of GaAs on expression of other cytokine mRNA in peritoneal leukocytes

RPL were harvested from GaAs-, latex bead-, and vehicle-exposed mice after 6 and 18 h, and total RNA was subjected to RPA. Cytokine mRNA levels are a percentage of GAPDH expression and are the mean \pm S.D. for three experiments. -Fold change is the ratio of cytokine expression in GaAs-exposed cells to that in vehicle controls.

Cytokine	Vehicle-Exposed Cells	Latex Bead-Exposed Cells	GaAs-Exposed Cells	Fold Change (GaAs vs. Vehicle)
6 h				
CCL3	49.0 \pm 12.0	47.1 \pm 6.5	72.0 \pm 12.0*	1.47
IL-6	3.20 \pm 0.50	2.6 \pm 0.8	11.9 \pm 1.60**	3.72
TNF- β	6.00 \pm 0.90	0.9 \pm 0.1	4.4 \pm 0.60*	0.73
18 h				
CCL3	0.10 \pm 0.10	9.8 \pm 5.3	14.9 \pm 2.4***	149.00
CCL4	0.20 \pm 0.10	4.2 \pm 0.8	13.0 \pm 3.3**	65.00
CCL1	0.90 \pm 0.40	4.8 \pm 1.6	5.3 \pm 0.3**	6.19
CCL5	2.9 \pm 1.8	5.6 \pm 0.2	9.6 \pm 0.3***	3.34
IFN- γ	1.4 \pm 0.2	1.0 \pm 0.1	6.4 \pm 1.1***	4.57
IL-6	1.9 \pm 0.2	0.9 \pm 0.2	6.1 \pm 1.0*	3.21
IL-12 p40	1.2 \pm 0.1	0.2 \pm 0.002	3.2 \pm 0.9*	2.67
IL-10	1.4 \pm 0.1	0.2 \pm 0.003	3.6 \pm 0.9**	2.57
IL-12 p35	1.7 \pm 0.1	0.3 \pm 0.003	4.0 \pm 0.8**	2.35

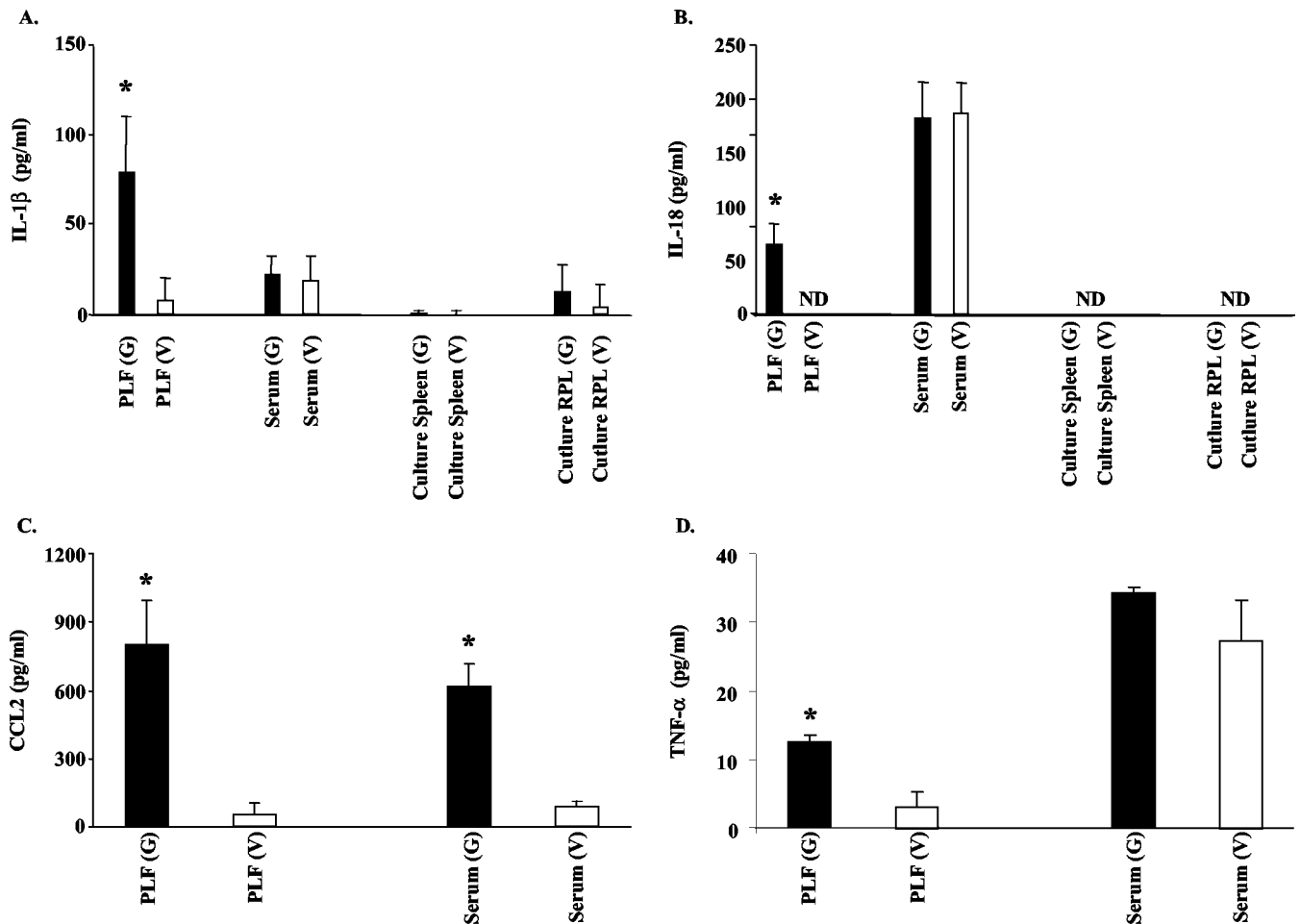
Significance between GaAs- and vehicle-exposed levels was determined by Student's *t* test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 7. Cytokine secretion by RPL after in vivo GaAs exposure. Biological fluid samples were prepared as described under *Materials and Methods* from GaAs (G)- (closed bars) and vehicle (V)- control (open bars) exposed mice 18 h after exposure. Samples were assayed for IL-1 β (A), IL-18 (B), CCL2 (C), and TNF- α (D) by ELISA. Data are mean \pm S.D. of three independent samples except for TNF- α , which is from two samples. ND (not detected) is below the sensitivity of the assay. PLF, peritoneal lavage fluid. *, Statistically different from vehicle control ($p < 0.05$).

enhanced expression of cathepsin L, a thiol protease, and invariant chain, a molecular chaperone (Harrison et al., 2003). However, in vitro exposure does not induce the complete activated macrophage phenotype that occurs after in vivo exposure. In striking contrast to in vivo chemical exposure, in vitro exposure does not stimulate secretion of IL-1 β ,

TNF- α , or IL-12 (Harrison et al., 2003). The differential impact of in vivo and in vitro exposures may be due to differences in the myeloid cells examined. Our in vitro model utilized macrophage cell lines and thioglycollate-elicited macrophages. An alternative is that other leukocytes and/or another organ system may influence cytokine production,

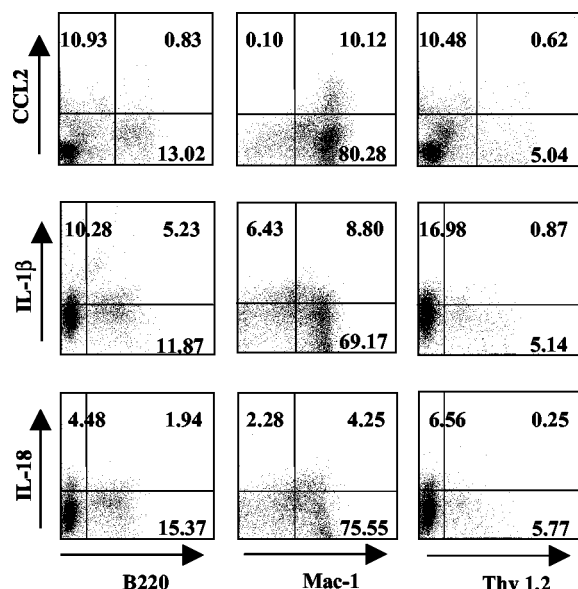


Fig. 8. Intracellular cytokine protein expression in GaAs-exposed RPL. Peritoneal leukocytes from GaAs-exposed mice were isolated after 18 h and stained for lineage-specific cell surface proteins (B220, Mac-1, Thy1.2). Production of CCL2 (top panels), IL-1 β (middle panels), and IL-18 (lower panels) was determined at the single cell level by intracellular cytokine staining and flow cytometry as described under *Materials and Methods*. Values in the upper and lower right quadrants represent the percentage of lineage marker positive cells with or without intracellular cytokine expression, respectively, in the total lymphocyte population. Experiments shown are representative of at least two repetitions.

monocyte migration, and myeloid cell activation during in vivo GaAs exposure.

Other chemotactic factors were up-regulated within the peritoneum following GaAs exposure, including CXCL1 and CCL5, which are chemotactic for neutrophils and T lymphocytes, respectively. However, we did not detect an increased proportion of either cell type 18 h after exposure. Perhaps, neutrophils entered the peritoneum at even earlier time points. In other inflammatory models, neutrophil influx occurs as early as 4 h after treatment (Schramm et al., 2002). Alternatively, neutrophil chemotaxis to CXC chemokines is dependent upon TNF- α (Schramm et al., 2000). Although increased TNF- α levels were present in peritoneal fluid from GaAs-exposed mice, the amount may be insufficient to support neutrophil diapedesis. Although substantial up-regulation of T lymphocyte chemokine CCL5 occurred, we found relatively minor induction of T cell-related cytokines (IL-18, IFN- γ , IL-12), suggesting that the role of T cells is minimal early after chemical exposure. Effector T cells are often found at sites of chronic inflammation, days or weeks after initiation. Hence, T lymphocytes may possibly infiltrate the peritoneum later. Ultimately, the role of T cells in the initiation and maintenance of GaAs-induced inflammation requires further study.

In conclusion, GaAs exposure quickly elevated mRNA expression of multiple proinflammatory cytokines, and production appeared to be mainly local, not systemic. Monocytes were recruited to the site of GaAs-mediated inflammation under the direction of CCL2, and may be activated primarily by IL-1. Recruited monocytes may become further stimulated by other cytokines. Although health risks to industrial workers exposed to GaAs remains unclear, animal studies have

established that chemical exposure causes detrimental biological effects. Intratracheal instillation and inhalation of GaAs results in pulmonary inflammation and edema in rodents (Webb et al., 1986; National Toxicology Program, 2000; Tanaka et al., 2000). Tissue damage is associated with accumulation of neutrophils and macrophages. Hence, the ability of GaAs to up-regulate proinflammatory cytokines may extend to the lungs, which could contribute to pulmonary damage. Identification of the genes whose expression is altered by GaAs exposure will lead to a better comprehension of the inflammatory reaction induced by the chemical.

Acknowledgments

We thank Dr. Kimber White, Jr. for helpful discussions.

References

- Bogdan C and Nathan C (1993) Modulation of macrophage function by transforming growth factor beta, interleukin-4 and interleukin-10. *Ann NY Acad Sci* **685**:713–739.
- Bonnefoy JY, Denoroy MC, Guillot O, Martens CL, and Banchereau J (1989) Activation of normal human B cells through their antigen receptor induces membrane expression of IL-1 alpha and secretion of IL-1 beta. *J Immunol* **143**:864–869.
- Burns LA, Sikorski EE, Saady JJ, and Munson AE (1991) Evidence for arsenic as the immunosuppressive component of gallium arsenide. *Toxicol Appl Pharmacol* **110**:157–169.
- Caffrey-Nolan RE and McCoy KL (1998) Direct exposure to gallium arsenide up-regulates costimulatory activity of murine macrophages. *Toxicol Appl Pharmacol* **151**:330–339.
- Fenton MJ, Clark BD, Collins KL, Webb AC, Rich A, and Auron PE (1987) Transcriptional regulation of the human prointerleukin 1 beta gene. *J Immunol* **138**:3972–3979.
- Fenton MJ, Vermeulen MW, Clark BD, Webb AC, and Auron PE (1988) Human pro-IL-1 beta gene expression in monocytic cells is regulated by two distinct pathways. *J Immunol* **140**:2267–2273.
- Ferreira KS, Almeida SR, Ribeiro CH, Mariano M, and Lopes JD (2003) Modulation of proliferation, differentiation and cytokine secretion of murine B-1 B cells by proteins of the extracellular matrix. *Immunol Lett* **86**:15–21.
- Gronich J, Konieczkowski M, Gelb MH, Nemenoff RA, and Sedor JR (1994) Interleukin 1 α causes rapid activation of cytosolic phospholipase A₂ by phosphorylation in rat mesangial cells. *J Clin Invest* **93**:1224–1233.
- Harrison MT, Hartmann CB, and McCoy KL (2003) Impact of in vitro gallium arsenide exposure on macrophages. *Toxicol Appl Pharmacol* **186**:18–27.
- Harrison MT and McCoy KL (2001) Immunosuppression by arsenic: a comparison of cathepsin L inhibition and apoptosis. *Int Immunopharmacol* **1**:647–656.
- Harrison RJ (1986) Gallium arsenide: state of the art reviews. *Occup Med* **1**:49–58.
- Hartmann CB and McCoy KL (1996) Gallium arsenide augments antigen processing by peritoneal macrophages for CD4⁺ helper T cell stimulation. *Toxicol Appl Pharmacol* **141**:365–372.
- Kabel PJ, de Haan-Meulman M, Voorbij HA, Kleingeld M, Knol EF, and Drexhage HA (1989) Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. *Immunobiology* **179**:395–441.
- Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, and Maeda N (1997) Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc Natl Acad Sci USA* **94**:12053–12058.
- Lewis TA, Hartmann CB, and McCoy KL (1998a) Gallium arsenide differentially affects processing of phagolysosomal targeted antigen by macrophages. *J Leukoc Biol* **63**:321–330.
- Lewis TA, Hartmann CB, and McCoy KL (1998b) Gallium arsenide modulates proteolytic cathepsin activities and antigen processing by macrophages. *J Immunol* **161**:2151–2157.
- Lewis TA, Munson AE, and McCoy KL (1996) Gallium arsenide selectively suppresses antigen processing by splenic macrophages for CD4⁺ T cell activation. *J Pharmacol Exp Ther* **278**:1244–1251.
- Luster MI, Portier C, Pait DG, White KL, Jennings C, Munson AE, and Rosenthal GJ (1992) Risk assessment in immunotoxicity I. Sensitivity and predictability of immune tests. *Fundam Appl Toxicol* **18**:200–210.
- Nakanishi K, Yoshimoto T, Tsutsui H, and Okamura H (2001) Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* **19**:423–474.
- National Toxicology Program (2000) NTP technical report on the toxicology and carcinogenesis studies of gallium arsenide (CAS No. 1303-00-0) in F344/N rats and B6C3F1 mice (inhalation studies). NTP-TR492/National Institutes of Health Publication No. 00-3951, NIH, Bethesda, MD.
- Schramm R, Liu Q, and Thorlacius H (2000) Expression and function of MIP-2 are reduced by dexamethasone treatment in vivo. *Br J Pharmacol* **131**:328–334.
- Schramm R, Schaefer T, Menger MD, and Thorlacius H (2002) Acute mast cell-dependent neutrophil recruitment in the skin is mediated by KC and LFA-1: inhibitory mechanisms of dexamethasone. *J Leukoc Biol* **72**:1122–1132.
- Siebert H, Sachse A, Kuziel WA, Maeda N, and Bruck W (2000) The chemokine receptor CCR2 is involved in macrophage recruitment to the injured peripheral nervous system. *J Neuroimmunol* **110**:177–185.
- Sikorski EE, Burns LA, McCoy KL, Stern ML, and Munson AE (1991a) Suppression of splenic accessory cell function in mice exposed to gallium arsenide. *Toxicol Appl Pharmacol* **110**:143–156.

- Sikorski EE, Burns LA, Stern ML, Luster MI, and Munson AE (1991b) Splenic cell targets in gallium arsenide-induced suppression of the primary antibody response. *Toxicol Appl Pharmacol* **110**:129–142.
- Sikorski EE, McCay JA, White KL, Bradley SG, and Munson AE (1989) Immunotoxicity of the semiconductor gallium arsenide in female B6C3F1 mice. *Fundam Appl Toxicol* **13**:843–858.
- Tanaka A, Hirata M, Omura M, Zhao M, Makita Y, Yamazaki K, Inoue N, Gotoh K (2000) Comparative study of the toxic effects of gallium arsenide, indium arsenide and arsenic trioxide following intratracheal instillations to the lung of Syrian golden hamsters. *Fukuoka Igaku Zasshi* **91**:21–33.
- Warren JS, Kunkel SL, Cunningham TW, Johnson KJ, and Ward PA (1988) Macrophage-derived cytokines amplify immune complex-triggered O₂-responses by rat alveolar macrophages. *Am J Pathol* **130**:489–495.
- Webb DR, Wilson SE, and Carter DE (1986) Comparative pulmonary toxicity of gallium arsenide, gallium (III) oxide, or arsenic (III) oxide intratracheally instilled into rats. *Toxicol Appl Pharmacol* **82**:405–416.
- World Health Organization (1992) Inorganic arsenic compounds other than arsine. WHO Health and Safety Guide No. 70, Geneva.
- Zhou LJ and Tedder TF (1996) CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc Natl Acad Sci USA* **93**:2588–2592.

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