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***In vitro* Atrazine Exposure Affects the Phenotypic and Functional Maturation of Dendritic Cells**

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Abstract

Recent data suggest that some of the immunotoxic effects of the herbicide atrazine, a very widely used pesticide, may be due to perturbations in dendritic cell (DC) function. As consequences of atrazine exposure on the phenotypic and functional maturation of DC have not been studied, our objective was, using the murine DC line, JAWSII, to determine whether atrazine will interfere with DC maturation. First, we characterized the maturation of JAWSII cells *in vitro* by inducing them to mature in the presence of growth factors and selected maturational stimuli *in vitro*. Next, we exposed the DC cell line to a concentration-range of atrazine and examined its effects on phenotypic and functional maturation of DC. Atrazine exposure interfered with the phenotypic and functional maturation of DC at non-cytotoxic concentrations. Among the phenotypic changes caused by atrazine exposure was a dose-dependent removal of surface MHC-I with a significant decrease being observed at 1 μ M concentration. In addition, atrazine exposure decreased the expression of the costimulatory molecule CD86 and it downregulated the expression of the CD11b and CD11c accessory molecules and the myeloid developmental marker CD14. When, for comparative purposes, we exposed primary thymic DC to atrazine, MHC-I and CD11c expression was also decreased. Phenotypic changes in JAWSII DC maturation were associated with functional inhibition of maturation as, albeit at higher concentrations, receptor-mediated antigen uptake was increased by atrazine. Thus, our data suggest that atrazine directly targets DC maturation and that toxicants such as atrazine that efficiently remove MHC-I molecules from the DC surface are likely to contribute to immune evasion.

Keywords

atrazine; pesticide immunotoxicity; dendritic cells; maturation; MHC-I

Introduction

Owing to its ability to control broadleaf weeds, atrazine [(2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine)], a broad-spectrum chloro-s-triazine herbicide, is one of the most widely used pesticides in the U.S. (Gianessi 2000, EPA 2002). Atrazine is the most frequently detected pesticide in ground and surface waters where it tends to persist for months (Koskinen

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and Clay 1997, Dorfler *et al.* 1997), particularly in waters with neutral or slightly basic pH (Cohen 1984). Although levels of atrazine in the water are typically in the low ppb range, they can reach several hundred $\mu\text{g/L}$ (Koskinen and Clay 1997, Dorfler *et al.* 1997). Besides the possibility of chronic, low-level exposure to atrazine in individuals consuming atrazine-containing well water, farmers, pesticide applicators, and other individuals in the vicinity of ongoing pesticide application, including children, may also be intermittently exposed to much higher levels of atrazine during application. Between 1993 and 1997, 14% of the farmers and commercial applicators in North Carolina and Iowa enrolled in the Agricultural Health Study (AHS) experienced unusually high pesticide exposure, with the percentage among the commercial applicators in Iowa being even higher (22%). Atrazine was one of only five pesticides that accounted for the majority of high pesticide exposure events (Storm *et al.* 2004).

Because of the atrazine's widespread use and the increased potential of exposure to it either chronically (water), or intermittently (during application), scientific interest pertaining to its toxic potential has expanded. Series of studies, including ours, have investigated effects of exposure to this herbicide on the nervous, endocrine, and reproductive systems (i.e., Cooper *et al.* 2000, Narotsky *et al.* 2001, Stoker *et al.* 2002, Stoker *et al.* 2000, Rodriguez *et al.* 2005, Filipov *et al.* 2007, Coban and Filipov 2007).

Importantly, several studies have investigated effects of exposure to this pesticide on the immune system. Thus, in a study mandated by the National Toxicology Program (NTP), 14-day oral gavage exposure to atrazine of adult female B6C3F₁ mice decreased spleen cell numbers and spleen and thymus weights (more sensitive than the spleen). Atrazine exposure did not affect the humoral immune response to a T cell dependent antigen, the cytotoxic T lymphocyte response, NK cell activity, or the proliferative response to the mitogens Con A and LPS. However, exposure to this herbicide resulted in a dose-dependent decrease in host resistance in the B16F10 melanoma challenge test (NTP 1994). Similar findings, i.e., reduced thymus and spleen weights, spleen cellularity, fixed macrophage function, as well as the host resistance to B16F10 melanoma, with the host resistance being compromised at levels of exposure (25 mg/kg) where no gross pathological effects of atrazine were observed, were reported in a different recent study (Karrow *et al.* 2005). Along the same lines, a single dose of atrazine (100 to 500 mg/kg) decreased thymic and splenic cellularity, affected lymphocyte subpopulations in the thymus and spleen, as well as the antibody responses to keyhole limpet hemocyanin (KLH) and NK cell activity (Pruett *et al.* 2003). Atrazine's immunotoxicity was also evaluated in developmental exposure paradigms and, interestingly, atrazine decreased the primary antibody and DTH responses only in the male offspring of Sprague-Dawley rats (Rooney *et al.* 2003), whereas developmental exposure of BALB/c mice (a Th2-skewed strain) to atrazine resulted in a significant increase in the number of antigen-specific IgM secreting B cells in the spleen of adult male offspring, a finding suggestive of possible Th1/Th2-specific effect of developmental atrazine exposure on dendritic cells (DC; Rowe *et al.* 2006).

Few *in vitro* studies have also assessed the immunotoxic potential of atrazine. From these studies, it appears that PHA-stimulated T cell proliferation is compromised by atrazine exposure, but only at relatively high concentrations (Pistl *et al.* 2003). On the other hand, much lower concentrations of atrazine (0.03–3 μM) substantially decreased the production of IFN- γ , IL-5, and TNF- α by human PBMC (Hooghe *et al.* 2000), a heterogeneous cell population that contains DC and their myeloid progenitors, monocytes.

During the last decade, DC, a family of bone marrow (BM)-derived antigen-presenting cells (APC) have come to be appreciated as critical controllers of the immune response. The unique capacity of DC to sample sites of pathogen entry, respond to microbial and viral signals, and activate naïve T and B cells suggests a critical role for these cells in initiating antimicrobial

and antiviral immunity (Steinman 1991, Banchereau and Steinman 1998, Palucka and Banchereau 1999). Extensive work in rodents and humans has demonstrated that the potent accessory properties of DC depend on a process of maturation (Larsen *et al.* 1990, Winzler *et al.* 1997). Immature DC efficiently process native antigens, but are relatively ineffective in activating naïve T cells (Cella *et al.* 1997a). In contrast, mature DC lose their capacity to efficiently process antigens, but have increased ability to activate T cells upon the first encounter with the antigen (Winzler *et al.* 1997, Cella *et al.* 1997b). In particular, DC can be triggered by proinflammatory stimuli such as TNF- α , IL-1, toll-like receptor (TLR) agonists-double stranded RNA, Poly I:C (TLR3) and LPS (TLR4) to mature and to upregulate adhesion and costimulatory molecules (CD40, CD80, CD86; De Smedt *et al.* 1996, Roake *et al.* 1995, Cella *et al.* 1997a, Cella *et al.* 1997b, Tsujimoto *et al.* 2006).

Although earlier data suggested that migration of immature DC from the periphery to the T cell areas of the secondary lymphoid organs only occurred in response to an antigen, more recent works indicate that migration of DC that contain apoptotic bodies occurs constitutively in the apparent absence of microbial stimulation and may contribute to the peripheral tolerance mechanisms (Huang *et al.* 2000, Steinman *et al.* 2003, Reddy *et al.* 2002). Moreover, in addition to immature “tolerogenic” DC, mature but resting DC can also induce tolerance *in vivo* (Albert *et al.* 2001) or deletion of antigen-specific CD4⁺ T cells *in vitro* (Finkelman *et al.* 1996, Hawiger *et al.* 2001, Probst *et al.* 2003). These data strongly imply that DC, depending on both their maturation status as well as their effector properties, induce immunity or tolerance through a process requiring a concerted action of multiple subsets of DC (Pulendran 2004, Pulendran 2005). Recent evidence also suggests that multiple subpopulations of DC differ in their phenotype, microenvironmental localization, migration potential, PRR expression, responsiveness to microbes, and finally their effector capacity to promote Th1, Th2 or regulatory type of T cell development (Pulendran 2004, Pulendran 2005, Reis e Sousa 2006). Therefore, it is important to define DC not only in terms of their maturation phenotypic properties but also in terms of their ontogeny and their effector properties (Reis e Sousa, 2006).

However, the difficulties in preparing DC in sufficient numbers in a reasonably pure form and the short life-span of DC in culture have greatly hindered the progress of knowledge of DC biology. Therefore, the establishment of DC lines has facilitated the generation of large numbers of DC in different stages of differentiation. In general, two types of DC lines have been generated: (i) immortalized DC lines which do not require continuous stimulation with growth factors for their propagation, and (ii) growth factor-dependent DC lines (Paglia *et al.* 1993, Lutz *et al.* 1999). An important limitation of most immortalized DC lines is that they retain an immature phenotype and cannot be stimulated to acquire a fully mature status. In contrast, growth factor-dependent DC lines can be more easily induced to mature *in vitro* and thus can more closely mimic the *in vivo* behavior of DC (Girolomoni *et al.* 1995).

In a recent *in vivo* study we demonstrated that atrazine exposure was detrimental to the immune system of juvenile mice by decreasing cellularity and affecting lymphocyte distribution, with certain effects persisting long after exposure has been terminated (Filipov *et al.* 2005). Interestingly, the proportion of mature splenic DC (CD11c^{high}) APC was also decreased; this decrease persisted for at least one week, suggesting that atrazine inhibited DC maturation (Filipov *et al.* 2005). These data, together with the fact that host resistance to a tumor challenge was compromised in atrazine-exposed adult mice (NTP 1994) and that the compromised host resistance to a tumor challenge was observed at doses of atrazine which did not decrease thymic/splenic weight and cellularity (Karrow *et al.* 2005), suggest that DC might be atrazine targets. In this regard, it is well established that DC are highly effective in generating anti-tumor immune responses (Avigan 2004). Data from the AHS also hint towards possible effects of atrazine exposure on DC. Thus, of about 40 different pesticides, atrazine was one of only

seven that were associated with wheeze in the AHS farmers (Hoppin *et al.* 2002, Storm *et al.* 2004). In this regard, it is well known that DC control pulmonary immune responses and antigen challenge is associated with a shift to a less mature phenotype (Bratke *et al.* 2007).

Considering the evidence hinting towards a possible effect of atrazine on DC and taking advantage of the utility and availability of a DC line, our primary objective in this study was to investigate the effects of *in vitro* atrazine exposure on the phenotypic and functional maturation of a growth factor-dependent, bone marrow-derived immature murine DC cell line, JAWSII. For comparative purposes, we also performed limited studies with primary thymic DC.

Materials and Methods

Dendritic Cell Line

JAWSII mouse DC line was obtained from the American Type Culture Collection (ATCC) and cultured according to the manufacturer's instructions. Briefly, cells were cultured in the Complete Growth Medium containing Minimum Essential Medium (MEM) alpha with ribonucleosides, deoxyribonucleosides, 4 mM L-glutamine and 1 mM sodium pyruvate (Gibco) supplemented with 20% fetal bovine serum (ATCC) and 5ng/ml murine recombinant GM-CSF (R&D Systems, Inc.). Culture medium was renewed once per week by replacing half of the medium. Prior to use, cell viability was assessed by using trypan blue and light microscopy.

Primary Thymic DC Preparation

Pan-DC populations from the male C57BL/6 mouse thymuses (8–12 week-old) consisting of CD11c+ and plasmacytoid DC (pDC) were positively selected by using PanDC microbeads conjugated to monoclonal anti-mouse CD11c (N418) and anti-mouse PDCA-1 antibodies, MACS separation columns and magnets (Miltenyi Biotech, Auburn, CA). Mice were handled according to Mississippi State University IACUC Guidelines. Magnetic cell separation technique was performed according to the manufacturer's instructions (Miltenyi Biotech). Briefly, isolated organs were placed in a Petri-dish with sufficient Collagenase D solution to completely cover the bottom of the dish. Mouse spleens and thymuses were injected with 500 µl of Collagenase D solution per organ followed by cutting the tissue into smaller pieces and 30 min incubation in Collagenase D solution at 37°C. The whole material including remaining fragments and Collagenase D-released cells were passed gently through cell strainers with plungers. Single cell suspensions were labeled with PanDC microbeads and positively selected by using magnetic separation with MS Columns.

Reagents

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; 98% purity; Lot 301-49A) was obtained from ChemServices (West Chester, PA). To promote DC line maturation LPS (*Escherichia coli* 0111: B4; Sigma, St. Louis, MO), GpG (Peptidoglycan from *Staphylococcus aureus*, Batch 77140, BioChemica, WA), CpG (Mouse CpG DNA, Batch HC4033-3422R16, HyCult Biotechnologies) and Poly I:C (Polyinosinic-polycytidinic acid sodium salt, Batch P0913, Sigma) were used.

Antibodies and Flow Cytometry

Fluorescein-conjugated mAbs to MHC class I (KH95), MHC class II (28-16-85), CD11b (M1/70), CD11c (HL3), CD14 (rmC5-3), and phycoerythrin-conjugated mAbs to CD8 (53-6.7), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), as well as isotype-matched controls were used. All conjugated mAbs were purchased from PharMingen/BD Biosciences (San

Diego, CA). Isotype-matched controls were purchased from ID Labs (Ontario, Canada). Fluorescein conjugated dextran (FITC-DX, $M_r = 68,100$, Sigma) and Lucifer Yellow CH (LY, Molecular Probes) were used for the endocytosis assay. Immunofluorescent staining and active endocytosis were analyzed using Cell Quest Version 3.3 Software (Becton Dickinson) and a FACS Calibur Flow Cytometer (Becton Dickinson). Cell staining and endocytosis were analyzed by measuring Mean Fluorescence Intensity (MFI) with Single Histogram Statistics.

Endocytosis Assay

The ability of cells to endocytose FITC-DX (a method used to determine a highly-selective, receptor-mediated antigen uptake mechanism) and Lucifer Yellow (LY; a method used to determine a non-selective, mainly macropinocytotic antigen uptake mechanism) was measured as described elsewhere (Sallusto *et al.* 1995, Pinchuk *et al.* 2003, Boyd *et al.* 2004). Briefly, cells were washed 3 times at 22° C and treated with FITC-DX (1 mg/ml) or LY (100 µg/ml) for 30 min at 37° C to measure active endocytosis and at 4° C to determine background levels of endocytosis (negative control). Cells were washed 4 times at 4° C by centrifugation (5 min; 300 × g) in cold PBS and analyzed using a FACS Calibur as described above.

Apoptosis Assay

Apoptosis in the JAWSII cell line was assessed by using Annexin-V Apoptosis Detection Kit following the manufacturer's instructions (BioVision, Inc., Mountain View, CA). Briefly, after respective exposures, cells (0.5×10^6 /ml) were incubated with Annexin-V-FITC and propidium iodide (PI) for 5 min at 22° C in the dark and analyzed by Flow Cytometry using two-color analysis with Dot Plot Quadrant Statistics. Staurosporine (10 µM, Sigma)-treated JAWII cells were used as positive control for apoptosis.

PCR Analysis

Total cellular RNA was extracted from JAWSII cells using the RNeasy Protect Mini Kit (Qiagen, Inc., Valencia, CA) and quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). PCR analysis was performed by using the OneStep RT-PCR System (Qiagen) and 1.2% agarose gels. PCR primers amplified the mRNA of mouse MHC class II H2-IA-alpha gene (b haplotype). Primer sequences were as follows: forward primer for MHC class II H2-IA-alpha, 5' - AAT AGC AAG TCA GTC GCA GAC GGT - 3'; reverse primer for MHC class II H2-IA-alpha, 5' - ATT CCA AGG GTG TGT GAG CTG TGA - 3'. β -actin mRNA was used as an internal standard (Takahashi *et al.*, 2003). The PCR amplification consisted of 40 cycles of 94 °C for 30 sec, 58 °C for 1 min and 72 °C for 1 min.

Real-time PCR Analysis

Total RNA was isolated from JAWSII in *RNA later* using the RNeasy Protect Mini Kit (Qiagen) and quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). Expression of mRNA was determined by real-time PCR using mouse specific primers and probes for β -2 microglobulin (MHC-I) and integrin- α M (CD11b). Primer and probe sequences were as follows: forward primer for β -2 microglobulin, 5' - ACC GGC CTG TAT GCT ATC CAG AAA - 3'; reverse primer for β -2 microglobulin, 5' - ATT TCA ATG TGA GGC GGG TGG AAC - 3'; TaqMan probe for β -2 microglobulin, 5' - TGG GAA GCC GAA CAT ACT GAA CTG CT - 3'; forward primer for integrin- α M (CD11b), 5' - ATC CTG TAC CAC TCA TTG TGG GCA - 3'; reverse primer for integrin- α M (CD11b), 5' - TCA TCA TGT CCT TGT ACT GCC GCT - 3'; TaqMan probe for integrin- α M (CD11b), 5' - ACT GCT GGC CTA TAC AAG CTT GGC TT - 3'. The quantification of each gene was normalized for differences in amount of total RNA in the reaction using the β -actin mRNA as an internal standard. The probe and primers for the β -actin gene were described by Takahashi *et al.* (2003). Reaction mixtures

were assembled in optical 96 well plates using the SuperScript™ III Platinum® One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). Amplifications were performed in an iCycler iQ (Bio-Rad) programmed for an initial step of 30 min at 50°C and 10 min at 95°C, followed by 40 or 50 cycles at 95°C for 15s and 1min at 60°C. Measurements were performed in duplicates and the average used for calculations. Relative quantification of gene expression was determined by the standard curve method (ABI PRISM 7700 sequence detection system, user bulletin #2).

Statistical Analysis

Data (3–4 independent experiments) were analyzed by analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison *post hoc* test and are presented as means $\pm$ SEM. The level of significance for all tests of effects was set at $P < 0.05$.

Results

Phenotypic and functional characterization of JAWSII mouse DC line

An extensive typically irreversible differentiation of DC in response to variable stimuli is called maturation, and the maturation stimulus influences the type of ensuing immune response (Pulendran *et al.* 2001, Pasare and Medzhitov 2004, Pulendran 2005). To test the possibility of using BM-derived JAWSII mouse DC line as a model system for studying the effects of atrazine on DC maturation *in vitro*, we investigated some phenotypic and functional properties of DC, including the surface expression of DC-specific markers and the ability of JAWSII DC to take up soluble antigens.

To assess the phenotypic changes in BM-derived DC in the presence of two potent maturational stimuli, LPS (a natural TLR4 agonist) and Poly I:C (a synthetic double stranded RNA-like TLR3 agonist), cells were stained with mAbs to key DC maturational markers, including costimulatory and accessory molecules (Figure 1A). After 24 h LPS or Poly I:C exposure, JAWSII cells significantly increased their surface levels of CD80 and CD86 costimulatory molecules suggesting the DC line was triggered by LPS/Poly I:C to undergo the maturation process (Figure 1A). In addition, surface expression of two myeloid-specific DC markers, CD11b and CD14, were significantly upregulated after exposure to LPS and Poly I:C *in vitro*, while the CD11c marker was downregulated by LPS but not by Poly I:C (Figure 1A). At the same time, LPS and Poly I:C exposure promoted moderate but significant decreases in the expression of MHC class I molecules (Figure 1A) and did not affect the expression of CD40 surface protein in mouse DC (data not shown). Importantly, surface MHC class II molecules were expressed at background levels in the DC line, and both maturational inducers triggered moderate but significant increases in their surface expression after 24 h exposure (Figure 1A). In order to determine the specific MHC class II mRNA expression in JAWSII DC, we used the OneStep semi-quantitative RT-PCR system and primers to amplify the mRNA of the mouse MHC class II H2-IA-alpha gene. As we expected, MHC class II transcripts were evident in the BM-derived DC line (Figure 1B).

To investigate the functional status of mouse DC exposed to maturational stimuli we examined selective and non-selective mechanisms of antigen uptake in JAWSII cells (Figure 2). Our data demonstrate that a substantial population of DC accumulated FITC-DX and LY when incubated at 37° C (Figure 2). LPS exposure significantly decreased both major mechanisms of antigen uptake in the DC line: a highly selective mechanism measured by FITC-DX uptake (Figure 2A) and a potent non-selective mechanism measured by LY uptake (Figure 2B). In addition to LPS and Poly I:C, we investigated the effect of other TLR agonists, GpG (Peptidoglycan from *Staphylococcus aureus*) and mouse CpG DNA, both known for their DC stimulatory effects on the phenotypic and functional maturation of mouse DC. GpG stimulation promoted morphological and functional changes in JAWSII cells that were similar to the effects

of LPS and Poly I:C (data not shown). In contrast to GpG, CpG DNA did not modulate any changes in phenotypes or active antigen uptake in mouse DC (data not shown).

Taken together, the morphological and functional data presented above suggest that JAWSII cells are immature DC that undergo maturation in the presence of LPS, Poly I:C, and GpG. Therefore, JAWSII cells can be used as an experimental model to study the effects of atrazine on DC maturation.

Cytotoxic effects of *in vitro* atrazine exposure on JAWSII DC

Our previous data indicate that atrazine exposure appears to be detrimental to the immune system of juvenile mice by decreasing cellularity and affecting lymphocyte distribution, with certain effects persisting long after exposure has been terminated (Filipov *et al.* 2005). Importantly, the proportion of mature dendritic cells (CD11c^{high}) was also decreased and it persisted for at least one week, suggesting that atrazine inhibited DC maturation. To investigate the possible direct cytotoxic effects of atrazine on the BM-derived immature DC line, JAWSII, we used several approaches. DC viability studies with trypan blue indicated that atrazine, at concentrations of up to 100 μ M was not cytotoxic to JAWSII DC (Figure 3A). Similar findings were obtained when cells were stained with propidium iodide (PI; data not shown). Finally, we performed an assessment of apoptosis in JAWSII DC line after *in vitro* exposure to atrazine using Annexin-V Apoptosis Detection Kit (Figure 3B). Our results indicate that atrazine, at concentrations of up to 200 μ M, did not induce significant increase in the number of JAWSII cells undergoing apoptosis, whereas the highest (300 μ M) concentration of atrazine increased modestly, but significantly, the percentage of Annexin-V positive cells (Figure 3B). The incubation of JAWSII DC with staurosporine (10 μ M; positive control) for up to 6 h revealed significant time-dependent increases in the number of apoptotic cells such that at 1 h post staurosporine treatment, the percentage of apoptotic cells increased more than 2.5-fold; at 6 h post staurosporine, close to 50% of the cells were apoptotic (data not shown).

Effect of *in vitro* atrazine exposure on the surface expression of MHC class I molecules on JAWSII DC

MHC class I molecules, expressed on the surface of professional APC, are essential for cell-cell interactions and antigen presentation (Inaba *et al.* 1997). Toxicants that efficiently remove MHC class I molecules from the surface of DC are very likely to contribute to immune evasion. Therefore, we examined the effect of *in vitro* atrazine exposure on MHC class I surface protein expression in DC (Figure 4A). When JAWSII cells were exposed for 24 h to atrazine, there was a dose-dependent down-regulation in the expression of the surface MHC-I molecules in the vast majority of cells (Figure 4, left panel) as significant decrease was observed at concentrations as low as 1 μ M (Figure 5A). To further investigate the effect of atrazine on the expression of MHC class I specific mRNA, we applied real time PCR with mouse specific primers and probe for the MHC-I invariant chain, β -2 microglobulin (Figure 5B). Atrazine exposure did not have any significant effects on the MHC class I specific mRNA expression in JAWSII cells (Figure 5B). However, we did detect a numerical, but non-significant increase in the MHC-I mRNA levels in DC treated with LPS (Figure 5B).

Effects of *in vitro* atrazine exposure on expression of accessory and costimulatory molecules on JAWSII DC

Integrin CD11b is an important adhesion molecule predominantly expressed on myeloid DC that plays a significant role in the stabilization of DC-specific naïve T cell complexes (Banchereau and Steinman 1998). Twenty-four h *in vitro* atrazine exposure induced a dose-dependent inhibition in the expression of the surface CD11b molecules in the whole cell population as significant decrease was observed at concentrations as low as 1 μ M (Figure 6A). Atrazine did not have any significant effects on the CD11b specific mRNA expression in

JAWSII cells at concentrations of up to 10 μM . However, at the 100 μM atrazine dose, the levels of CD11b mRNA were significantly enhanced (Figure 6B). We also detected markedly increased mRNA levels of CD11b in JAWSII cells treated with LPS (Figure 5B) and Poly I:C (data not shown).

The expression of CD11c, an important mouse maturation DC-specific marker, the expression levels of which reflect the maturation status of DC (immature DC expressing the lowest levels of CD11c and mature DC expressing the highest), was also decreased by 24-h atrazine exposure (Figure 6C). Significant decreases in the CD11c expression were observed at 10 and 100 μM concentrations of atrazine (Figure 6C). Unlike the decreases in the expression of CD11b, the CD11c decrease was limited to a rather small cell population within JAWSII cells (Figure 4, middle panel). Furthermore, atrazine exposure at concentrations 10 μM and higher significantly decreased the expression of the myeloid DC marker CD14 (Table 1). Finally, we investigated the effects of atrazine exposure on the surface expression of costimulatory molecules in JAWSII DC. Short-term atrazine exposure decreased the levels of CD86 in the whole population (Figure 4, right panel) at high concentrations (100 and 300 μM) and did not change the expression of CD80 (Table 1).

In vitro atrazine exposure enhances selective and non-selective mechanisms of antigen uptake in JAWSII DC

To further investigate the effects of atrazine on the functional maturation of murine DC line, we analyzed receptor-mediated endocytosis and macropinocytosis in JAWSII cells by flow cytometry. Incubation with atrazine for 24 h resulted in a significant increase of JAWSII DC antigen uptake ability mediated by selective and non-selective mechanisms. Both receptor-mediated endocytosis (Figure 7A) and macropinocytosis (Figure 7B) were increased by atrazine exposure, with the effect being statistically significant only for FITC-DX uptake, further indicating that atrazine interferes with DC maturation by promoting the features of immature DC. In particular, FITC-DX uptake in mouse DC showed numerical but non-significant increases at doses 1–10 μM , was significantly enhanced at doses 50–200 μM , followed by the decrease at concentration 300 μM (Figure 7A). Atrazine exposure also induced slight numerical, non-significant increases in macropinocytosis of JAWSII cells (Figure 7B).

Effects of in vitro atrazine exposure on expression of MHC class I and CD11c molecules on primary thymic DC

To determine whether the effects of atrazine exposure on JAWSII DC will also be observed in primary DC, we performed experiments with thymic DC isolated from C57BL/6 mice. Similar to the decreases in the expression of MHC class I in JAWSII cells, atrazine exposure also decreased the surface expression of this molecule on thymic DC (Figure 8A). The other subset of thymic DC that was affected by atrazine exposure were the CD11c⁺/CD8⁺ cells and they were decreased (Figure 8B). One μM was the lowest concentration of atrazine that caused the decreases of these two surface molecules on thymic DC.

Discussion

We previously observed that *in vivo* atrazine exposure decreased the proportion of CD11c^{high} cells (a mature DC population) in the spleen, which suggested atrazine inhibited DC maturation (Filipov *et al.* 2005). This finding, together with other evidence of possible effects of atrazine on DC (NTP 1994, Karrow *et al.* 2005) and the lack of data pertaining to direct effects of atrazine exposure on DC maturation, prompted us to undertake the current study.

First, we assessed the feasibility of using a mouse growth factor-dependent BM-derived DC line, JAWSII, as a model system to investigate the effects of toxicant exposure on the phenotypic and functional maturation of DC. Multiple reports suggest that the most studied and best characterized pathogen-associated molecular pattern, LPS, and synthetic TLR agonists promote DC maturation, which is an extensive irreversible differentiation of DC that results in the enhanced antigen presentation, costimulatory molecule expression, and decreased antigen uptake by DC (Pulendran *et al.* 2001, Pasare and Medzhitov 2004, Pulendran 2005, Whitmore *et al.* 2004). Based on phenotypic and functional changes in the presence of potent maturational stimuli (LPS, Poly I:C, and GpG), i.e., enhanced expression of costimulatory and MHC class II molecules combined with decreased uptake of FITC-DX and LY, we conclude that JAWSII cells are immature DC that undergo an extensive maturation process in the presence of LPS and synthetic TLR agonists. Significant upregulation of two myeloid markers, CD11b and CD14, in the presence of several maturational agents, in the presence of LPS demonstrate the myeloid origin for the JAWSII DC line (Rissoan *et al.* 1999).

Most studies on enhanced antigen presentation during DC maturation indicate that LPS and several other TLR agonists increase the surface expression of MHC class II molecules (De Smedt *et al.* 1996, Roake *et al.* 1995, Cella *et al.* 1997a, Cella *et al.* 1997b, Tsujimoto *et al.*, 2006). In our study, exposure to LPS, Poly I:C, and GpG for 24 h promoted moderate but significant increases in the expression of MHC class II molecules in mouse GM-CSF-dependent immature DC line, JAWSII. Further, moderate but significant decreases in MHC class I molecule expression was also observed. Although growth factor-dependent DC lines or *in vitro* generated monocyte- or bone marrow-derived DC can be induced to mature and thus closely mimic the *in vivo* behavior of DC, there will always be some differences between *in vitro* stimulated DC and DC that were exposed to maturation stimuli *in vivo* (Giolomoni *et al.* 1995). In our study, these differences mostly include low surface expression levels of MHC class II proteins even after stimulation with LPS and TLR-agonists, although the MHC class II-specific mRNA is expressed at high levels in stimulated and non-stimulated JAWSII DC (Table 2). In addition, the levels of surface expression of several other markers also suggest incomplete maturation of the JAWSII cell line in the presence of a maturational stimulus, such as LPS (Table 2). Therefore, based on the prototypical phenotypic and functional changes that result from the presence of potent maturational stimuli, JAWSII DC can be used as an experimental model to study the *in vitro* effects of atrazine and other immunotoxicants on the DC maturation process. There are ongoing efforts to improve and standardize primary DC cultures for immunotoxicity research, including human DC (Hymery *et al.* 2006a). Primary DC cultures have been used successfully for the assessment of the effects of trichothecenes (Hymery *et al.* 2006b) and PAHs (Laupeze *et al.* 2002) on DC. However, the distinct advantages of using JAWSII DC over primary DC include homogeneity, availability, ease of handling, and the ability for long-term culture.

Our previous observation that the proportion of mature CD11c^{high} DC was decreased after *in vivo* atrazine exposure suggested that atrazine inhibited DC maturation (Filipov *et al.* 2005). In this study, we demonstrate that *in vitro* atrazine exposure at concentrations 200 μ M and greater were required to exert cytotoxicity to JAWSII cells. As we observed several significant effects of atrazine on the DC maturation process at concentrations that were not cytotoxic, our *in vitro* data suggest that the mechanisms of atrazine immunotoxicity are not mediated via direct cytotoxicity. Among the phenotypic changes caused by atrazine exposure, the effects on MHC class I surface expression were the most substantial. Atrazine exposure efficiently removed surface MHC class I proteins in a dose-dependent manner with the lowest effective concentration being 1 μ M. In addition, atrazine exposure decreased the expression of the costimulatory molecule CD86, significantly downregulated the levels of CD11b and CD11c accessory molecules, and decreased the expression of the myeloid developmental marker

CD14. Importantly, the effects of atrazine exposure on morphological DC maturation were confirmed by effects on functional maturation as the antigen uptake was increased in the atrazine-exposed DC. Major effects of atrazine exposure on JAWSII DC are summarized in Table 2. Of note, the concentrations of atrazine that increased antigen uptake were significantly higher than the concentrations that inhibited the expression levels of several surface markers suggesting that phenotypic maturation molecules are more sensitive to this immunotoxicant than cytoskeletal rearrangement-related mechanisms of receptor-mediated and non-selective mechanisms of antigen uptake (Racoosin and Swanson 1992, Watts and Marsh 1992, Norbury, 2006)

It is difficult to estimate how the effective concentrations of atrazine used in this study relate to *in vivo* research with this herbicide, or, to human exposure. Nevertheless, our effective concentrations are in line with the study of Hooghe *et al.* (2000) where atrazine exposure *in vitro* decreased the production of IFN- γ , IL-5, and TNF- α by human PBMC. In a preliminary pharmacokinetic study with mice that has now been expanded to determining tissue levels of atrazine and its metabolites in target tissues, we determined that plasma levels of atrazine and its major metabolite, 2-Chloro-4,6-diamino-S-triazine (DACT) after a single oral dose of 5 mg/kg atrazine were approximately 0.2 and 10 μ M, respectively (Ross and Filipov 2006). Exposure to the highest (250 mg/kg) dose in this study resulted in plasma levels of 1.2 and 92.5 μ M, for atrazine and DACT, respectively. In this regard, immunotoxic effects of *in vivo* exposure (single, or 14-day) to atrazine in mice have been observed in the range of 25 to 250 mg/kg (NTP 1994, Pruett *et al.* 2003, Karrow *et al.* 2005, Filipov *et al.* 2005). Of note, documented exposures of over 200,000 people to atrazine levels above the acute Reference Dose (RfD), which is derived from a NOAEL of 10 mg/kg/day and a LOAEL of 70 mg/kg/day have been reported (EPA 2002). Additional consideration for the relevancy of the *in vitro* concentrations of atrazine used in the present study, particularly to occupational exposures, is also the fact that while in our mouse studies we have been able to detect the urinary metabolite of atrazine, atrazine-mercapturate, only in mice acutely exposed to the 125 and 250 mg/kg doses (Ross and Filipov 2006), this metabolite is readily detectable in the urine of pesticide applicators (Lucas *et al.* 1993). Moreover, atrazine was readily detected in the saliva of pesticide applicators indicating significant exposures during spraying with this pesticide (Hines *et al.* 2006) and, interestingly, while the urinary metabolite of atrazine was substantially higher in fathers, mothers and children living on a farm compared with non-farm households, regardless of pesticide application, recent atrazine application by fathers resulted in increased urinary atrazine in their children (Curwin *et al.*, 2007).

Several disparate toxicants have been reported to affect DC and, in certain cases, these effects are similar to the effects of atrazine observed in the present study. For example, exposure to polycyclic aromatic hydrocarbons (PAH) caused an inhibition of functional differentiation and maturation of human monocyte-derived DC (Laupeze *et al.* 2002). More specifically, important monocyte-derived DC “maturation” markers such as CD1a, CD80, CD40 surface expression and IL-12 production were markedly inhibited after *in vitro* exposure to PAH (Laupeze *et al.* 2002). On the other hand, the immunosuppressive effects on DC caused by 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) appear to involve the phenomenon of “pseudo maturation”, which consists of inappropriate activation of DC, their premature deletion, and prematurely terminated DC-dependent immune responses (Vorderstrasse and Kerkvliet, 2001). Our data suggest that unlike TCDD, atrazine directly targets DC maturation by decreasing the levels of their MHC class I, costimulatory and accessory molecules, and by enhancing selective and non-selective antigen uptake mechanisms. These are all phenotypes exhibited by immature DC. Inhibited DC maturation leads in turn to impaired antigen presentation (Steinman 1991, Banchereau and Steinman 1998, Palucka and Banchereau 1999).

MHC class I molecules, which are expressed on the surface of DC and other APC, are critical for cell-cell interactions, including thymic T-cell development, and antigen presentation (Inaba *et al.* 1997). Hence, toxicants such as atrazine that efficiently remove MHC class I molecules from the surface of DC are very likely to contribute to immune evasion. In this regard, decreased MHC class I on the DC surface may have contributed to the compromised host resistance to a tumor challenge reported after exposure to atrazine *in vivo* (NTP 1994, Karrow *et al.* 2005). The exact molecular mechanism(s) that cause decreased expression of MHC class I and other surface molecules on DC following atrazine exposure is unclear at present and will be the subject of future investigations. One possible mechanism, however, may involve atrazine acting in a way similar to common anti-inflammatory agents, such as salicylic acid (SA; aspirin). SA, at pharmacological concentrations, inhibited the *in vitro* maturation and *in vivo* immunostimulatory function of murine (Hackstein *et al.* 2001) and human (Matasic *et al.* 2000, Ho *et al.* 2001) myeloid DC. Among the effects elicited by SA, a profound inhibition of CD40, CD80, CD86, and MHC class II expression on the surface of murine DC, generated from BM progenitors in the presence of GM-CSF and IL-4, was observed (Hackstein *et al.* 2001). In addition, SA-treated DC exhibited impaired IL-12 expression after LPS stimulation and an inability to induce normal cell-mediated contact hypersensitivity *in vivo* (Hackstein *et al.* 2001). SA exposure *in vitro* inhibited DC-dependent T cell proliferation and severely suppressed the levels of IL-12 (Matasic *et al.* 2000). One of the pharmacological effects of SA at the molecular level is the effective inhibition of nuclear factor kappa B (NFκB), a key intracellular switch for many molecular events, including surface molecules and cytokine expression, by DC and other cells (McCarty and Block 2006). A very recent study demonstrated that *in vivo* and *in vitro* exposure to atrazine was very effective in inhibiting Poly I:C-induced IL-6 production (Pruett *et al.* 2006). Importantly, atrazine also appeared to dampen Poly I:C-induced NFκB activation, albeit to a smaller extent than when combined with exposure to another pesticide, dieldrin (Pruett *et al.* 2006). Interestingly, MHC-I surface expression is rapidly upregulated in viral infections such as West Nile Virus (Cheng *et al.* 2004, Kesson and King 2001), and the mechanism of this upregulation is, at least in part, dependent upon NFκB (Cheng *et al.* 2004).

In summary, our data suggest that atrazine has direct effects on DC and that interference with DC maturation, the surface expression of MHC class I in particular, is one main consequence of exposure to this herbicide.

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Figure 1.

Phenotypic characterization of the JAWSII mouse dendritic cell (DC) line. **(A)**. Surface marker expression. JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to 100 ng/ml of lipopolysaccharide (LPS, a natural TLR4 agonist) or 50 μ g/ml of Poly I:C (a synthetic double stranded RNA-like TLR3 agonist). At the end of the incubation period, cells were stained with mAbs to key DC maturational markers, including costimulatory and accessory molecules (MHC-I, MHC-II, CD11b, CD11c, CD14, CD80, CD86) as described in the Materials and Methods. Data ($n = 3-4$ independent experiments/condition) are presented as Mean Fluorescence Intensity (MFI $\pm$ SEM). ^{a, b} Presence of letters on top of bars indicate treatment differences in the MFI within a phenotypic marker with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$). **(B)**. Semi-quantitative RT-PCR analysis for MHC-II mRNA. JAWSII cells were exposed to vehicle, LPS, or Poly I:C as described in **(A)** for 6 h. Following exposure, total RNA was extracted, quantified, and semi-quantitative RT-PCR analysis for mouse MHC class II H2-IA-alpha gene (b haplotype) and β -actin (internal standard) performed as described in the Materials and Methods section. Lanes 1, 2, 3, 4, and 5 represent the ladder, negative

control, vehicle-, LPS-, and Poly I:C-exposed cells, respectively of one representative experiment (out of 3). Upper bands: MHC class II mRNA; lower bands: β -actin (internal standard, run separately on the same samples).

Figure 2.

Functional characterization of the JAWSII mouse dendritic cell (DC) line. JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to 100 ng/ml of lipopolysaccharide (LPS, a natural TLR4 agonist) or 50 μ g/ml of Poly I:C (a synthetic double stranded RNA-like TLR3 agonist). At the end of the incubation period cells were washed and incubated with FITC-DX (selective uptake; 1 mg/ml, **A**) or Lucifer Yellow (LY, non-selective uptake; 100 μ g/ml, **B**) for 30 min at 37° C to measure active endocytosis or at 4° C to determine background levels of endocytosis (negative control) as described in the Materials and Methods. Data ($n = 3-4$ independent experiments/condition) are presented as the difference in the Mean Fluorescence Intensity ($MFI \pm SEM$) between 37 and 4° C. ^{a, b}

Presence of letters on top of bars indicate treatment differences in the in the FITC-DX and LY uptakes with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$).

Figure 3.

Cytotoxic (**A**) and apoptotic (**B**) effects of 24 h exposure to a concentration range of atrazine (1–300 μM). JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to atrazine. At the end of the incubation period, dead and apoptotic cells were determined with the Trypan Blue and Annexin-V tests, respectively, as described in the Materials and Methods section. Each bar (mean $\pm$ SEM) on the figures represents a minimum of three independent experiments with each experimental condition run in duplicate. ^{a, b} Presence of letters on top of bars indicate treatment differences due to atrazine exposure with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$).

Figure 4.

Representative dot plots depicting the effects of 24 h exposure to atrazine (ATR; 100 μ M), LPS (100 ng/ml), or control vehicle (CTL) on the surface expression of MHC-I, CD11c, or CD86. JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to atrazine or LPS. At the end of the incubation period, cells were stained with a mAb to MHC-I, CD11c, or CD86. Then, surface expression of these molecules was determined as described in detail in the Materials and Methods. Numbers in the upper left quadrants represent % positive cells.

Figure 5.

Effects of 24 h exposure to a concentration range of atrazine (1–100 μM) on the surface expression of MHC-I (**A**) and on the mRNA levels of β-2 microglobulin (MHC-I invariant chain; **B**). JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to atrazine. At the end of the incubation period, cells were either stained with a mAb to MHC-I (**A**), or total RNA was extracted (**B**). Then, surface expression of MHC-I or the mRNA levels of β-2 microglobulin was determined as described in detail in the Materials and Methods. Each bar (mean ± SEM on the figures represents a minimum of three independent experiments with each experimental condition run in duplicate. ^{a, b, c} Presence of letters on top of bars (**A**) indicate treatment differences due to atrazine exposure with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$).

Figure 6.

Effects of 24 h exposure to a concentration range of atrazine (1–100 μM) on the surface expression (A), and mRNA levels (B) of CD11b, as well as on the surface expression of CD11c (C). JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to atrazine. At the end of the incubation period, cells were either stained with mAbs to CD11b (A) and CD11c (C), or, total RNA was extracted (B) and surface expression of CD11b and CD11c or the mRNA levels of CD11b determined, respectively, as described in detail in the Materials and Methods. Each bar (mean $\pm$ SEM) on the figures represents a minimum of three independent experiments with each experimental condition run in duplicate. ^{a, b} Presence of letters on top of bars indicate treatment differences due to atrazine

or LPS (**B**) exposure with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$).

Figure 7.

Effects of 24 h exposure to a concentration range of atrazine (1–300 μM) on the receptor-mediated endocytosis (FITC-DX uptake; **A**) or macropinocytosis (LY uptake; **B**) JAWSII dendritic cells. JAWSII cells (0.5×10^6 cells/ml) were plated overnight in 24-well plates and then exposed for additional 24 h to atrazine. At the end of the incubation period cells were washed and incubated with FITC-DX (1 mg/ml, **A**) or LY (100 μg/ml, **B**) for 30 min at 37°C to measure active endocytosis or at 4°C to determine background levels of endocytosis (negative control) as described in the Materials and Methods. Data (n = 3–4 independent experiments/condition) are presented as the difference in the Mean Fluorescence Intensity (MFI ± SEM) between 37 and 4°C. ^{a, b}Presence of letters on top of bars indicate treatment differences due to atrazine exposure with bars with different letters being different from each other and from bars without a letter designation (P < 0.05).

Figure 8.

Effects of 24 h exposure to a concentration range of atrazine (1–10 μM) on the surface expression of MHC-I (**A**) and CD11c+/CD8+ (**B**) in primary thymus-derived dendritic cells (0.5×10^6 cells/ml) exposed for 24 h to a concentration range of atrazine. At the end of the incubation period, cells were stained with mAbs to MHC-I (**A**), or CD11c and CD8 (**B**) and their surface expression of was determined as described in detail in the Materials and Methods. Each bar (mean $\pm$ SEM) on the figures represents a minimum of three independent experiments with each experimental condition run in duplicate. ^{a, b} Presence of letters on top of bars indicate treatment differences due to atrazine exposure with bars with different letters being different from each other and from bars without a letter designation ($P < 0.05$).

Flow Cytometry analyses of the surface expression of accessory and co-stimulatory molecules on JAWSII dendritic cells (0.5×10^6 cells/ml) exposed to a concentration range of atrazine for 24 h. Data are from a minimum of four independent experiments and are represented as mean fluorescence intensity (MFI, mean $\pm$ SEM).

Table 1.

Marker	Control	1 μ M	10 μ M	100 μ M	300 μ M
CD14	14.7 $\pm$ 0.43	13.9 $\pm$ 0.58	13.0 $\pm$ 0.25 ^a	12.8 $\pm$ 0.67 ^a	12.8 $\pm$ 0.64 ^a
CD80	68.7 $\pm$ 0.27	70.7 $\pm$ 5.79	65.1 $\pm$ 3.67	64.1 $\pm$ 4.26	64.3 $\pm$ 9.61
CD86	31.9 $\pm$ 1.56	30.9 $\pm$ 0.69	32.3 $\pm$ 2.45	28.0 $\pm$ 1.14 ^a	25.1 $\pm$ 2.40 ^b

^{a, b} Presence and different letters within a row indicate differences due to atrazine exposure with means with different letters being different from each other and from means without a letter designation ($P < 0.05$).

Table 2.

Summary of surface molecule and antigen uptake characteristics of immature dendritic cells (IDC); resulting changes caused by DC maturation *in vivo* (mature DC; MDC), as well as the major alterations in JAWS II DC resulting from *in vitro* exposure to a maturational stimulus (JAWS II DC + MS) or to atrazine (JAWS II + atrazine).

Marker/Uptake	IDC	MDC	JAWS II DC + MS	JAWS II DC + Atrazine
MHC I	+++	<i>nc</i> ↓	↓	↓↓
MHC II	+	↑↑↑	↑	<i>nc</i>
CD11b	+++	<i>nc</i> ↑	↑↑	↓↓
CD11c	+	↑↑	<i>nc</i> ↓	↓
CD14	-/+	<i>nc</i> ↑	↑	↓
CD40	+	↑↑	<i>nc</i>	<i>nc</i>
CD80	-/+	↑↑↑	↑	<i>nc</i>
CD86	-/+	↑↑↑	↑↑	↓
FITC uptake	+++	↓↓↓	↓↓	↑
LY uptake	+++	↓↓↓	↓	↑

-/+ , +++ Presence and number of + indicates the presence and the relative amount of surface molecule expression or the antigen uptake ability of IDC.

nc
no change

↑ increased surface expression or uptake ability caused by *in vivo* maturation or *in vitro* treatment

↓ decreased surface expression or uptake ability caused by *in vivo* maturation or *in vitro* treatment. Number of arrows represents the relative magnitude of the maturational changes