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THE EFFECTS OF ESTROGEN IN ATRAZINE-MEDIATED FOXP3 INDUCTION AND INHIBITION OF CD4+ T EFFECTOR CELLS

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THE EFFECTS OF ESTROGEN IN ATRAZINE-MEDIATED FOXP3 INDUCTION AND INHIBITION OF $CD4^+$ T EFFECTOR CELLS

By

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The Effects of Estrogen in Atrazine-mediated Foxp3 Induction and Inhibition of $CD4^+$ T effector Cells

Atrazine (ATR) is a chlorotriazine herbicide that is heavily used in agricultural areas. Atrazine was banned in Europe in 2006 but it is still used in the United States. It is also the most common drinking water contaminant in the United States. Atrazine has been linked to adverse health effects and displays immunotoxicity. It is a potent phosphodiesterase inhibitor and has been shown to induce aromatase activity leading to elevated estrogen levels. Previous studies demonstrated that *in vitro* atrazine exposure inhibits $CD4^+$ T cell activation and proliferation and increases the frequency of $Foxp3^+$ $CD4^+$ T cells with more severe phenotypes in male-derived cells. The decreased proliferation and activation of $CD4^+$ T cells was not replicable by pharmacologically increasing cAMP. This, along with the sex bias, suggested that ATR elevation of estrogen could mediate an increased severity in T cell proliferation and activation, specifically through GPER-1. We show that treatment with the GPER-1 agonist G-1 can mimic effects seen with low concentrations of ATR but blockade of GPER-1 with the antagonist G-36 does not alleviate ATR-mediated effects on $CD4^+$ T cells. We also show that estrogen can synergize with ATR to further decrease $CD4^+$ T cell proliferation and activation upon challenge with antigen. Overall, GPER-1 does not appear to be involved in the ATR mediated decrease in $CD4^+$ T cell proliferation, activation, or increase in the frequency of $Foxp3^+$ T_{regs} *in vitro*.

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Atrazine Exposure and Regulations

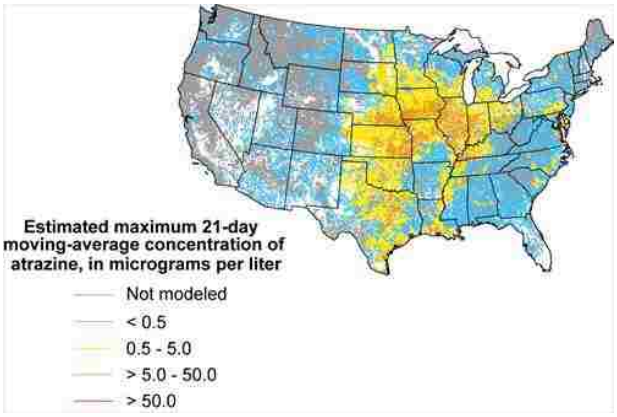


Figure 1: 2007 estimates of maximum 21-day average Atrazine concentrations in streams. The colors on the map refer to the concentration of Atrazine found (blue=lowest, orange=highest). Higher concentrations of Atrazine are found in streams near where Atrazine is most heavily used**. Provided by the USGS.**

Atrazine (ATR) is a chlorotriazine herbicide that kills broadleaf and grassy weeds by inhibiting photosynthesis (1). It inhibits electron transport by blocking electron flow from plastoquinone A to Q_B in photosystem II (2). It is estimated that 76.4 million pounds are applied annually on corn, sorghum and sugarcane crops in the United States (U.S.) (1). The large-scale use of this herbicide makes it the most common contaminant of ground and drinking water with more than half of the U.S population exposed (3). Atrazine is sprayed onto crops allowing for the wind to spread droplets to areas outside of the application site. Water run-off also spreads atrazine into aquifers and near-by streams allowing for a larger area of contamination and contamination of drinking water. About 75% of stream samples (40% containing more than 0.1ppb ATR) and 40% of groundwater samples (more than 10% containing more than 0.1ppb ATR) in agricultural areas of the U.S. contained ATR between 1992 and 2001 with slight elevations seen in more recent years (4).

The U.S. maximum containment level (MCL), which is the highest concentration allowed in drinking water, of atrazine is 3 parts per billion (ppb) (5). Since ATR is not classified as a carcinogen (6), the MCL is determined by taking the safe dose (the

lowest dose thought to not cause adverse effects) and dividing it by five. The division by five is done to account for other modes of exposure such as from food, air or skin absorption (7,8).' MCLs are based on yearly averages, which can allow for huge concentration spikes over the MCL during the spraying season. It is estimated that over 200,000 people are exposed to levels above the MCL (9). The No-observed-adverse-effect-level (NOAEL) is 10mg/kg/day while the Lowest-observed-adverse-effect-level (LOAEL) is 70/mg/kg/day) (9). In spite of potential health effects, atrazine regulation has become a very controversial topic because it is cheap and effective. The European Union (EU) banned its use in 2006 because of potential health effects and the inability to reduce water contamination below 0.1ppb (which they required because they do not recognize a safe level in drinking water) (4, 26).

Even with regulations set in place, the concentration of ATR in bodies of water and rain vary drastically. Levels as high as 4,000 ppb have been reported in runoff from treated fields and 2.5ppb in rainfall around agricultural and non-agricultural areas (10). Even though the use of atrazine is regulated, one of the main concerns lies with those that actually apply it to crops. Atrazine applicators are at risk of directly exposing themselves and their families to hazardous levels of atrazine. ATR has been found to contaminate dust and air within farming homes leading to increased levels of atrazine and its metabolites in urine. A significant level of atrazine in urine is also found in non-farming families because atrazine is easily spread from the initial application site (11).

Biochemical activities of Atrazine

Atrazine is known as an endocrine disrupting compound (EDC) because it can alter testosterone and estrogen levels. Atrazine can induce expression of aromatase,

which converts androgens to estrogens, and ultimately increases estrogen levels (12). ATR has also been shown to interfere with testosterone synthesis that ultimately causes a decrease in serum and plasma levels of testosterone (13).

Due to ATR's endocrine disrupting activity, its effects can vary depending on the concentrations used. Traditional, monotonic, dose-response curves have a defined relationship between the dose and the biological effect as seen in Figure 2. However, EDCs have been shown to exhibit non-monotonic dose responses, meaning that the relationship between dose and effect is not linear (14). This means that in some instances lower doses of EDCs may have more severe effects than higher doses, as displayed in Figure 2 (15).

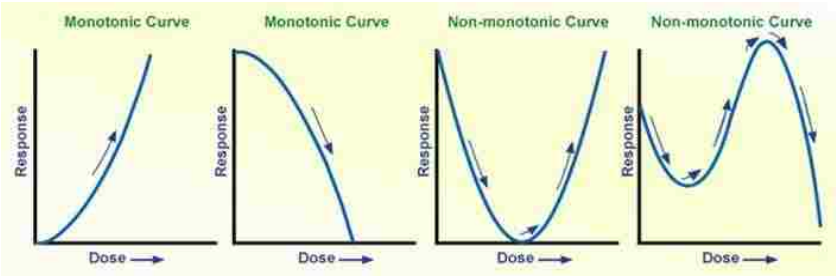


Figure 2: Monotonic versus Non-monotonic dose curves. Adapted from (15).

Along with ATR's endocrine disrupting function, it is also known to be a very potent cyclic adenosine monophosphate (cAMP) phosphodiesterase (PDE) inhibitor (16). Phosphodiesterase inhibition leads to an increase of intracellular cAMP, which has also been linked to aromatase induction (17). ATR is a more potent PDE inhibitor than the well-characterized, non-selective PDE inhibitor isobutyl methylxanthine (IBMX). While IBMX displayed significant PDE inhibition down to 500nM, ATR was active down to 5nM demonstrating a 100-fold increase in potency versus IBMX (17).

The increase of intracellular cAMP has many different biological effects. In $CD4^+$ T lymphocytes elevated cAMP stabilizes the transcription of forkhead box protein 3 (Foxp3). Foxp3 is the master transcriptional regulator of regulatory T cells (T_{regs}). Within the first intron of Foxp3 is a CpG island that, if unmethylated, is bound by cAMP response element binding protein (CREB), which maintains Foxp3 expression. About 45% of naïve $CD4^+$ T cells have methylated CpG regions within the Foxp3 gene whereas $CD4^+$ $CD25^+$ regulatory cells displayed no methylation (18). The demethylated region in naïve cells could potentially give rise to Foxp3 expressing T_{reg} cells if they experienced an increase in intracellular cAMP.

Atrazine Metabolism

There are twelve known metabolites of atrazine with the most common being desethyl atrazine (DE), desisopropyl atrazine (DIP) and diaminochlorotriazine (DACT) (19). Atrazine is not known to bioaccumulate (20, 21) and its half life in the environment varies depending on whether it is in water, soil or the body. The half life of atrazine in soil is 146 days and 742 days in water (4, 22). A study done by Ross *et al.* looked at the concentration of atrazine and its metabolites in urine, plasma, and various body tissues of mice. Mice were administered one dose of atrazine, ranging from 5mg/kg to 250mg/kg, via oral gavage. Mice exposed to the highest level of atrazine had detectable levels of atrazine and its metabolites in their plasma and urine for 48 to 72 hours. DACT was found at higher levels (50µM in urine) than all other metabolites and lasted the longest in the body (up to 96 hours). ATR levels peaked 1 hour after exposure and were found at 28µM in urine and 11µM in the spleen and thymus after 4 hours. ATR levels declined between 24 and 48 hours (19). Although the highest dose (250mg/kg) is higher

than the LOAEL (70mg/kg/day), the study only looked at short-term atrazine exposure. It is currently unclear what the levels of ATR and its metabolites in plasma, urine, or body tissue during chronic exposure to lower levels of ATR. Such experiments would better mimic human exposure patterns.

The adverse effects of ATR metabolites have not been studied extensively. The main metabolite, DACT, has been shown to decrease levels of testosterone more severely than treatment with ATR (13). Treatment with 100mg/kg or 200mg/kg of DACT or ATR decreased transcription levels of proteins associated with testosterone synthesis in the testis of male mice. DACT reduced transcript levels more severely than ATR but that could be due to the breakdown of ATR into many different metabolites versus pure levels of DACT. DACT suppresses luteinizing hormone (LH) release in mice (23). LH triggers ovulation in females and testosterone production in males. A reduction of LH can delay puberty in both sexes, which can be confirmed as a side-effect of ATR treatment or ATR metabolite treatment in male and female rats (24, 25).' Most of the metabolite effects studied have focused on changes in tissues and endocrine disruption, but metabolite effects on the immune system have yet to be examined.

Environmental and Health Effects of Atrazine

Multiple studies have linked atrazine to increases in prostate and breast cancer (27, 28, 29).'' Males employees working in atrazine production plants had higher incidences of prostate cancer (28), while breast cancer incidence correlate with atrazine application sites (29). In contrast to these studies, a 2011 study from Freeman *et al.* found no significant increases in cancer amongst atrazine applicators, with the

exception of a small increase in thyroid cancer (27). These, and other, conflicting results lead the IARC to conclude atrazine is “not classifiable as a human carcinogen.”

In addition to cancer, atrazine exposure is linked to fetal developmental problems such as low birth weight, fetal limb defects, and pre-term delivery (30, 31, 32).” A study done by Winchester *et al*. found a correlation between increased usage of atrazine between April and July and birth defects in children conceived during that time. Men were also found to have abnormal sperm during the same months (33). Women exposed to ATR during their third trimester resulted in a 17-19% increase of small-for-gestational-age babies (low birth weight) and exposure over the entire pregnancy significantly increased these chances by 11% (30). Most pregnancy complications, such as pre-term delivery and small for gestational age, arose when the mother was pregnant with a boy (32, 30). The effects on male fetuses could be due to atrazine’s effect as an endocrine disrupting compound.

Atrazine in the environment has also been the focus of a lot of research. The most widely reported environmental concern regarding ATR is the feminization of frogs and fish exposed to ATR. Due to ATR’s ability to induce aromatase and increase estrogen, frogs grown in the presence of ATR have decreased testosterone levels and feminization of their gonads (production of female oocytes in testes) (34). As study done by Hayes *et al*. found that in leopard frogs exposed to 0.1ppb of atrazine, 12% of males had under-developed testes with low to no production of germ cells. Around 8% of frogs treated with 25ppb displayed sex-reversal and production of female oocytes. These findings were confirmed at high usage agricultural sites. For one high-usage site near the North Platte River in Wyoming, 92% of sampled males showed signs of sex-reversal

(35). Another study looking at the effect of atrazine at or above the MCL shows that frogs exposed to 3ppb of ATR had lower survival rates than frogs exposed to higher concentrations (36). Although this result appears counterintuitive, it displays ATR's non-monotonicity. The results of these studies show that ATR can severely alter the sex ratios and survival rates of amphibians in the wild. This can directly skew reproduction and population numbers and pose an ecological threat to frogs.

Immunotoxicity of Atrazine

Immunotoxic effects of atrazine have previously been observed. Treatment with ATR inhibits natural killer (NK) cell lytic granule release, compromising the ability to kill target cells. Cell-to-cell contact and concentrations of lytic proteins remained unchanged but the exocytosis of lytic proteins was inhibited in ATR cultures (37). Atrazine treatment decreased dendritic cell (DC) maturation in primary murine DC and the murine dendritic cell line JAWSII (38). The percentage of mature dendritic cells (defined as CDllc high) decreased 24 hours after treatment with 1µM ATR compared to controls. A study done by Filipov *et al.* looked at immunotoxic effects of short term ATR exposure *in vivo*. Male mice were exposed to 5, 25, 125, or 250mg/kg of ATR daily via oral gavage for 14 days and then analyzed at 1 day, 1 week, and seven weeks after the last ATR exposure. Mice treated with 125 mg/kg/day had significantly decreased thymus and spleen weights and thymus and spleen cellularity were reduced with concentrations as low as 25/mg/kg/day ATR. The reduced splenic cellularity lasted up to seven weeks. Higher levels of ATR (125 and 250mg/kg/day) decreased the number of splenic and circulating naïve $CD4^+$ T cells and increased the percentage of highly activated cytotoxic/memory T cells (39). These studies suggest that ATR is potentially immunosuppressive but

much remains unknown about how ATR affects adaptive immunity, specifically $CD4^+$ T cells.

T cell Functions

$CD4^+$ T cells are essential components in the generation of a protective adaptive immune response. $CD4^+$ T cells are divided into functionally distinct effector subtypes based upon cytokine products and gene expression patterns. The currently well characterized $CD4^+$ T cell subsets are T helper type 1 (T_H1), T helper type 2 (T_H2), T helper type 17 (T_H17), T follicular helper cells (T_{FH}) and regulatory T cells (T_{regs}). T_H1 $CD4^+$ T cells are associated with cell-mediated immunity and defense against intracellular pathogens (40). T_H1 cells are associated with delayed-type hypersensitivity and the production of pro-inflammatory cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) (41). T_H2 $CD4^+$ T cells are associated with humoral immunity and defense against extracellular pathogens by encouraging antibody production (40). T_H2 responses are also associated with allergic inflammation and asthma (42). T_H17 $CD4^+$ T cells help defend against parasites and are known to strongly influence gut-associated immunity (43). T_{FH} cells are found in lymphoid follicles and function to help B cells undergo antibody class switching and somatic hypermutation to create higher affinity antibodies (44).

In contrast to the previously mentioned $CD4^+$ T cell subsets, regulatory T cells (T_{regs}) suppress immune responses in order to maintain self-tolerance. The most well characterized T_{reg} population expresses the transcription factor Foxp3, and the interleukin 2 receptor α chain (CD25). They are defined as $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg}.

Foxp3 is the master transcriptional regulator of T_{regs} and its expression drives expression of a set of genes that are associated with T_{reg} function. As mentioned above, Foxp3 expression can be induced via elevated cAMP. T_{regs} naturally have higher levels of cAMP and can use this to suppress effector T cells (T_{eff}) (45). In fact, elevated cAMP is also an effector mechanism of T_{regs} as T_{regs} directly transfer cytoplasmic cAMP via gap-junctions into effector T cells (45). The subsequent increase of cAMP in effector T cells increases the level of inducible cAMP early repressor protein (ICER), which then inhibits proliferation and IL-2 synthesis resulting in decreased activation (46). T_{regs} can also mediate suppressive action through contact independent mechanisms, including the secretion of anti-inflammatory cytokines like IL10 and TGFβ (47, 48).

The balance of the frequency of T_{regs} to effector T cells is critical in maintaining immune homeostasis. Decreased frequencies of T_{regs} correlate with increases in autoimmune diseases, such as rheumatoid arthiritis (49) and atopic dermatitis (50), due to the lack of suppression. On the other hand, increased T_{reg} frequencies can also lead to disease progression by inhibiting protective responses against pathogens like *Mycobacterium tuberculosis* (51). In this case, the increased number of T_{regs} suppresses the active immune response against the pathogen, preventing the pathogen from being cleared and allowing continued infection. Increased frequencies of T_{regs} can occur through two methods: proliferation and expansion of natural T_{regs} (nT_{regs}, derived from the thymus) (52) or induction of effector T cell conversion into T_{regs} (iT_{regs}, made outside of the thymus) (53). Both nT_{reg} and iT_{reg} populations are potent inhibitors of immune responses. Major disruption in the balance of T_{reg} populations can have detrimental

effects on self-tolerance, pathogen clearance and can also favor the development of cancer (54).

$CD4^+$ T cell Subset	Transcription factor	Characteristic cytokine	Characteristic function
T_H1	T-bet	IFNγ	Intracellular pathogens
T_H2	Gata-3	IL-4	Extracellular pathogens
T_H17	RORγt	IL-17	Extracellular bacteria, parasites
T_{FH}	Bcl-6	IL-21	Antibody class switching, B cell interactions
T_{reg}	Foxp3	TGFβ	Immune tolerance and regulation

Table 1: CD4+ T cell subsets and functions. (55)

Effects of Atrazine on CD4$^+$ T cells

Previous work in the Wetzel laboratory investigated the effects of ATR on CD4$^+$ T cells. Figure 3 shows that treatment with 30µM atrazine consistently reduced the number of CD4$^+$ T cells in culture, with a mean reduction of 70.6%. This reduction was due to decreased CD4$^+$ T cell proliferation (Figure 4). CD4$^+$ T cells cultured in the presence of 30µM ATR were stained with carboxyfluorescein succinimidyl ester (CFSE) to monitor proliferation. CFSE non-specifically labels intracellular proteins and upon division the fluorescence for each daughter cell is only half as bright as the parent cell. Proliferating cells have decreased CFSE whereas non-proliferating cells retain a high CFSE signal. As seen in Figure 4, 30µM ATR severely reduced proliferation compared to the untreated and EtOH-only cultures.

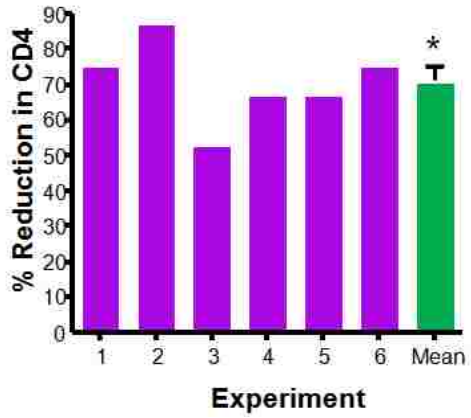


Figure 3: Atrazine decreases the amount of CD4+ T cells in culture. The percent reduction in the absolute number of CD4$^+$ T cells in the 30µM ATR-treated cultures compared to the EtOH-only control cultures is shown for 6 separate experiments. Cells were stimulated with 2.5µM MCC peptide. The ATR-associated mean reduction in CD4$^+$ T cells for these 6 experiments was statistically significant * *p=0.0058* **.**

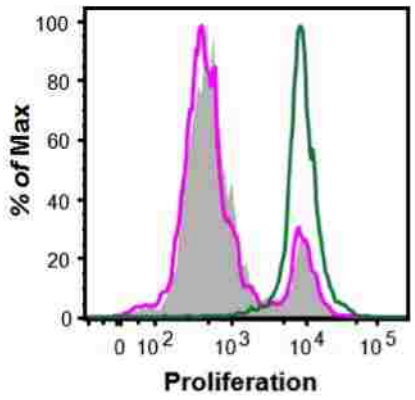


Figure 4: Treatment with 30µM ATR (green line) decreases CD4+ T cell proliferation compared to the untreated (pink line) and EtOH only controls (shaded grey). Cells were stimulated with 2.5µM MCC peptide. **(Thueson et al., in revision).**

Along with decreased proliferation, 30µM ATR exposure also decreased $CD4^+$ T cell activation (Figure 5, Top row). Activation was assessed by staining CD25 and CD69 after 4 days in culture. Expression of both CD25, the interleukin 2 receptor alpha chain (56), and CD69, a cell surface glycoprotein, increase upon activation (57). The frequency of $CD25^+$ $CD69^+$ cells in the EtOH culture was 77.4%, which was significantly higher than 13.2% of $CD25^+$ $CD69^+$ cells found in the ATR culture. Interestingly, the decreased expression of CD25 and CD69 correlated with an increase in the frequency of $Foxp3^+$ regulatory T cells (T_{regs}) (Figure 5, Bottom row). 30µM ATR exposure typically increases the frequency of Foxp3 by 2-5 fold. The observed decrease in the activation and proliferation of the $CD4^+$ T cells in ATR treated cultures may be due to the functions of increased numbers of T_{regs} in the cultures.

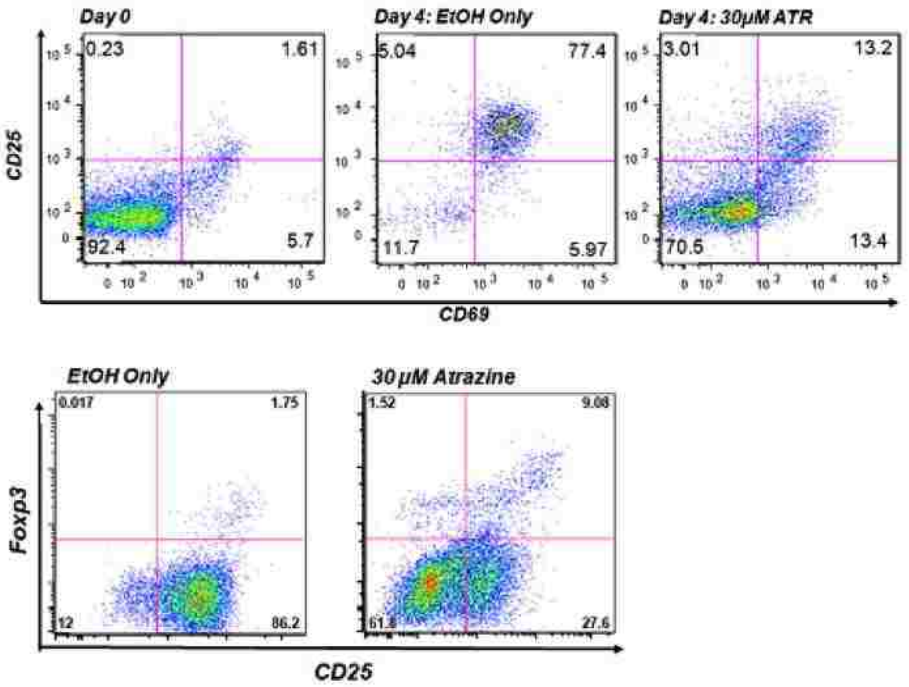


Figure 5: Treatment with 30μM ATR decreases CD4+ T cell activation. Cells were stimulated with 2.5μM MCC peptide. Activation was determined by staining CD25 and CD69. 30μM ATR has 13.2% of CD25+ CD69+ T cells (Top, right) while the EtOH only control has 77.4% (Top, middle). Treatment with 30μM ATR also increased the frequency of Foxp3+ CD25+ T cells by more than 5 fold (Bottom, left) compared to the EtOH control (Bottom, right). **(Thueson et. al., in revision)**

As mentioned earlier, increases in cAMP can stabilize expression of Foxp3. Thus the rise in Foxp3 expression could be mediated through ATR's PDE inhibitor function. In order to test this hypothesis, cells were treated with the PDE inhibitor pentoxyfilline (PTX). Upon treatment with PTX, $CD4^+$ T cells displayed both decreased proliferation (data not shown) and decreased activation (Figure 6). 11.4% of $CD4^+$ T cells treated with PTX were $CD25^+$ $CD69^+$ while 6.2% were $CD25^+$ $CD69^+$ in the atrazine treated cultures. While supportive of a role for elevated cAMP in the ATR immunotoxic effects, the difference between ATR and PTX suggest additional factors may be involved. Coincidentally it was observed that cells derived from male mice were more sensitive to ATR than cells derived from female mice (Figure 7). Male $CD4^+$ T cells did not proliferate as well as the female $CD4^+$ T cells upon treatment with the same concentration of ATR (30μM). Since ATR decreased activation more than that seen with

a PDE inhibitor alone and male cells were more affected than female cells, it raises the possibility that ATR's endocrine disrupting function could be playing a role in the effects on $CD4^+$ T cell proliferation and activation.

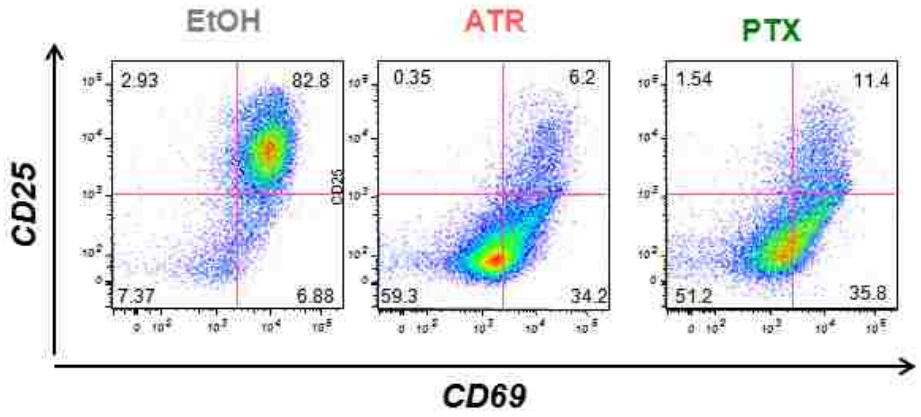


Figure 6: Treatment with 250 µM PTX can reduce proliferation (data not known) and activation of CD4+ T cells. 30µM ATR has 6.2% CD25+ CD 69+ T cells compared to 11.4% in the presence of PTX and 82.8% in the ethanol control. (Thueson et al., in revision)

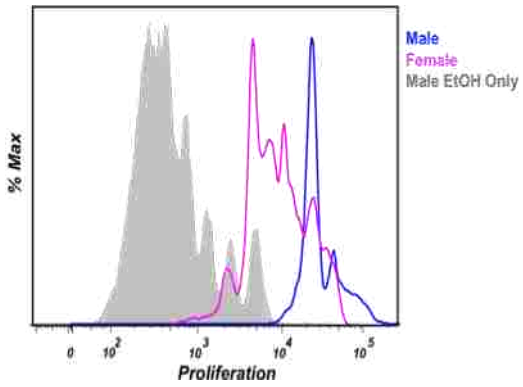


Figure 7: Effects of 30 µM ATR on male (blue) and female (pink) CD4+ T cells. Male and female cells display decreased proliferation compared to the EtOH vehicle control (shaded grey), although male cells do not proliferate as many times as the female cells. (Thueson et al., in revision)

Estrogen as an Immunomodulator

Since ATR can increase levels of estrogen, elevated levels of estrogen could potentially be causing the increased severity of ATR seen in Figure 6. Estrogen is known to be a very potent immunomodulator. Antigen stimulated human T cells cultured in the presence of estrogen (1000-1500 pg/ml) proliferate poorly compared to controls

and addition of exogenous IL-2 did not rescue this proliferation defect. The expression of CD25 was also decreased in estrogen treated cultures (58). Since CD25 is part of the IL-2 receptor, lower expression could lead to a diminished response to IL-2. Estrogen has also been shown to directly interfere with the IL-2 receptor expression at the mRNA level (59). Treatment with estrogen, or specific agonists for either Estrogen Receptor α (ERα) or Estrogen Receptor β (ERβ), increases levels of the cAMP response element modulator α (CREMα) in T cells. CREMα is a transcriptional repressor that suppresses IL-2 transcription and cytokine production. The suppression of IL-2 transcription by CREMα occurred more frequently in cells derived from females than cells derived from males (59).

Estrogen can also expand the frequency of $CD4^+$ $CD25^+$ T_{regs} in vivo (60). Mice treated with time-release estrogen pellets had significantly increased levels of $Foxp3^+$. Increases in Foxp3 expression correlated with increases in CD25 expression and the induction of experimental autoimmune encephalomyelitis (EAE, mouse model of multiple sclerosis) in estrogen treated mice resulted in lower disease severity scores compared to controls (60). Overall, estrogen appears to have an immunosuppressive effect on T cells by causing an increase in T_{regs} and directly decreasing the activation and proliferation of effector T cells.

Estrogen can also affect other cells of the immune system. Estrogen has been shown to increase levels of toll-like receptor 4 (TLR-4) on macrophages and increase the production of pro-inflammatory cytokines (61). Targeted disruption of ERα abolished this effect showing that estrogen was acting through ERα to mediate this effect. In

contrast to these results, signaling through G-protein coupled estrogen receptor 1 (GPER-1, formerly known as GPR30) on macrophages decreased expression of TLR-4 and inhibited a LPS-induced pro-inflammatory response (62). These results show that different estrogen receptors can cause different effects on the same cell type and that treatment with estrogen (specifically 17-β-estradiol) can give rise to many different outcomes depending on the context of the signaling and the receptors involved.

Interaction of Atrazine and Estrogen Receptors

Although ERα and ERβ are classical nuclear estrogen receptors, additional receptors including the more recently identified estrogen receptors like G-protein coupled estrogen receptor 1 (GPER-1) may play a role in immune modulation. Signaling through GPER-1 leads to rapid signaling and transcriptional events (63). It is expressed in the central and peripheral nervous systems and has been linked to attenuating serotonin receptor signaling (63). GPER-1 can also be found in cardiovascular tissue where it causes vasodilation and can decrease blood pressure (63). GPER-1 signaling is also involved in thymic atrophy and double positive thymocyte apoptosis (64). GPER-1 knockout mice treated with estrogen had a reduction in double positive thymocyte apoptosis compared to ERα and ERβ knockout mice. This provides evidence that T cells can express GPER-1 although expression of GPER-1 has not yet been assessed for single positive T cells that have exited the thymus. Previous studies have looked at the effects of GPER-1 on various tissues, but its effects on $CD4^+$ T cell biology are less understood.

A study by Yates *et al*. examining the protective effects of estrogen on EAE disease severity identified GPER-1 as a potential mediator of that protection. They

found that ERα- knockout mice treated with estrogen still displayed reduced disease severity, but when GPER-1 knockout mice were treated with estrogen the protective effect was lost. This led them to conclude that estrogen mediated the protective effect via GPER-1 (65). In subsequent experiments, Blasko e*t al*. treated mice with the GPER-1 agonist G-1 and found that it decreased EAE disease severity and pro-inflammatory cytokine production (66). These results suggest that activation of GPER-1 may be immunosuppressive.

The possibility exists that atrazine and its metabolites could physically interact with estrogen receptors like ERα, ERβ or GPER-1 to mediate our previously observed effects. However, competitive binding experiments involving these estrogen receptors have shown that ATR does not interact with ERα or ERβ (17), but it can weakly interact with GPER-1 (67). ATR metabolites have not been shown to interact with ERα or ERβ although studies have not examined metabolite interactions with GPER-1 (17). These findings, as well as the potential for ATR to increase levels of estrogen, strongly suggest that ATR exposure could trigger GPER-1 signaling. Our previous observations have shown that phosphodiesterase inhibition alone does not fully replicate the decreased $CD4^+$ T cell activation seen in ATR treated cultures (Figure 6) and male-derived cells are more sensitive to ATR treatment than female-derived cells (Figure 7). These results, as well as the fact that ATR is an endocrine disrupting compound, suggest that the more severe ATR-mediated decrease in $CD4^+$ T cell proliferation and activation and increase in the frequency of $Foxp3^+$ T cells may involve GPER-1.

Thesis Hypothesis and Project Rationale

ATR is the most common contaminant of ground and drinking water in the United States (1). It can last for extended time periods in soil and water and is easily spread far from application sites (22). Biochemically, ATR is a potent PDE inhibitor that increases cAMP levels. ATR is also an endocrine disrupting compound that is capable of inducing aromatase expression and increasing estrogen levels (12). Previous studies in the Wetzel laboratory have investigated the effects of ATR's PDE inhibitor activity on $CD4^+$ T cells and found that pharmacological agents that increase cAMP can inhibit $CD4^+$ T cell activation and proliferation. The PDE inhibitor, Pentoxyfilline (PTX), and the non-cleavable cAMP, dibutyryl cAMP, were used to mimic PDE inhibitor effects of ATR on $CD4^+$ T cells. In PTX and dibutyryl cAMP treated cultures, inhibition of $CD4^+$ T cell proliferation was comparable to that seen in 30µM ATR treated cultures. However, the frequency of activated $CD4^+$ T cells was lower in ATR exposed cultures compared to PTX (Figure 6) and dibutyryl cAMP (data not shown) exposed cultures. It was also discovered that male-derived cells were more sensitive to ATR exposure than female-derived cells (Figure 7). Since treatment with ATR displayed a sex bias and decreased the frequency of activated $CD4^+$ T cells more than that seen by pharmacologically increasing cAMP, we hypothesized that ATR's EDC activity was involved in the decrease of activated $CD4^+$ T cells and the increase in $Foxp3^+$ regulatory T cells. Since ATR is known to via aromatase induction and increase estrogen levels (12), we further hypothesized that ATR was acting, in part, via elevated estrogen.

ATR has not been known to interact with ERα or ERβ but it has been shown to weakly interact with GPER-1 (67). GPER-1 became the receptor of interest because it

impacts immune cells via decreased TLR4 expression on macrophages (62), and GPER-1 triggering by the specific agonist G-1 was protective in experimental autoimmune encephalomyelitis (EAE, a mouse model of multiple sclerosis) (66). GPER-1 triggering also increased cAMP (63), which may be complementing ATR's PDE inhibitor activity. GPER-1 signaling appears to have immunosuppressive effects, but how it impacts $CD4^+$ T cells remain elusive. From these observations, we hypothesized that the ATR-mediated decrease in $CD4^+$ T cell activation and proliferation and increase in frequency of $Foxp3^+$ $CD4^+$ T cells is, in part, due to ATR-mediated elevation of estrogen, which then triggers GPER-1 signaling.

Materials and Methods

Cell Culture Reagents

Murine splenocytes were cultured in complete RPMI 1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum. (Atlanta, Biologicals, Atlanta, GA). RPMI was supplemented with L-glutamine, sodium pyruvate, Penicillin G, Streptomycin, Gentamycin, phenol red, and MEM essential and non-essential amino acids (Sigma, St. Louis, MO). Media was sterile filtered through a 0.2µm filter into an autoclaved 1 liter glass bottle. It was stored at 4°C and warmed in a 37°C water bath prior to use.

Red blood cells were lysed by incubation in a hypotonic buffer for 5 to 10 minutes at room temperature. This buffer, Gey's solution, was made by mixing 200ml of Solution A (35g NH_4Cl, 1.85g KCl, 0.595g anhydrous Na_2HPO_4, 0.12g KH_2PO_4, 5g Glucose, 50mg phenol red in 1L of Millipore water), 50ml of Solution B (1.05g $MgCl\bullet 6H_2O$, 0.35g $MgSO_4\bullet 7H_2O$, 0.85g anhydrous $CaCl_2$ in 250ml Millipore water) and 50ml Solution C (5.63g $NaHCO_3$ in 250ml Millipore water). The volume of the final 1X Gey's solution was adjusted to 1L with 700 ml Millipore water before it was sterile filtered using a 0.2µm filter. This final solution was stored at 4°C.

Staining Antibodies

The following purified, fluorescent-conjugated or biotinylated antibodies were purchased from BioLegend (San Diego, CA): CD3 (145.2C11), CD4 (GK1.5), CD25 (3C7 and PC61), CD28 (37.51), CD62L (MEL-14), CD69 (H1.2F3) , PD-1 (RMP1-30 and 29F.1A12), and Foxp3 (150D). In addition, antibodies specific for Vβ3 (KJ25) and

CD69 (H1.2F3) were purchased from BD Biosciences (San Jose, CA). Staining for Foxp3 was done using the BioLegend AlexaFluor 488 Anti-mouse/rat/human Foxp3 Flow Kit. To monitor proliferation, cells were stained with 5-(and 6-)carboxy-2',7'-dichlorofluorscein diacetate succinmidyl ester (CFSE) or Cell Trace Violet (CTV) (Life Technologies, Eugene, OR) according to the manufacturer's protocol on day 0. Day 0 stains (stained, unstained, isotype) were also removed from the whole spleen preparation before CFSE/CTV staining. A single-color control for CFSE and CTV was made by removing 10^6 cells from an untreated culture on day 1. Live cells were assessed by flow cytometry or cells were fixed for 30 minutes using 4% paraformaldehyde (PFA) and 0.5% Glutaraldehyde in PBS. Fixative was washed out using FACS buffer (PBS with 2% and bovine serum albumin (BSA) and 0.1% sodium azide) and resuspended in 300µL FACS buffer. This sample stored at 4°C in the dark until analyzed. Day 0 samples were, fixed and stored at 4°C in the dark until analyzed with day 4 samples. Single color controls were made using UltraComp ebeads (eBioscience, San Diego, CA).

Mice

B10.BR mice were purchased from (Jackson Labs, Bar Harbor, ME.). AD10 mice, specific for pigeon cytochrome C peptide 88-104 presented by I-E^k and reactive against moth cytochrome peptide 88-103 (68) were provided by Dr. David Parker at Oregon Health and Science University.

AD10 mice were maintained as heterozygotes. To identify transgenic offspring, AD10 pups were genotyped by PCR using primers specific for the recombined Vβ3 and

J2 of the AD10 TCR β chain. Toe tissue samples were taken from AD10 x B10.BR litters before 7 days of age for identifying and typing purposes. Toe tissue was digested using 40µL Toe/Ear digestion buffer and 2µL proteinase K. After a 1 hour incubation at 55°C, 158µL of autoclaved water was added, the sample was lightly vortexed and then heated at 95°C for 10 minutes to inactivate the proteinase K. Samples were then stored at -20°C until PCR analysis. For long-term storage, DNA samples were stored at -80°C.

B10.BR and AD10 mice were kept in specific pathogen free (SPF) housing at the University of Montana and allowed food and water *ad libitum*, in accordance with the UM Institutional Animal Care and Use Committee (IACUC) guidelines.

Preparation of Atrazine, Estrogen, and Experimental compounds

To prepare a 60 mM stock solution of atrazine, 250mg of atrazine (Chem Service, Chester PA) was resuspended in 19.3ml of 100% ethanol (Sigma, St. Louis, MO). After resuspending the atrazine, the solution was vortexed until a homogenous suspension was made. The stock was then aliquoted and stored at -80°C. When used in experiments, an aliquot of the stock solution was thawed and vortexed. It was then diluted 1:2 in 100% ethanol to form a 30 mM working solution. This solution was diluted 1:1000 in complete RPMI in the 6-well plates, giving a final concentration in the wells of 30µM in 0.1% ethanol. For the vehicle controls, EtOH was added to a final concentration of 0.1%.

The phosphodiesterase inhibitor Pentoxyfilline was purchased from Tocris Biological (Minneapolis, MN) and resuspended in ethanol to create a stock solution of 100mM. For experiments the final solution in the wells was a 250µM, which was

generated by addition of 12.5µL of the stock 100 mM solution was added to 5ml cell cultures.

To prepare the estrogen stock solution, 17-β-estradiol (E2) was purchased from (Sigma, St. Louis, MO) and resuspended in 100% ethanol. It was then further diluted with PBS to make the stock a 500ng/ml concentration. For experiments, the 500 ng/ml stock solution was diluted in complete RPMI to a final concentration of 5, 10, or 25 ng/ml. Note, new estrogen stock solutions were made monthly.

The Estrogen receptor α agonist PPT (4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol) was purchased from Tocris Biologicals (Minneapolis, MN). It has a 410 fold selectivity for ERα over ERβ and has an EC_{50} of ~200 pM. To prepare a 5mM stock solution, 10 mg were resuspended in 5.1ml of 100% ethanol. Working 1000X solutions were prepared by dilution in 100% ethanol and these solutions were then diluted 1:1000 into RPMI so that the final concentration of ethanol was 0.1%.

The aromatase inhibitor YM511 (4-[[(4-Bromophenyl)methyl]-4*H*-1,2,4-triazol-4-ylamino]benzonitrile) was purchased from Tocris Biologicals (Minneapolis, MN) and resuspended in 100% ethanol to create a 20mM stock solution. The stock solution was diluted to 10µM or 5µM in 100% ethanol and diluted 1000 X in complete RPMI to give the desired final concentration. YM511 has an IC_{50} of 0.4nM in rat ovaries and decreases estrogen levels with an IC_{50} of 0.13nM (69).

GPER-1 agonist and antagonist solution preparation

The GPER-1 agonist G-1 and the GPER-1 antagonists G-15 and G-36 were purchased from purchased from Tocris or Azano Biotech (Albuquerque, NM). 10mM stock solutions of each of these reagents was made by resuspending in an appropriate amount of dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). For G-1 and G-36, 10mg were resuspended in 2.4 ml DMSO. For G-15, 10mg was resuspended in 2.7 ml DMSO. These 10mM solutions were diluted to 1000X working solutions in DMSO and then diluted 1:1000 into complete RPMI so that the final solutions contained 0.1% DMSO. G-1 has a K_i of 11nM and an EC_{50} of 2nM and displays no activity with ERα or ERβ up to 10μM (70). G-15 has a half maximal inhibitory concentration (IC_{50}) of 190nM (71). G-36, a newly generated and more specific variant of G-15 has an IC_{50} of 112nM (72).

In vitro exposure primary cultures

In the experiments described in this thesis, primary murine splenic cultures were stimulated in the presence of atrazine, the indicated compounds, or vehicle-only controls for 4 days. To establish single-cell splenocyte cultures on day 0, spleens were harvested from mice over 6 weeks of age and placed in Hanks' Balanced Salt Solution (Sigma, St. Louis, MO). The spleen was then placed in a sterile petri dish and mechanically ruptured by gentle grinding between the frosted sides of two sterile glass microscope slides in 10ml of RPMI. Cells were centrifuged at 500x*g* for 7 minutes. Supernatant was aspirated off and red blood cells were subsequently lysed using 5ml of hypotonic Gey's solution. After 5 minutes in Gey's solution, 5ml of RPMI was added and cells were centrifuged for 500x*g* for 7 minutes. Supernatant was aspirated off and cells

were resuspended in 10ml RPMI. A small aliquot of cells was then diluted with trypan blue and directly counted using a hemocytometer.

AD10 cells were stimulated with 2.5 µM Moth Cytochrome C (MCC_{88-103}) peptide, while non-transgenic B10.BR cells were stimulated using plate bound anti-CD3 (145-2C11) and anti-CD28 (37.51) antibodies, both at 10µg/ml. To coat plates with antibodies 6-well tissue culture dishes (Greiner, Monroe, NC) were incubated with anti-CD3 and anti-CD28 in PBS for 2 hours at 37°C. The plates were then sealed to prevent evaporation and stored at 4°C until used. Before use, the antibody solution was removed and discarded. The wells were washed with 3ml of PBS and allowed to dry at room temperature before cells were added.

Cells were resuspended at $1.6x10^6$ or $2x10^6$ cells per ml and 5ml were plated in six well tissue culture dishes and the indicated treatment compounds were added. Vehicle-only control cultures were established containing 0.1% EtOH or 0.1% DMSO, which corresponded to the final concentration of vehicle in experimental samples. The control and treatment cultures were maintained at 37°C with 5% CO_2 for 4 days. On day 4, a small aliquot of cell supernatant was collected and filtered to remove any cells and stored at -20°C for analysis of estrogen levels. Viable lymphocytes were recovered via density centrifugation using LymphoLyte M (Cedar Lane, Burlington, NC). Cells were counted using a hemocytometer and Fc Receptors were blocked by addition of anti-CD16 antibodies (Fc Block, 1:100 dilution, BioLegend, San Diego, CA) for 15 minutes. Cells were then stained with 1:100 dilutions of various combinations of extracellular stains (CD4, CD69, CD25, Vβ3, CD62L, and PD-1) for 25 minutes. When necessary, cells were stained with fluorochrome-conjugated secondary antibodies for an additional

15 minutes and washed once with FACS buffer. In some experiments, cells were fixed and stained for intracellular Foxp3 using the Foxp3 staining kit (BioLegend, San Diego, CA.). Briefly, cells were incubated for 20 minutes in 1X Fix/Perm buffer before 3 washes with FACS buffer. The cells were incubated an additional 15 minutes in 1X Perm buffer, washed and stained with a 1:20 dilution of anti-Foxp3 antibody for 30 minutes. Fixed samples were stored for up to 3 days in the dark at 4°C before analysis using a FACSAria IIu (Becton Dickenson, San Jose, CA) in the University of Montana Fluorescence Cytometry Core facility. If cells were antibody stimulated, 10,000 $CD4^+$ cells were collected from each sample. If cells were stimulated via peptide, then 10,000 $CD4^+$ $V\beta3^+$ cells were collected from each sample. Data was analyzed after using FlowJo Software version 8.8.7 (Treestar, Inc, Ashland, OR).

Statistical Analysis

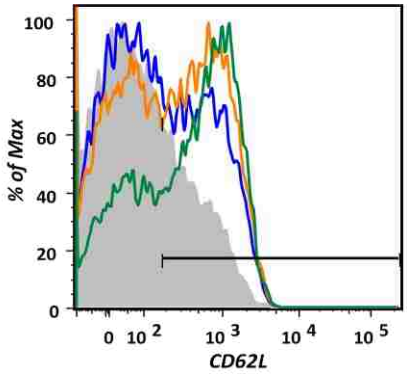
To compare different treatment groups, data was analyzed using an unpaired student's t test on Microsoft Excel 2007. Values of $p \leq 0.05$ were considered statistically significant.

Results

Atrazine prevents the down-regulation of CD62L and up-regulation of Programmed Death 1 (PD-1)

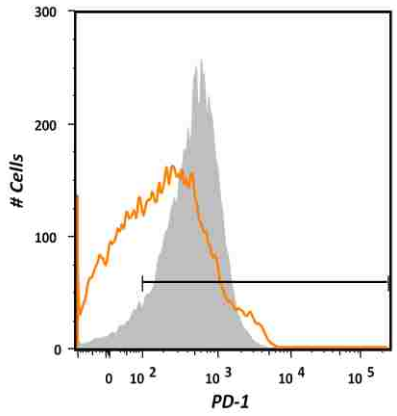
Our laboratory has previously found that *in vitro* exposure to 30µM ATR during antigen-recognition significantly reduced expression of CD25 and CD69 on $CD4^+$ T cells (Thueson et. al. in revision). To further characterize the activation status of our $CD4^+$ T cells in culture, we examined expression of CD62L and programmed death-1 (PD-1), (Figures 8 and 9). CD62L is expressed at high levels on naïve cells and expression decreases upon activation. PD-1 is a negative co-stimulatory molecule on T_{regs} and $CD4^+$ T cells whose expression is up-regulated upon activation (73). Primary splenocytes were antibody stimulated in the presence of 50µM, 30µM, 10µM ATR or the ethanol only vehicle control (Figure 8). As seen in Figure 8, there is a dose-dependent increase in CD62L, indicating that at higher ATR concentrations fewer T cells were activated. Upon treatment with 50 µM ATR, 66.5% of cells expressed CD62L whereas with the ethanol control only 22.8% were $CD62L^+$. In addition, 50µM of ATR significantly reduced T cell recovery from the cultures (data not shown). The 30µM ATR treatment showed a moderate ATR phenotype with 53.3% of cells being $CD62L^+$, while 10µM ATR treatment resulted in a minimal ATR effect with 38.3% $CD62L^+$. Based upon these and previous observations from the Wetzel laboratory, 30µM of ATR was chosen for subsequent experiments, unless otherwise noted, because it resulted in the most severe phenotype without significantly increasing cell death (data not shown). Treatment with 30µM of ATR decreased expression of PD-1, with only 66.8% of $CD4^+$ T cells expressing PD-1 compared to 92.7% in the ethanol only control (Figure 9). The

results from Figures 8 and 9 are consistent with our previous CD25 and CD69 data that showed that ATR exposure inhibited $CD4^+$ T cell activation *in vitro*.



Treatment	MFI	% CD62L +
50 uM ATR	319	66.5%
30 uM ATR	204	53.3%
10 uM ATR	161	38.3%
EtOH	91.3	22.8%

Figure 8: *In vitro* ATR exposure inhibits CD62L down-modulation in a dose-dependent manner. Cells were stimulated with anti-CD3ε and anti-CD28. Antibodies and gated on $CD4^+$ T cells. B10.BR splenocytes were exposed to 50 µM (green), 30 µM (orange), or 10µM (blue) ATR or the ethanol vehicle control (shaded grey). CD62L expression on Day 4 is displayed in the left panel. The region marker indicates the $CD62L^+$ population. Table indicates the Mean Fluorescence Intensity (MFI) and the frequency of $CD62L^+$ cells. Data representative of two separate experiments.

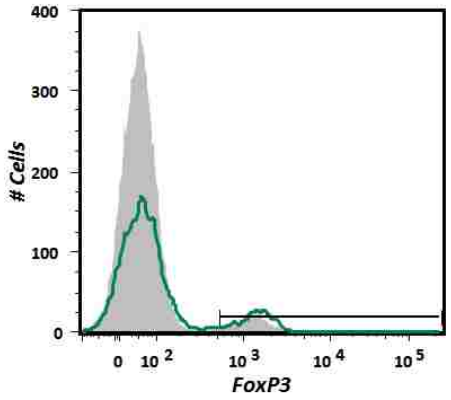


Treatment	MFI	% PD-1+
30 µM ATR	192	66.8%
EtOH	433	92.7%

Figure 9: *In vitro* ATR exposure inhibits PD-1 expression. Cells were stimulated with plate bound antibodies and gated on $CD4^+$ $V\beta3^+$ T cells. AD10 splenocytes were exposed to 30µM ATR (orange) or the ethanol vehicle control (shaded grey). PD-1 expression on Day 4 is displayed in the left panel. The region marker indicates the $PD\text{-}1^+$ population. Table indicates the Mean Fluorescence Intensity (MFI) and the frequency of $PD\text{-}1^+$ cells. Data representative of three separate experiments.

PTX causes an increase in the frequency of Foxp3$^+$ CD4$^+$ T cells

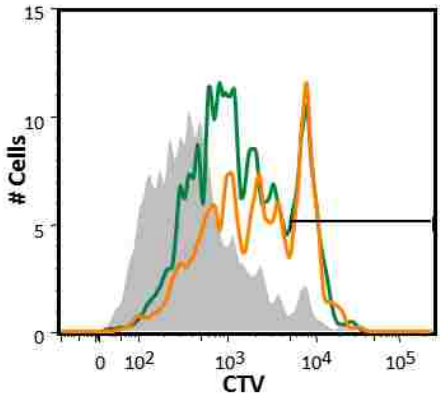
On a biochemical level, atrazine is a potent phosphodiesterase inhibitor, which results in significant increases in cAMP (16). Previous experiments used the chemical PDE inhibitor pentoxyfilline (PTX) and cell permeant, non-cleavable cAMP analog dibutyryl cAMP (dbc-AMP) to increase the level of intracellular cAMP to assess the role of elevated cAMP in the ATR-associated T cell phenotype (Figure 6). Those experiments showed that the ATR phenotype of decreased T cell activation and proliferation could partially, but not completely, be replicated by elevating cAMP levels pharmacologically. The frequency of Foxp3$^+$ cells was not previously assessed. Here we observed that the increase in Foxp3$^+$ regulatory T cells (T_{regs}) seen with ATR treatment was replicated by PTX as seen in Figure 10. The CD4$^+$ T cells from the 250µM PTX exposed cultures had 2.3 times more Foxp3$^+$ CD4$^+$ T cells compared to the ethanol control. This is comparable to, and consistent with, the 2-5 fold increase in Foxp3$^+$ cells seen in cultures treated with 30µM ATR.



Treatment	MFI	% Foxp3+
250 uM PTX	95.3	12.3%
EtOH	64.6	5.4%

Figure 10: *In vitro* PTX exposure increases the frequency of Foxp3$^+$ T cells. AD10 splenocytes were exposed to 250µM PTX (green) or the ethanol vehicle control (shaded grey). Foxp3 expression on day 4, CD4$^+$Vβ3$^+$ gated T cells is displayed in the left panel. The region marker indicates the Foxp3$^+$ population. Table indicates the Mean Fluorescence Intensity (MFI) and the frequency of Foxp3$^+$ cells. Data representative of two separate experiments.

To examine the underlying mechanism for the observed 2-5 fold increase in the frequency of Foxp3$^+$ cells in the PTX and ATR cultures, we examined the proliferation of the T_{regs}. The Foxp3$^+$ CD25$^+$ T_{regs} proliferated, although not to the same extent as cells in the ethanol-only control (Figure 11). Since the proliferation of T_{regs} is less than the ethanol controls but the frequency of T_{regs} is increased 2-5 fold, the increase in the T_{reg} population is not due solely to T_{reg} proliferation. The increase in Foxp3$^+$ T_{reg} frequency, therefore, is likely due to a combination of the expansion of natural, thymus-derived T_{reg} (nT_{reg}) and conversion of CD4$^+$ T_{eff} cells into T_{regs} (induced T_{regs}, iT_{reg}).



Treatment	MFI	% Undivided
30 uM ATR	1886	29.2%
250 uM PTX	1425	22.9%
EtOH	441	5.1%

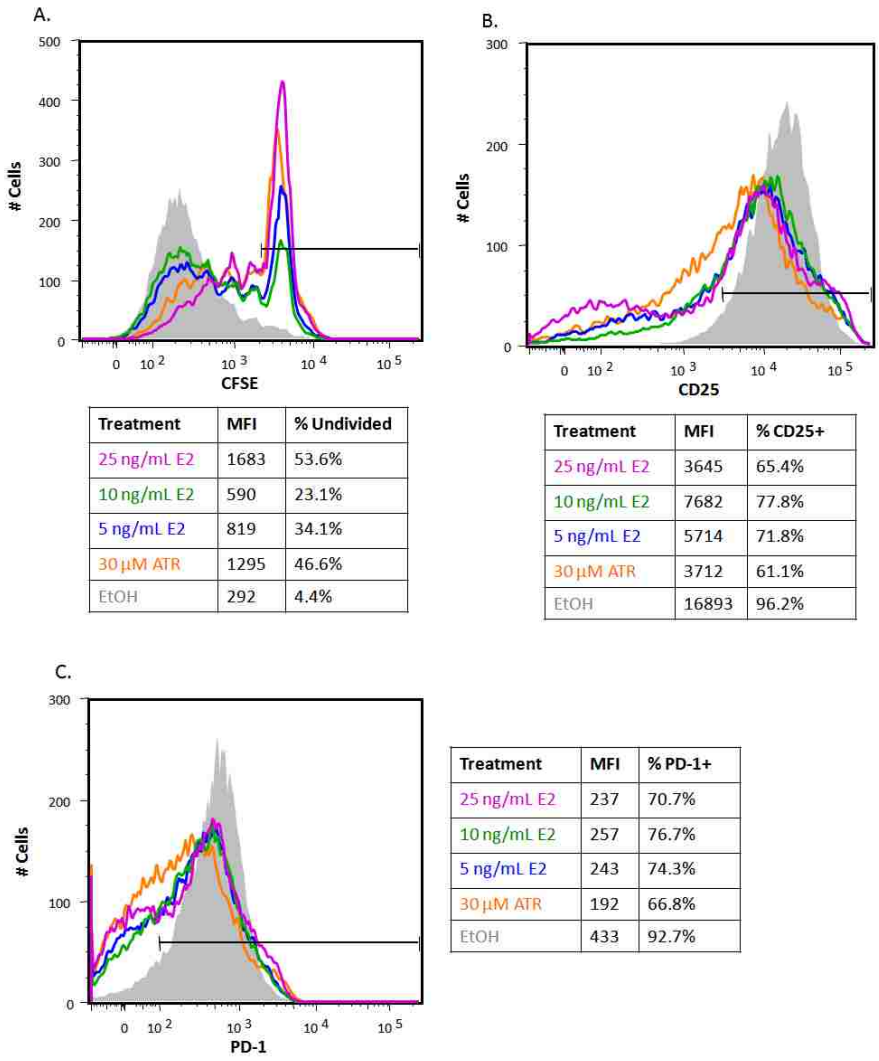
Figure 11: *In vitro* ATR and PTX exposure decreases proliferation of CD25$^+$ Foxp3$^+$ CD4$^+$ T cells. AD10 splenocytes were exposed to 250µM PTX (green) or 30µM (orange) ATR or the ethanol vehicle control (shaded grey). Proliferation of CD4$^+$Vβ3$^+$ CD25$^+$ Foxp3$^+$ gated T cells on day 4 is displayed in the left panel. Cells were stained with CTV to monitor proliferation. The region marker indicates the undivided population. Percent undivided indicates cells that have not divided or have divided only a few times. Table indicates the Mean Fluorescence Intensity (MFI) and frequency of undivided cells. Data representative of two separate experiments.

Estrogen causes a decrease in proliferation and activation of CD4$^+$ T cells.

Our previous results show that cells from male mice are more severely affected by ATR exposure than cells from female mice (Figure 7). Combined with differences in proliferation and activation upon treatment with PTX and ATR, this suggested that increased cAMP was not solely responsible for the observed ATR effects. Because of

the observed male/female difference and the fact that ATR is an estrogen disrupting compound that is known to elevate estrogen levels (74), we explored the possibility that estrogen could be involved in the atrazine-mediated effects. To determine whether estrogen could affect the activation of $CD4^+$ T cells, estrogen was added to the cell cultures at 5 ng/ml, 10 ng/ml and 25 ng/ml on day 0. The results in Figure 12 confirm that elevated estrogen can inhibit $CD4^+$ T cell activation and proliferation. Figure 12A shows that 25ng/ml estrogen decreased T cell proliferation 12-fold, which is comparable to the effects of 30µM ATR. The addition of 10ng/ml and 5ng/ml of estrogen also decreased proliferation (5 fold and 7.8 fold, respectively), although not as severely as 25ng/ml. Interestingly, 5ng/ml of estrogen was slightly more potent than 10ng/ml, consistent with non-monotonic effects (14).

In addition to proliferation, we examined the activation of T cells in the presence of estrogen. As seen in Figure 12B, 25ng/ml reduced the frequency of $CD25^+$ cells. This is similar to the effects observed with 30µM ATR. The 5ng/ml and 10ng/ml estrogen treatments also decreased the MFI of CD25 and increased the frequency of $CD25^-$ cells, but not as severely as 25ng/ml. Figure 12C shows that all three estrogen concentrations tested also decreased expression of PD-1 compared to the ethanol only control. As with proliferation and CD25 expression results, the frequency of $PD\text{-}1^+$ cells in the 25ng/ml group was comparable to that seen with the 30µM ATR treatment. Together, the data from Figure 12 showed 25ng/ml of estrogen decreased T cell activation and proliferation to a comparable extent with 30µM ATR. This finding supports the hypothesis that estrogen is contributing to the observed ATR phenotype.



Treatment	MFI	% Undivided
25 ng/mL E2	1683	53.6%
10 ng/mL E2	590	23.1%
5 ng/mL E2	819	34.1%
30 µM ATR	1295	46.6%
EtOH	292	4.4%

Treatment	MFI	% CD25+
25 ng/mL E2	3645	65.4%
10 ng/mL E2	7682	77.8%
5 ng/mL E2	5714	71.8%
30 µM ATR	3712	61.1%
EtOH	16893	96.2%

Treatment	MFI	% PD-1+
25 ng/mL E2	237	70.7%
10 ng/mL E2	257	76.7%
5 ng/mL E2	243	74.3%
30 µM ATR	192	66.8%
EtOH	433	92.7%

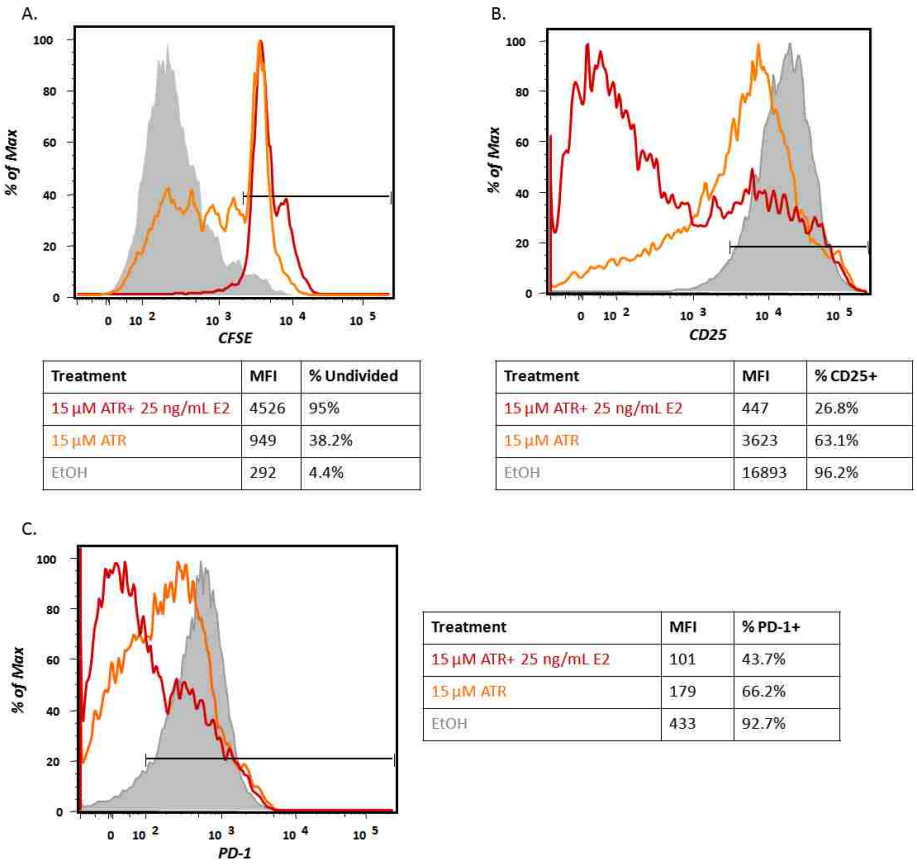
Figure 12: *In vitro* estrogen exposure causes decreased proliferation and activation. AD10 splenocytes were exposed to 25ng/ml (pink), 10ng/ml (green), 5ng/ml (blue) or 30µM (orange) ATR or the ethanol vehicle control (shaded grey). A. Cells were stained with CFSE to monitor proliferation. Estrogen was diluted with PBS. Table below shows MFI and percentage of undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. B and C. CD25 and PD-1 were used to show T cell activation within each treatment group. Table below B shows MFI and percentage of $CD25^+$ T cells. Table to the right of C shows MFI and percentage of $PD\text{-}1^+$ T cells. Cells were stimulated with plate-bound anti-CD3ε and anti-CD28. Data representative of 2 separate experiments.

Estrogen synergizes with Atrazine to further decrease $CD4^+$ T cell activation and proliferation

Our previous results show that use of pharmacological reagents that increase cAMP and addition of exogenous estrogen mimicked the ATR effects on $CD4^+$ T cells. Since it would be expected, based upon its biochemical activities, that ATR treatment

would lead to increases in both cAMP and estrogen, we next combined these treatments to see if there was any additive or synergistic effects. Cells were activated in the presence of a suboptimal concentration of ATR (15µM) with or without addition of 25ng/ml of estrogen. As seen in Figure 13, 15µM ATR treatment resulted in 38.2% of cells remaining undivided. Addition of 25ng/ml of estrogen to the 15µM ATR cultures inhibited prolferation in 95% of the cells. By comparison, 53.6% of undivided cells in 25ng/ml estrogen cultures alone did not divide (Figures 12A and 13A). The significant increase in the percentage of undivided cells in the cultures containing 15µM ATR and 25ng/ml estrogen are indicative of a synergistic effect between these compounds.

In additon to examining the effects on proliferation, the effects of 15µM ATR and 25ng/ml estrogen on the activation state of $CD4^+$ T cells, as measured by CD25 and PD-1 expression, was also assessed. As with proliferation, addition of 25mg/ml estrogen with 15µM ATR decreased expression levels of CD25 by an additional 8 fold and PD-1 by 1.5 fold on the activated $CD4^+$ T cells compared to 15µM ATR alone (Figures 13B & 13C). The region marker in Figure 13B indiates the $CD25^+$ T cell population. Upon treatment with 15µM ATR, 63.1% of the cells were $CD25^+$ while the addition of 25ng/ml of estrogen to 15µM ATR reduced that to 26.8% $CD25^+$ T cells (Figure 13B). As with the frequency of $CD25^+$ cells, the frequency of PD-1^+ cells was 1.5 fold lower in the 25ng/ml estrogen and 15µM ATR treatment group (43.7% PD-1^+) compared to the 15µM ATR treatment (66.2% PD-1^+) or 25ng/ml estrogen treatment (70.7%, Figure 12C). The decreased levels of CD25 and PD-1 in the culture containing estrogen and atrazine, along with the proliferation data in Figure 13A, show that estrogen can further decrease proliferation and activation in the presence of atrazine.



Treatment	MFI	% Undivided
15 µM ATR+ 25 ng/mL E2	4526	95%
15 µM ATR	949	38.2%
EtOH	292	4.4%

Treatment	MFI	% CD25+
15 µM ATR+ 25 ng/mL E2	447	26.8%
15 µM ATR	3623	63.1%
EtOH	16893	96.2%

Treatment	MFI	% PD-1+
15 µM ATR+ 25 ng/mL E2	101	43.7%
15 µM ATR	179	66.2%
EtOH	433	92.7%

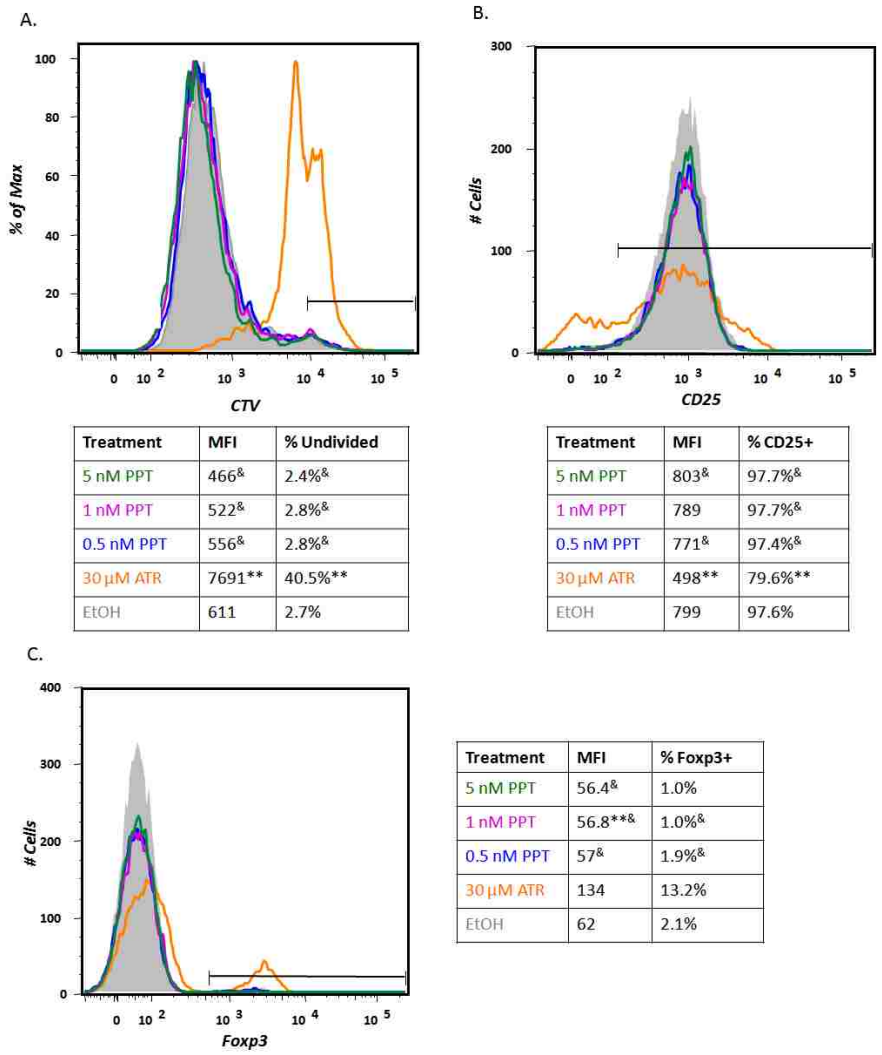
Figure 13: Cells cultured with Atrazine + estrogen display decreased proliferation and activation. B10.BR splenocytes were exposed to 25ng/ml estrogen (pink), 15µM ATR + estrogen (red), 15µM ATR (orange), or an EtOH only control (shaded grey). Cells were stimulated with plate-bound anti-CD3ε and anti-CD28 and for 4 days and then gated on live $CD4^+$ T cells. A. Cells were stained with CFSE to monitor proliferation (left panel). Table shows MFI and percentage of undivided cells. Region markers indicated percentage of undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. B and C. CD25 and PD-1 expression on cells from the cultures in A. Tables indicate MFI and percent of $CD25^+$ and $PD\text{-}1^+$ cells (right panels). Region markers indicate percent of $CD25^+$ and $PD\text{-}1^+$ cells. Data representative of 2 separate experiments.

Signaling through GPER 1, but not ERα, mimics the decreased activation and proliferation seen with Atrazine

Figures 12 and 13 suggest that elevated estrogen levels inhibit $CD4^+$ T cell activation and proliferation and that this might play a role in the observed ATR phenotype. To begin to understand this potential mechanism, we next examined which estrogen receptor(s) might be involved. Previously published reports have suggested both estrogen receptor α (ERα) and the G-protein coupled estrogen receptor (GPER-1)

are involved in estrogen-mediated control of T cell activity (75, 66).' We began by culturing cells in the presence of 5nM, 1nM, or 0.5nM of the selective ERα agonist PPT to determine if signaling through ERα could inhibit proliferation and/or activation. PPT has an EC 50 of 200pM and is 410 times more selective for ERα over estrogen receptor β (ERβ) (76).

As shown in Figure 14, PPT treatment resulted in no significant changes in $CD4^+$ T cell proliferation, CD25 expression, or the frequency of $Foxp3^+$ T cells. The $CD4^+$ T cells in the PPT cultures proliferated nearly identically to the ethanol control (Figure 14A) and had similar CD25 expression (Figure 14B). The frequency of Foxp3 was slightly lower than the ethanol control although the difference was not significant (Figure 14C). In contrast, the 30μM ATR treatment left 40.5% of cells undivided, reduced the frequency of $CD25^+$ T cells by 1.2 fold and increased the frequency of $Foxp3^+$ T cells more than 6-fold. Based on the data from Figure 14, ERα stimulation does not appear to mimic the ATR effects seen on $CD4^+$ T cells.



Treatment	MFI	% Undivided
5 nM PPT	466[&]	2.4%[&]
1 nM PPT	522[&]	2.8%[&]
0.5 nM PPT	556[&]	2.8%[&]
30 μM ATR	7691**	40.5%**
EtOH	611	2.7%

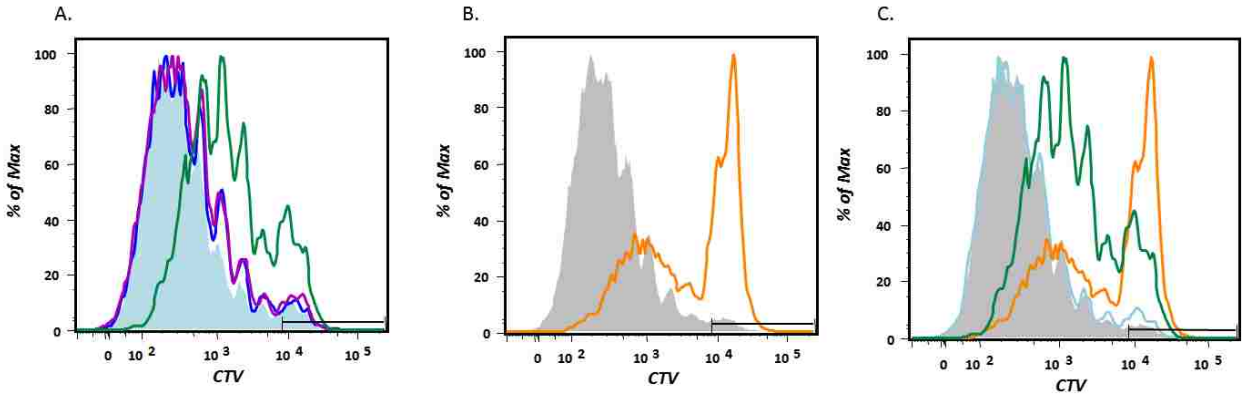
Treatment	MFI	% CD25+
5 nM PPT	803[&]	97.7%[&]
1 nM PPT	789	97.7%[&]
0.5 nM PPT	771[&]	97.4%[&]
30 μM ATR	498**	79.6%**
EtOH	799	97.6%

Treatment	MFI	% Foxp3+
5 nM PPT	56.4[&]	1.0%
1 nM PPT	56.8**[&]	1.0%[&]
0.5 nM PPT	57[&]	1.9%[&]
30 μM ATR	134	13.2%
EtOH	62	2.1%

Figure 14: Cells cultured with the ERα agonist PPT did not display decreased proliferation and activation. AD10 splenocytes were exposed to 5nM PPT (green), 1nM PPT (pink), 0.5nM PPT (blue), 30μM ATR (orange), or the EtOH control (shaded grey). Cells were gated on $CD4^+$ $V\beta3^+$ T cells. A. Cells were stained with CTV to monitor proliferation (left panel). Table shows MFI and percentage of undivided cells. Region marker indicates percentage of undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. B. CD25 was used to indicate T cell activation (left panels). Table indicates MFI and percent of $CD25^+$ cells (right panel). Region marker indicates percent of $CD25^+$ cells. C. Foxp3 was stained using Biolegend's Foxp3 staining kit (left panel). Table indicates MFI and percent of $Foxp3^+$ cells (right panel). Region marker indicates $Foxp3^+$ cells. Data representative of 2 separate experiments. ** p≤0.05 compared to EtOH, [&] p≤0.05 compared to ATR

Since triggering ERα using PPT didn't affect activation or proliferation of the $CD4^+$ T cells, we next examined the potential role of GPER-1. The GPER-1 agonist, G-1, has been shown to decrease the severity of EAE in mice by increasing the production

of anti-inflammatory cytokines (65). Since GPER1 triggering has immunosuppressive effects, it is possible that it may be playing a role in the observed ATR-associated, $CD4^+$ T cell inhibition. To test this possibility, spleen cells were treated with 10nM, 100nM, 1µM G-1 or DMSO vehicle control. During the 4 day activation culture. As seen in Figure 15A, treatment with 10nM and 100nM of G-1 did not affect $CD4^+$ T cell proliferation. However, treatment with 1µM G-1 increased the frequency of non-dividing $CD4^+$ T cells by approximately 4 fold compared to the DMSO control. In comparison, the atrazine control increased the frequency of dividing cells by approximately 22 fold compared to the ethanol control (Figure 15B). The effects of 1µM G-1 data are similar to the results of treatment with the suboptimal 3µM concentration of ATR (data not shown). G-1 can mimic the effects we see with lower concentrations of ATR suggesting that it has inhibitory effects on $CD4^+$ T cells. A direct comparison of $CD4^+$ T cell proliferation in the 1µM G-1 and ATR cultures is shown in Figure 15C. The mean fluorescence intensity (MFI) of 1µM G-1 and 30µM ATR were statistically significant compared to their respective vehicle controls. It is interesting to note that $CD4^+$ T cell recovery was decreased in both 1µM G-1 and ATR cultures.

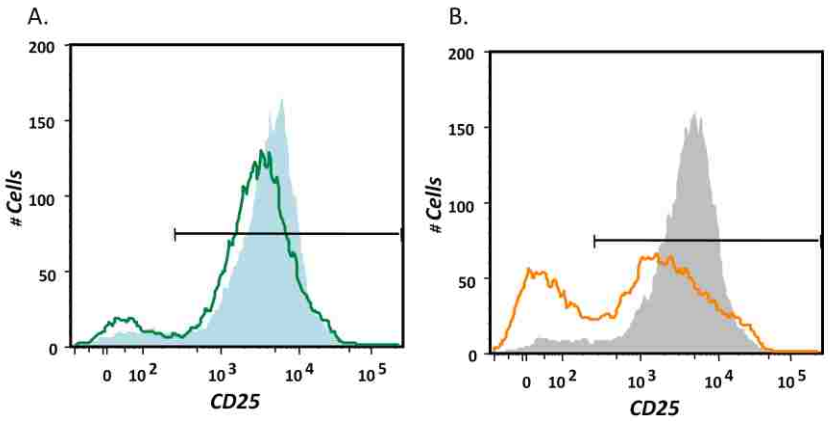


Treatment	MFI	% Undivided
1 µM G1	1470*&	13.7%
100 nM G1	432&	4.4%&
10 nM G1	419&	3.9%&
DMSO	361	3.5%

Treatment	MFI	% Undivided
30 µM ATR	3807**	46.6%**
EtOH	335	2.1%

Figure 15: Cells cultured with the GPER-1 agonist, G-1, display decreased proliferation. Cells were stimulated with 2.5µM MCC peptide and gated on $CD4^+$ $V\beta3^+$ T cells. AD10 splenocytes were exposed to 1µM G-1 (green), 100nM G-1 (pink), 10nM G-1 (blue), 30µM ATR (orange), an EtOH control (shaded grey) or a DMSO vehicle control (shaded cyan, cyan line in Part C). Cells were stained with CTV to monitor proliferation. A and B. Tables shows MFI and percentage of undivided cells. Region markers indicate percentage of undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. C. Overlay of 1µM G-1 and 30µM ATR treatment groups with EtOH and DMSO controls. Data representative of 2 separate experiments. * p≤0.05 compared to DMSO, ** p≤0.05 compared to EtOH, & p≤0.05 compared to ATR

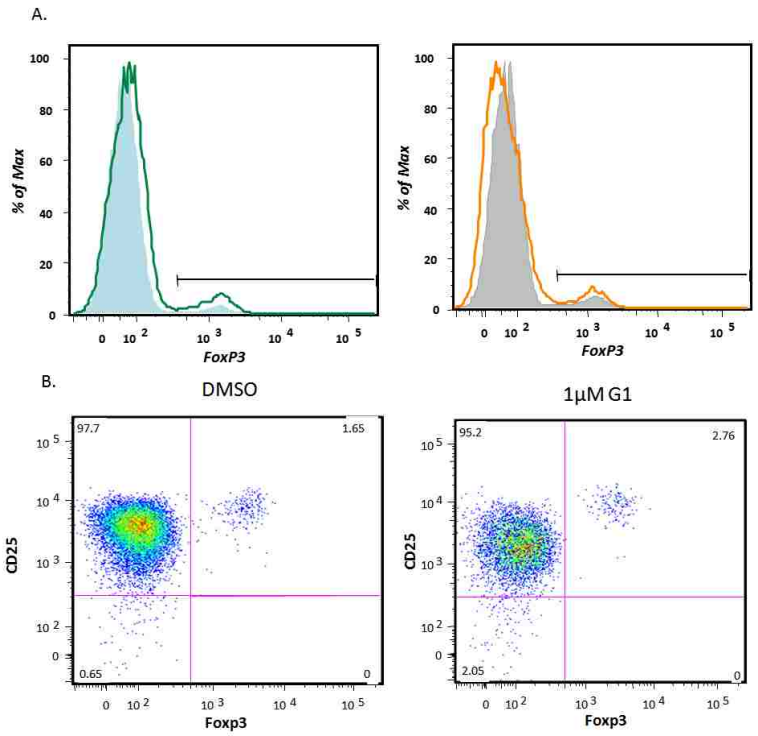
We next assessed the role GPER-1 signaling may have on T cell activation by looking at CD25 expression. Figure 16A shows that exposure to 1µM G-1 did not significantly alter T cell activation, as measured by the frequency of $CD25^+$ $CD4^+$ T cells. There were 90.3% $CD25^+$ T cells in the G-1 culture compared to 93.5% for the DMSO control. In comparison, 30µM ATR treatment resulted in a bimodal population with 34% of cells being $CD25^-$ (Figure 16B). GPER-1 signaling with G-1 does not significantly decrease expression of CD25 on $CD4^+$ T cells, suggesting that is does not directly impact T cell activation.



Treatment	MFI	% CD25+
1 µM G1	2190*	90.3%
DMSO	3109	93.5%
30 µM ATR	688**	66.1%**
EtOH	3208	94.5%

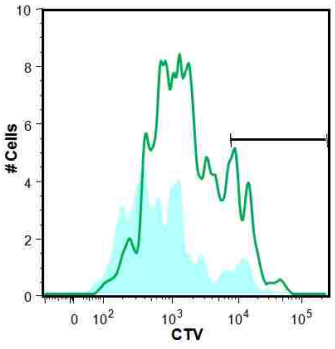
Figure 16: Treatment with 1µM G-1 (left, A) and ATR (middle, B) decrease the expression of CD25 in the cell culture. Cells were gated on $CD4^+ V\beta3^+$ T cells. AD10 splenocytes were exposed to 1µM G-1 (green), 30µM ATR (orange), an EtOH control (shaded grey) or a DMSO vehicle control (shaded cyan). Table on the right shows MFI and percentage of $CD25^+$ cells. Region markers indicate $CD25^+$ cells. *p≤0.05 compared to DMSO, ** p≤0.05 compared to EtOH, $^{\&}$ p≤0.05 compared to ATR

We also examined whether 1µM G-1 treatment would alter the frequency of $Foxp3^+$ $CD4^+$ T cells. Figure 17A shows that treatment with G-1 increased the frequency of $Foxp3^+$ $CD4^+$ T cells 2 fold (7.3% compared to 3.4%). Unfortunately the increase in $Foxp3^+$ cells upon treatment with G-1 was variable, as seen in Figure 17B. In some experiments, G-1 did not significantly alter $Foxp3^+$ cell frequencies. Thus, while we can confidently conclude that 1µM G-1 inhibits $CD4^+$ T cell proliferation, we cannot conclude that GPER-1 stimulation with G-1 increases the frequency of $CD4^+$ $Foxp3^+$ T_{regs}.



Treatment	MFI	% Foxp3+
1 µM G1	88[&]	7.26%
DMSO	64.9	3.41%
30 µM ATR	65.6	6.26%
EtOH	71.5	4.88%

Figure 17: Varied effects of Foxp3 expression upon treatment with 1µM G-1. Cells were gated on $CD4^+$ $V\beta3^+$ T cells. AD10 splenocytes were cultured with 1µM G-1 (green), 30µM ATR (orange), DMSO control (shaded cyan), or EtOH control (shaded grey). A. 1µM G-1 (left) and 30µM ATR (right) increased the frequency of $Foxp3^+$ T cells (left panel). Table indicates MFI and percent of $Foxp3^+$ cells. Region marker indicates $Foxp3^+$ cells. B. Treatment with 1µM G-1 did not significantly increase the frequency of $Foxp3^+$ $CD25^+$ T_{regs} compared to the DMSO control. Data representative of 2 separate experiments. *p≤0.05 compared to DMSO, ** p≤0.05 compared to EtOH, [&] p≤0.05 compared to ATR



Treatment	MFI	% Undivided
1uM G1	1773	16.3%
DMSO	762	8.8%

Figure 18: *In vitro* G-1 exposure slightly decreases proliferation of $CD25^+$ $Foxp3^+$ $CD4^+$ T cells. Cells were gated on $CD4^+V\beta3^+$ $CD25^+$ $Foxp3^+$ T cells. AD10 splenocytes were exposed to 1µM G-1 (green) or DMSO (shaded cyan). Proliferation on Day 4 is displayed in the left panel. Cells were stained with CTV to monitor proliferation. The region marker indicates the undivided population. Percent undivided indicates cells that have not divided or have divided only a few times. Table indicates the Mean Fluorescence Intensity (MFI) and frequency of undivided cells. Data representative of two separate experiments.

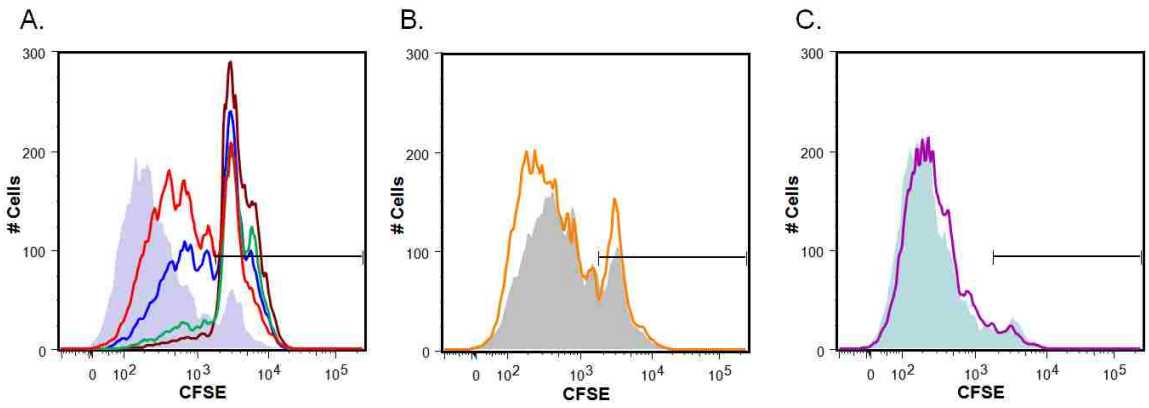
Since we observed a slight defect in T_{reg} proliferation upon treatment with PTX and ATR (Figure 11), we assessed whether this defect was present in 1µM G-1 treated cultures. As seen in Figure 18, T_{reg} proliferation was slightly decreased (1.86 fold increase in percentage of undivided cells, 2.3 fold higher MFI) compared to the DMSO control. As seen in Figure 11, PTX and ATR increased the MFI of proliferation by 3-4 fold indicating that T_{regs} underwent fewer divisions than the ethanol control. The difference in T_{reg} proliferation upon GPER-1 triggering with G-1 was comparable to the differences seen in T_{reg} proliferation upon treatment with ATR and PTX. Thus, in experiments where G-1 treatment increased the frequency of $Foxp3^+$ $CD4^+$ T cells, it negatively affected T_{reg} proliferation.

The GPER-1 agonists, G-15 and G-36, do not alleviate the ATR-mediated effects on $CD4^+$ T cells

To further investigate the potential of GPER-1 signaling in ATR treated cultures, we antagonized GPER-1 with the antagonist G-15. We reasoned that if GPER-1 was involved in the ATR-mediated decrease in activation and proliferation then antagonizing the receptor should alleviate the effect. Since ATR is still inhibiting PDE and elevating cAMP, we predicted that there would be a small phenotypic rescue instead of a complete reversal of the ATR phenotype. In these experiments we used 15µM ATR rather than 30µM because it gave a more moderate phenotype, which would likely be more sensitive to GPER-1 blockade than the much more severe 30µM treatment phenotype. Cells were stimulated with anti-CD3 and anti-CD28 in the presence of 15µM ATR and various concentrations of the GPER-1 antagonist G-15 for four days. Of note, upon analysis of the vehicle controls, the antibody stimulation resulted in suboptimal T

cell stimulation (Figures 19 and 20) since proliferation and CD69 expression were slightly inhibited compared to antigen stimulated cultures (Figures 14,15,and 16).

Contrary to the prediction, the results in figures 19 and 20 show that addition of G-15 to 15µM ATR actually potentiated the ATR phenotype rather than reversing the ATR effects. Treatment with 15µM ATR typically gives an intermediate phenotype, but when combined with G-15, it mimicked the 30µM ATR results. The dose response in the G-1 and 15µM ATR cultures showed that 10nM G-15 + 15µM ATR inhibited $CD4^+$ T cell proliferation 2.8 fold more than the 15µM ATR + DMSO control (Figure 19). Since G-15 has been shown to lose GPER-1 selectivity at higher concentrations (77), the 1µM G-15 treatment may be triggering different estrogen pathways causing decreased severity compared to lower G-15 concentrations. The increased severity of 10nM G-15 in 15µM ATR cultures may also be the result of a non-monotonic (14) dose response since its K_i is relatively low (K_i=20nM) (78). These results indicate that treatment with G-15 increased the severity of our ATR treatment, but higher concentrations of G-15 were less potent. Inhibiting GPER-1 with G-15 in the absence of atrazine had no adverse effect on T cell proliferation (5.5% undivided) compared to the DMSO control (7% undivided, Figure 19C).



Treatment	MFI	% Undivided
1µM G15+ 15µM ATR	1461	51.7%
100nM G15+ 15µM ATR	2832	80.4%
10nM G15+ 15µM ATR	3615	92.4%
1µM G15 only	283	5.5%
15µM ATR	520	21%
15µM ATR+ DMSO	862	32.5%
EtOH	602	20.7%
DMSO	260	7%
EtOH+ DMSO	322	12.2%

Figure 19: The GPER-1 agonist (G-15) in the presence of ATR, severely inhibits CD4$^+$ T cell proliferation. Cells were stimulated with plate bound anti-CD3ε and anti-CD28 antibodies and gated on CD4$^+$ T cells. B10.BR splenocytes were cultured with 1µM G-15 + 15µM ATR (blue), 100nM G-15 + 15µM ATR (green), 10nM G-15 + 15µM ATR (brown), 1µM G-15 only (pink), 15µM ATR (orange), 15µM ATR + DMSO (red), EtOH (shaded grey), DMSO (shaded cyan), or EtOH + DMSO (light purple). A. Cells were stained with CTV to monitor proliferation. Treatment with G-15 + ATR decreased the proliferation of CD4$^+$ T cells more than ATR treatment alone (Part B). C. Treatment with 1µM G-15 alone had no adverse effects on CD4$^+$ T cells. Table shows MFI and percentage of undivided cells. Region markers indicate percent of undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. Data representative of 2 separate experiments.

In addition to inhibiting proliferation, treatment with 15µM ATR and G-15 also inhibited CD69 expression (Figure 20), with 10nM G-15 and 15µM ATR having the fewest CD69$^+$ CD4$^+$ T cells and a significantly reduced per cell CD69 expression (MFI) (Figure 20A). Treatment with 10nM G-15 + 15µM ATR increased the frequency of CD69$^-$ CD4$^+$ T cells 4.9 fold while 1µM G-15 + 15µM ATR increased the frequency of CD69$^-$ CD4$^+$ T cells by 2.1 fold compared to the 15µM ATR + DMSO control. 100nM G-15 and 15µM ATR gave an intermediate phenotype between the 10nM G-15 and 15µM ATR and 1µM G-15 and 15µM ATR cultures. Again, this is consistent with a non-

monotonic dose-response curve. The frequency of $CD69^-$ cells in the G-15 treated cultures was also much higher than the frequency $CD69^-$ cells in the 15µM ATR (17.4%, Figure 20B) and EtOH + DMSO control (27.2%, Figure 20A) cultures. Treatment with 15µM ATR and DMSO did not alter the 15µM ATR effect showing that the increased severity caused by 15µM ATR and G-15 is not due to addition of DMSO into ATR cultures, but is due to the G-15. Interestingly, treatment with 1µM G-15 alone did not affect $CD4^+$ T cell proliferation or activation showing that antagonizing GPER-1 with G-15 in the absence of ATR had no effect on $CD4^+$ T cells (Figure 20C). Figures 19 and 20 suggest that G-15 treatment did not antagonize GPER-1. This may have been due to and non-specific binding of G-15 to other estrogen receptors on the $CD4^+$ T cells.

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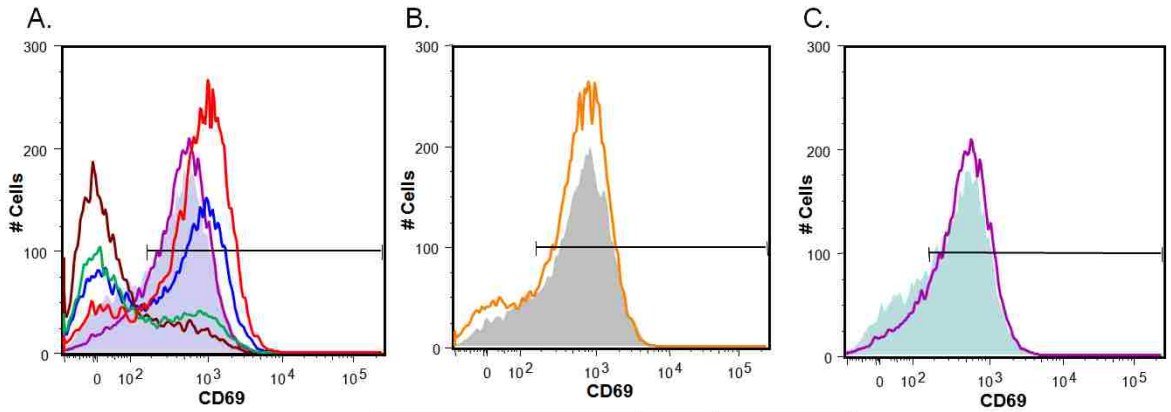


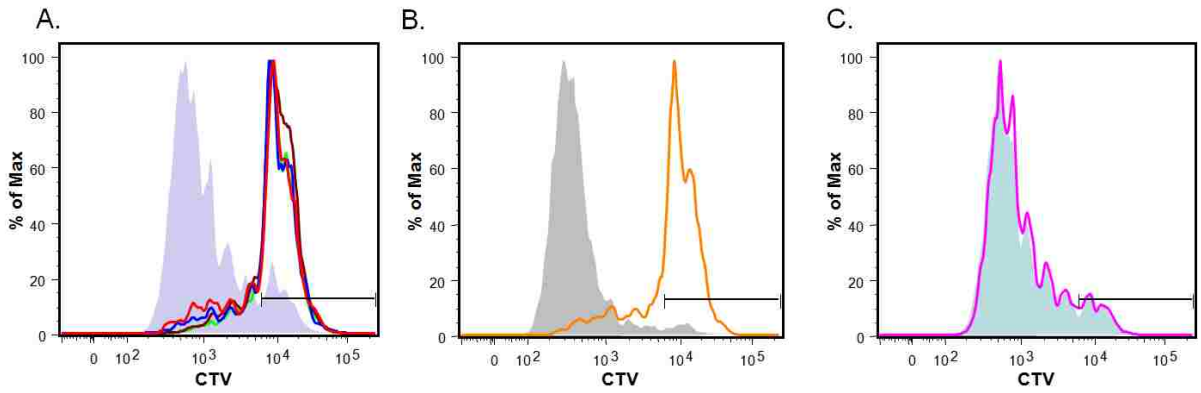
Treatment	MFI	% CD69+
1μM G15+ 15μM ATR	300	65.2%
100nM G15+ 15μM ATR	119	36.9%
10nM G15+ 15μM ATR	55.6	20.6%
1μM G15 only	396	83.6%
15μM ATR	465	82.6%
15μM ATR+ DMSO	557	83.5%
EtOH	510	83.9%
DMSO	304	72.8%
EtOH+ DMSO	314	72.8%

Figure 20: The GPER-1 agonist, G-15, severely inhibits CD69 expression. Cells were stimulated with plate bound anti-CD3ε and anti-CD28 antibodies and gated on $CD4^+$ T cells. B10.BR splenocytes were cultured with 1μM G-15 +15μM ATR (blue), 100nM G-15 + 15μM ATR (green), 10nM G-15 + 15μM ATR (brown), 1μM G-15 only (pink), 15μM ATR (orange), 15μM ATR + DMSO (red), EtOH (shaded grey), DMSO (shaded cyan), or EtOH + DMSO (light purple). A. Treatment with G-15 + ATR decreased the activation of $CD4^+$ T cells more than ATR treatment alone (Part B). C. Treatment with 1μM G-15 alone had no adverse effects on $CD4^+$ T cells. Table shows MFI and percentage of $CD69^+$ cells. Region markers indicate $CD69^+$ cells. Data representative of 2 separate experiments.

While the G-15 data was initially difficult to decipher, the structure of G-15 allows it to interact with ERα and ERβ, triggering them while blocking GPER-1 (77). Thus drawing conclusions from the G-15 results is difficult. To better test the role of GPER1 by antagonizing the receptor we used a new, selective GPER-1 antagonist, G-36, that was recently synthesized and became available in April 2014 (77). G-36 has an additional functional group that has been shown to limit interaction with other estrogen receptors (77). The G-15 GPER1 antagonism experiments were repeated with G-36

although 30μM ATR was used instead of 15μM ATR in order to allow for a direct comparison with our well characterized ATR phenotype.

As with G-15, when G-36 was added to cultures containing 30μM ATR, it did not reverse the ATR-associated decrease in $CD4^+$ T cell proliferation (Figure 21). With 30μM ATR treatment 76.2% of cells that did not divide (Figure 21B). This was comparable to the 1μM G-36 + 30μM ATR, 500nM G-36 + 30μM ATR, and 10nM G-36 + 30μM ATR treatments (Figure 21A). Treatment with ATR and lower levels of G-36 actually increased the amount of undivided cells, although the increase was not significant. Treatment with 1μM G-36 only did not affect $CD4^+$ T cell proliferation showing that blocking GPER-1 is not detrimental to $CD4^+$ T cell proliferation (Figure 21C).



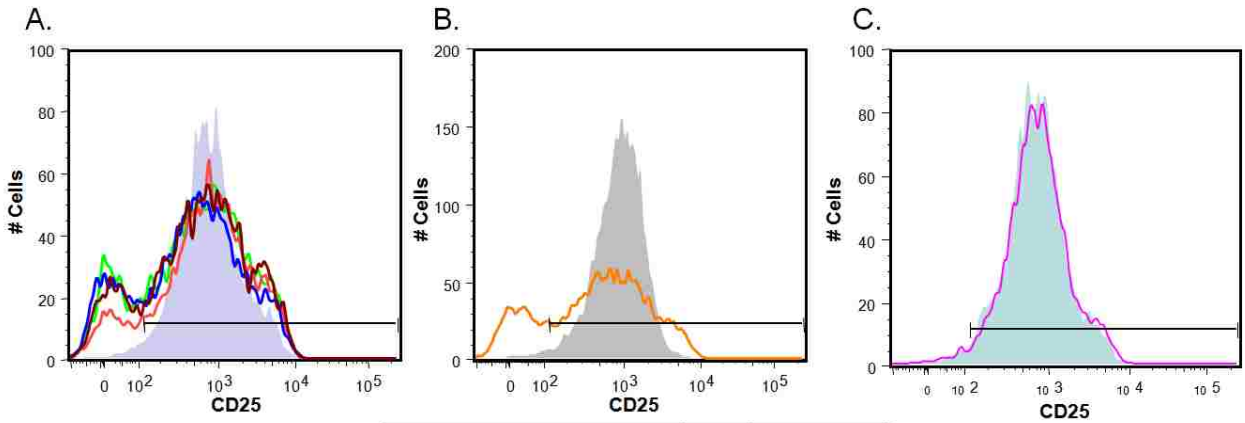
Treatment	MFI	% Undivided
1μM G36+ 30μM ATR	8190	78.2%
500nM G36+ 30μM ATR	9467	82.1%
100nM G36+ 30μM ATR	9394	83.1%
1μM G36 only	1026	9.6%
30μM ATR	7550	76.2%
30μM ATR+ DMSO	7351	73.5%
EtOH	425	2.9%
DMSO	957	9.7%
EtOH+ DMSO	1135	12.3%

Figure 21: The GPER-1 agonist, G-36, does not alter the effects of ATR on CD4$^+$ T cells. Cells were gated on CD4$^+$ Vβ3$^+$ T cells. AD10 splenocytes were cultured with 1μM G-36 + 30μM ATR (blue), 500nM G-36 + 30μM ATR (red), 100nM G-36 + 30μM ATR (green), 1μM G-36 only (pink), 30μM ATR (orange), 30μM ATR + DMSO (brown), EtOH (shaded grey), DMSO (shaded cyan), or EtOH + DMSO (light purple). A. Cells were stained with CTV to monitor proliferation. Treatment with G-36 + ATR did not alleviate the ATR phenotype (Part B). C. Treatment with 1μM G-36 alone had no adverse effects on CD4$^+$ T cells. Table shows MFI and percentage of undivided cells. Region markers indicate undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. Data representative of 3 separate experiments.

Treatment with ATR and G-36 did not alleviate the ATR-mediated reduction in the activation status of the T cells or reduce the frequency of Foxp3$^+$ T cells (Figures 22 and 23). Comparing the frequencies of CD25$^+$ T cells in the 30μM ATR and G-36 cultures to the 30μM ATR control (76.6% CD25$^+$) shows that there is no significant difference between the atrazine treatment and the atrazine + G-36 treatments. Cultures containing 30μM ATR or 30μM ATR and G-36 had about a 1.9 fold decrease in the MFI of CD25 and about a 1.2 fold decrease in the frequencies of CD25$^+$ T cells. The addition of DMSO into ATR treated cultures had no adverse effects on T cell activation as seen

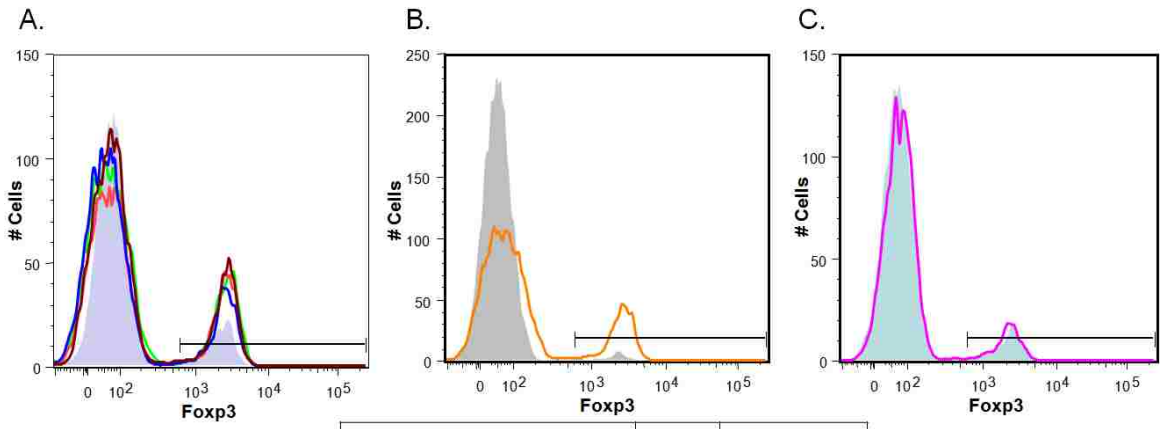
by the 30μM ATR and DMSO control. Treatment with 1μM G-36 alone did not affect the activation status of $CD4^+$ T cells showing that blocking GPER-1 is not detrimental to $CD4^+$ T cell activation. These results show that antagonizing GPER-1 does not significantly alter $CD4^+$ T cell activation in ATR treated cultures.

Treatment with G-36 did not significantly decrease or increase the frequency of $Foxp3^+$ $CD4^+$ T cells compared to the 30μM ATR culture (Figure 23). 30μM ATR increased the frequency of $Foxp3^+$ T_{regs} by 9.6 fold. The DMSO and EtOH + DMSO controls contained higher frequencies of $Foxp3^+$ T cells compared to the ethanol control. It is unclear why the increase of $Foxp3^+$ T cells is higher in DMSO containing cultures, but 30μM ATR and 500nM G-36 still increased the frequency of $Foxp3^+$ T cells by 1.8 fold compared to the EtOH + DMSO control. In this case, the 30μM ATR and 500nM G-36 and 30μM ATR and 100nM G-36 treatments increased the frequency of $Foxp3^+$ T cells more than 30μM ATR alone, although the difference is negligible. It is also interesting to note that T cell recovery was lower in the 30μM ATR and 30μM ATR + G-36 cultures (data not shown). Since 30μM ATR is a potent PDE inhibitor, which can result in increased frequencies of $Foxp3^+$ T cells (Figures 5 and 10), blocking GPER-1 may not be potent enough to reverse the ATR effect. Signaling through GPER-1 can moderately inhibit $CD4^+$ T cell proliferation and activation, but we cannot confidently say that GPER-1 is involved in the ATR-mediated phenotype since we did not see any alleviation of the ATR phenotype upon antagonizing the receptor.



Treatment	MFI	% CD25+
1μM G36+ 30μM ATR	436	78.4%
500nM G36+ 30μM ATR	641	87.1%
100nM G36+ 30μM ATR	498	80.6%
1μM G36 only	739	97.7%
30μM ATR	426	76.7%
30μM ATR+ DMSO	530	82.4%
EtOH	845	98.4%
DMSO	708	97.8%
EtOH+ DMSO	772	97.8%

Figure 22: The GPER-1 agonist, G-36, does not increase CD25 expression in ATR treated cultures. Cells were gated on $CD4^+$ $V\beta3^+$ T cells. AD10 splenocytes were cultured with 1μM G-36 + 30μM ATR (blue), 500nM G-36 + 30μM ATR (red), 100nM G-36 + 30μM ATR (green), 1μM G-36 only (pink), 30μM ATR (orange), 30μM ATR + DMSO (brown), EtOH (shaded grey), DMSO (shaded cyan), or EtOH + DMSO (light purple). A. Treatment with G-36 + ATR had no effect on CD25 expression compared to the 30μM ATR culture (Part B). C. Treatment with 1μM G-36 alone had no adverse effects on $CD4^+$ T cells. Table shows MFI and percentage of $CD25^+$ cells. Region markers indicate $CD25^+$ cells. Data representative of 3 separate experiments.



Treatment	MFI	% Foxp3+
1µM G36+ 30µM ATR	125	18.1%
500nM G36+ 30µM ATR	160	22.7%
100nM G36+ 30µM ATR	154	21.4%
1µM G36 only	112	10.3%
30µM ATR	145	18.8%
30µM ATR+ DMSO	160	21.7%
EtOH	52.3	1.96%
DMSO	100	8.56%
EtOH+ DMSO	120	12.6%

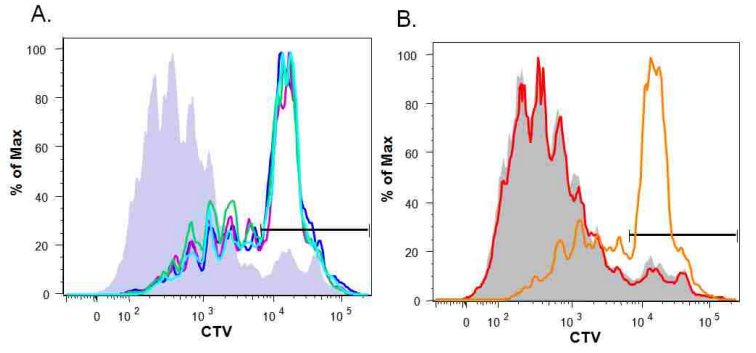
Figure 23: The GPER-1 agonist, G-36, does not affect Foxp3 expression in ATR treated cultures. Cells were gated on $CD4^+ V\beta3^+$ T cells. AD10 splenocytes were cultured with 1µM G-36 + 30µM ATR (blue), 500nM G-36 + 30µM ATR (red), 100nM G-36 + 30µM ATR (green), 1µM G-36 only (pink), 30µM ATR (orange), 30µM ATR + DMSO (brown), EtOH (shaded grey), DMSO (shaded cyan), or EtOH + DMSO (light purple). A. Treatment with G-36 + ATR had no effect on Foxp3 expression compared to the 30µM ATR culture (Part B). C. Treatment with 1µM G-36 alone did not affect Foxp3 expression. Table shows MFI and percentage of $Foxp3^+$ cells. Region markers indicate $Foxp3^+$ cells. Data representative of 3 separate experiments.

The aromatase inhibitor, YM511, does not alleviate the ATR phenotype

Since ATR is known to induce expression of aromatase and subsequently increase levels of estrogen, we cultured cells in 30µM ATR and the aromatase inhibitor YM511. The hypothesis was that inhibition of aromatase would prevent any estrogen elevation, which would reduce the ATR effect if estrogen was playing a role in the ATR phenotype. As seen in Figure 25, the addition of 30µM ATR + 5nM YM511 had no significant effect on $CD4^+$ T cell proliferation (65.6% of undivided cells) versus 30µM ATR alone (61.5% of undivided cells). Treatment with 0.5nM and 1nM YM511 in ATR

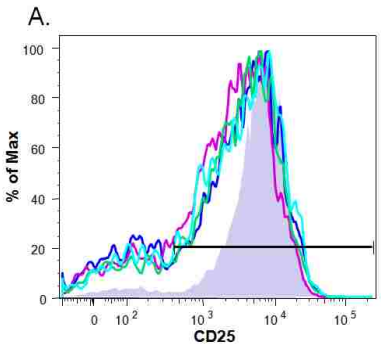
cultures slightly decreased the percentage of undivided cells more than the 0.1nM and 5nM YM511 treated cultures suggesting that the aromatase inhibitor could follow a non-monotonic dose response (14). Treatment with 5nM YM511 alone did not affect $CD4^+$ T cell proliferation showing that it does not interfere with $CD4^+$ T cell expansion.

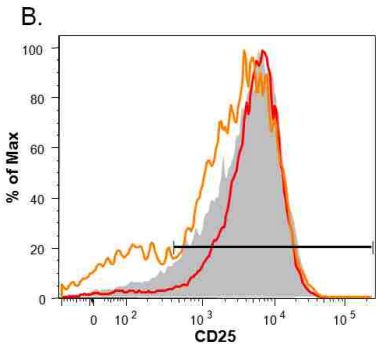
As seen in Figure 25A, addition of 5nM YM511 into ATR treated cultures did not increase the percentage of $CD25^+$ T cells compared to the 30μM ATR culture (Figure 25B). Treatment with 0.1nM YM511 in ATR cultures decreased the frequency of $CD25^+$ T cells more than the 30μM ATR control but the difference was not significant. The addition of YM511 alone did not affect the frequency of $CD25^+$ T cells showing that it does not interfere with $CD4^+$ T cell activation. These results suggest that aromatase induction may not be involved in the ATR-mediated phenotypes, although further tests need to be done to test YM511 aromatase inhibitor activity.



Treatment	MFI	% Undivided
30μM ATR+ 5nM YM511	7621	65.6%
30μM ATR+ 1nM YM511	5934	57%
30μM ATR+ 0.5nM YM511	6583	57.4%
30μM ATR+ 0.1nM YM511	7472	65%
30μM ATR	6725	61.5%
5nm YM511 only	588	6.9%
7.5μL EtOH	693	9.7%
5μL EtOH	611	10.1%

Figure 24: Inhibiting aromatase does not alleviate the proliferation inhibition caused by ATR exposure. Cells were stimulated with plate bound anti-CD3ε and anti-CD28 antibodies and gated on $CD4^+$ T cells. B10.BR splenocytes were cultured with 30μM ATR + 5nM YM511 (cyan), 30μM ATR + 1nM YM511 (green), 30μM ATR + 0.5nM YM511 (pink), 30μM ATR + 0.1nM YM511 (blue), 30μM ATR (orange), 5nM YM511 only (red), 7.5uL EtOH (shaded light purple), or 5uL EtOH (shaded grey). Cells were stained with CTV to monitor proliferation (top panels). Table shows MFI and percentage of undivided cells. Region markers indicate undivided cells. Percent undivided indicates cells that have not divided or have divided only a few times. Data representative of 2 separate experiments.





Treatment	MFI	% CD25
30μM ATR+ 5nM YM511	2243	85.2%
30μM ATR+ 1nM YM511	2205	85.5%
30μM ATR+ 0.5nM YM511	1779	85.2%
30μM ATR+ 0.1nM YM511	1909	80.5%
30μM ATR	1916	82.3%
5nm YM511 only	4504	92.5%
7.5μL EtOH	5027	93.4%
5μL EtOH	3423	92.9%

Figure 25: Inhibiting aromatase does not alleviate the inhibition of activation caused by ATR exposure. Cells were stimulated with plate bound anti-CD3ε and anti-CD28 antibodies and gated on $CD4^+$ T cells. B10.BR splenocytes were cultured with 30μM ATR + 5nM YM511 (cyan), 30μM ATR + 1nM YM511 (green), 30μM ATR + 0.5nM YM511 (pink), 30μM ATR + 0.1nM YM511 (blue), 30μM ATR (orange), 5nM YM511 only (red), 7.5uL EtOH (shaded light purple), or 5uL EtOH (shaded grey). CD25 expression was not affected compared to the 30μM ATR culture. Treatment with 5nM YM511 has no adverse effect on $CD4^+$ T cell activation (Part B). Table shows MFI and percentage of $CD25^+$ cells. Region markers indicate $CD25^+$ cells. Data representative of 2 separate experiments.

Discussion

ATR is the most common drinking water contaminant in the United States and it has been linked to adverse human health effects and environmental effects, such as increases in cancer (28, 29) and feminization of amphibians (34). However, the potential effects of ATR on adaptive immunity are not well understood. Our previous studies showed that ATR exposure decreased $CD4^+$ T cell proliferation (Figure 4) and activation (Figure 6). It was also shown to increase the frequency of $Foxp3^+$ T_{regs} (Figure 5). ATR is a PDE inhibitor, but the decreased activation status of the $CD4^+$ T cells was not completely mimicked by pharmacologically increased cAMP (Figure 6). In addition, a sex bias upon treatment with ATR emerged (Figure 7) with male-derived cells being more adversely affected. These latter results suggested that, as an endocrine disrupting compound, ATR may be increasing estrogen levels *in vitro* and causing a more severe phenotype than that seen with PDE inhibition alone. Our focus turned to the G-protein coupled estrogen receptor, GPER-1 because it has demonstrated immunosuppressive effects in experimental autoimmune encephalomyelitis (EAE, mouse model of multiple sclerosis) (66) and can increase cAMP (63). This led us to examine the role of estrogen and GPER-1 signaling in ATR-mediated effects of decreased $CD4^+$ T cell activation, proliferation and increase in the frequency of $Foxp3^+$ $CD4^+$ T cells.

We began these studies by extending the previous results showing that ATR exposure decreased $CD4^+$ T cell proliferation and activation (Figure 6) and increased the frequency of $Foxp3^+$ T_{regs} (Figure 5). We found that $CD4^+$ T cells activated in the presence of atrazine had a decrease in the expected CD62L down-regulation (Figure 7)

and decreased PD-1 expression (Figure 8). This phenotype correlates with decreased activation because CD62L is highly expressed on naïve cells and decreases upon activation (79), while PD-1 expression increases upon activation and has been shown to increase in the presence of estrogen (80). The effects on CD62L expression were concentration dependent (Figure 8), confirming that ATR treatment prevents T cell activation and that higher concentrations are more toxic.

Our previous results have shown that treatment with the phosphodiesterase inhibitor Pentoxyfilline inhibited T cell proliferation (Figure 4) and activation (Figure 6), but was not as potent as 30μM ATR. Here, we examined the effect of PTX treatment on Foxp3 expression. PTX increased the frequency of $Foxp3^+$ T cells by 2 fold (Figure 10). This is similar to the effects of 30μM ATR, which increases the frequency of $Foxp3^+$ T cells by 2-5 fold. This suggests that a significant elevation of intracellular cAMP is sufficient to stabilize Foxp3 expression. It also suggests that the PDE inhibitory activity of ATR is sufficient for the increase in the frequency of $Foxp3^+$ T_{regs}. In contrast, the inhibition of T cell proliferation and activation by PTX did not completely mimic the effects of ATR suggesting that elevated cAMP alone is not sufficient to decrease activation in this system and is consistent with the hypothesis that ATR is functioning via elevated estrogen levels.

To explore the possible effects of estrogen in the ATR-mediated decrease in proliferation, activation and increase in $Foxp3^+$ T cells, primary splenocytes were cultured in the presence of estrogen. In our experiments, addition of exogenous estrogen (17β-estradiol) inhibited T cell activation and proliferation following a non-monotonic dose response. Non-monotonic dose responses are exhibited by endocrine

hormones and endocrine disrupting compounds and display a non-linear relationship between dose and effect (14). Estrogen at 5ng/ml inhibited $CD4^+$ T cell proliferation and CD25 expression slightly more than 10ng/ml of estrogen, but 25ng/ml was more suppressive than 5ng/ml (Figure 12). These results are consistent with non-monotonic dose response curves for endocrine disruptors. Endocrine disrupting compounds, like estrogen, often display effects at low doses and follow non-monotonic dose response curves (36). Since estrogen can interact with multiple estrogen receptors, it is also possible that estrogen can bind different estrogen receptors and subsequently exert differential effects on target cells. Because the mice we used are wild type and express multiple estrogen receptors, these results cannot distinguish whether the estrogen effect is due to triggering of ERα, ERβ, and/or GPER-1.

When cells were cultured with both ATR and estrogen, the estrogen and ATR synergized to further decrease activation and proliferation more than ATR or estrogen alone (Figures 12 and 13). This unexpected result raises the possibility that ATR may not significantly elevate estrogen levels *in vitro* because we would expect the increased estrogen to decrease $CD4^+$ T cell activation and proliferation more than what has been observed with 30µM ATR treated $CD4^+$ T cells. Experiments to determine the estrogen concentrations in the various cultures have failed due to technical difficulties.

As mentioned above, there are multiple estrogen receptors that may be responsible for the observed estrogen effects. To determine which are playing a central role in the estrogen and ATR phenotype, we began by testing whether ERα stimulation could mimic the inhibitory effects seen with ATR treatment. Cells were exposed to the ERα agonist PPT during a 4 day activation culture. As seen in Figure 14, PPT did not

decrease proliferation or activation of $CD4^+$ T cells. It also did not increase the frequency of $Foxp3^+$ T cells. These results suggest that stimulation of ERα is not playing an important role in the ATR-mediated effects.

A second estrogen receptor that may be mediating the ATR effect is GPER-1. Previously published studies have shown that treatment with the GPER-1 specific agonist, G-1, is capable of decreasing the disease severity of experimental autoimmune encephalomyelitis (EAE, a mouse model of multiple sclerosis) by inducing T_{regs} and increasing the production of anti-inflammatory cytokines (66). Another factor favoring the idea of GPER-1 involvement in the ATR–mediated phenotype is that GPER-1 stimulation leads to an increase in cAMP (63). However, elevated concentrations of PTX do not fully mimic the ATR effects (Thueson, *et al.* in revision).

Signaling through GPER-1 via the agonist G-1 causes a moderate decrease in proliferation and activation although not a consistent increase in Foxp3 (Figures 15, 16, and 17). This is the first set of experiments showing that treatment with G-1 negatively impacts the *in vitro* $CD4^+$ T cell response. If G-1 triggering of GPER-1 worked as predicted, we would expect a significant decrease in CD25 expression and decreased frequency of $Foxp3^+$ $CD4^+$ T_{regs}. However, the only phenotype observed was a significant decrease of $CD4^+$ T cell proliferation. Treatment with G-1 *in vitro* appears to negatively impact proliferation of $CD4^+$ effector T cells. It has recently been shown that G-1 may bind a variant of ERα called estrogen receptor alpha-36 (ER-α36)(88, 89). This makes our interpretations of the G-1 data more difficult.

One interesting discovery was that T_{regs} in the presence of ATR, PTX and the GPER-1 agonist G-1 did not proliferate as well as T_{regs} treated with vehicle controls. In EtOH controls, T_{regs} proliferated similar to the $CD4^+$ effector T cells. T_{regs} in the presence of PTX, ATR or G-1 proliferated more than the $CD4^+$ T effector cells, but not nearly as well as the T_{regs} in the ethanol and DMSO vehicle-control cultures (Figures 11 and 18). The inhibition of proliferation of effector $CD4^+$ T cells was consistent with the observed increase in the T_{reg} population. It is in the nature of regulatory T cells to suppress an immune response, but it is unclear what is altering the proliferation status of the T_{regs} *in vitro*. It is known that increased levels of cAMP can inhibit effector lymphocyte proliferation (81), but T_{regs} naturally harbor increased levels of intracellular cAMP and even use this to suppress conventional T cells (82). Thus T_{regs} may not be as sensitive as $CD4^+$ T effector cells to increased cAMP levels. This would allow T_{regs} to proliferate better than effector T cells in conditions where there is an increase in cAMP. Since $CD4^+$ effector T cells don't normally have high levels of cAMP, they may be more sensitive to cAMP increases which, in turn, may prevent effector T cell proliferation. This could explain why we saw almost complete inhibition of $CD4^+$ effector T cell proliferation and minimal inhibition of T_{reg} proliferation. This effect could also be mediated through antigen presenting cells, such as dendritic cells (DCs) (83), although previous studies have shown that DC phenotypes were not altered in these ATR treated cultures (data not shown).

Since we saw a decrease in the overall number of $CD4^+$ T cells in culture upon treatment with ATR, PTX, and G-1 (data not shown), the increased frequency of Foxp3 expression may be due to a combination of natural T_{reg} (nT_{reg}) proliferation and the

conversion of $CD4^+$ effector T cells into T_{regs} (iT_{regs}). The nT_{reg} proliferation in ATR cultures was not as robust as that seen in ethanol cultures yet there was still an increased frequency of $Foxp3^+$ T cells upon treatment with ATR. The ATR-associated increase in $Foxp3^+$ T cell frequency may be due to the appearance of iT_{regs}, which could be mediated through the increase in cAMP levels (18). The results from Figures 11 and 18 suggest that expansion of nT_{regs} and induction of iT_{regs} may be occurring in ATR treated cultures. In the presence of ATR, T_{regs} proliferated more than the $CD4^+$ effector T cells showing that the natural Treg population was expanding. However, this expansion was not comparable to the proliferation of Tregs in the EtOH vehicle control (Figure 11), where the proliferation of T_{regs} was similar to the proliferation of $CD4^+$ effector T cells. The slight inhibition of T_{reg} proliferation and increase in overall frequency of $Foxp3^+$ T cells in ATR treated cultures may indicate that conversion of $CD4^+$ effector T cells into $Foxp3^+$ T_{regs} is occurring.

In order to further investigate the effects of GPER-1 signaling in ATR-mediated effects, we antagonized GPER-1. If GPER-1 signaling was involved, then antagonizing GPER-1 should alleviate the ATR-mediated effects. Initial attempts to antagonize GPER-1 with G-15 were difficult as G-15 has off-target effects due to its ability to stimulate ERα and ERβ (77). Non-specific binding of G-15 to other estrogen receptors may explain why treatment with 15μM ATR and G-15 increased the severity of the ATR-mediated effects (Figure 19). There have been many studies using G-15 where they conclude that it can effectively block GPER-1 stimulation, but our results suggest otherwise. Treatment with 15μM ATR and G-15 further decreased $CD4^+$ T cell proliferation (Figure 19) and activation (Figure 20) compared to the 15μM ATR control.

Since G-15 can bind other estrogen receptors, we further explored antagonizing GPER-1 with the more selective antagonist, G-36 (77). G-36 is similar to G-15, but it has an additional functional group that prevents it from interacting with ERα and ERβ (77). Antagonizing GPER-1 with G-36 did not modulate ATR effects on $CD4^+$ T cell proliferation or activation, or alter the frequency of $Foxp3^+$ T cells (Figures 21, 22, and 23). If G-36 had worked as predicted, significant increases in the frequency of proliferating and activated $CD4^+$ T cells should have been observed, as well as no increase in the $Foxp3^+$ frequency. Thus, our results appear to support the conclusion that GPER-1 signaling is likely not involved in ATR-mediated effects on $CD4^+$ T cells.

ATR elevates estrogen by stimulating the expression of the *aromatase II* gene. In order to assess whether ATR-induction of aromatase was mediating an effect on $CD4^+$ T cells by elevating estrogen levels, cells were stimulated in the presence of ATR with or without the aromatase inhibitor YM511. According to our hypothesis, if aromatase induction was inhibited there would be significantly lower levels of estrogen present in the ATR cultures and there would be increased $CD4^+$ T cell proliferation and activation and a decrease in the frequency of $Foxp3^+$ T cells. As seen in Figures 24 and 25, the presence of the aromatase inhibitor did not decrease the severity of ATR. $CD4^+$ T cells still exhibited decreased proliferation and decreased expression of CD25. This suggests that ATR may not be inducing aromatase or that induction of aromatase has no effect on $CD4^+$ T cells. Before coming to a conclusion, the functionality of the aromatase inhibitor in this system needs to be confirmed.

In summary, here we show that ERα stimulation does not inhibit $CD4^+$ T cell proliferation or activation and does not increase the frequency of $Foxp3^+$ T cells. While

ERα had no effect, GPER-1 signaling with G-1 inhibited $CD4^+$ T cell proliferation, but did not significantly reduce the frequency of activated $CD4^+$ T cells or consistently increase the frequency of $Foxp3^+$ T cells *in vitro*. Blocking GPER-1 with the antagonist G-15 potentiated the ATR phenotype while antagonizing GPER-1 with G-36 did not significantly alleviate the ATR-mediated effects on $CD4^+$ T cells. Treatment with the aromatase inhibitor YM511 also did not alleviate the ATR-mediated effects on $CD4^+$ T cells. Overall, the results presented in this thesis suggest that ATR may not be working to inhibit $CD4^+$ T cells through increasing estrogen levels *in vitro.* Further, elevated estrogen levels and GPER-1 signaling does not appear to be involved in the ATR-mediated decrease in $CD4^+$ T cell activation and proliferation and increase in frequency of $Foxp3^+$ T cells.

Future Directions

In order to further the understanding of estrogen-mediated effects in ATR treated cultures, it would be beneficial to examine a few more parameters in future experiments. In order to assess whether ATR is increasing the levels of estrogen *in vitro*, an estrogen enzyme-linked immunosorbent assay (ELISA) should be used to analyze estrogen concentrations in ATR and ethanol cultures. We have saved serum samples from ATR and ethanol treated cultures to determine the levels of estrogen present in each culture on day 4. We have recently purchased an estrogen ELISA and will analyze serum samples as soon as the kit arrives. This piece of data will strongly determine if elevated estrogen levels are mediating the observed effects on our $CD4^+$ T cells in ATR treated cultures.

Even if ATR is not elevating estrogen *in vitro*, it is possible that estrogen signaling is still playing a role in the ATR mediated decrease in $CD4^+$ T cell activation and proliferation and increase in the frequency of $Foxp3^+$ T_{regs}. The involvement of GPER-1 still remains promising because stimulation with G-1 displayed similar results on $CD4^+$ T cells as did ATR treatment. It has also been shown that ATR can weakly interact with GPER-1 (67) showing that even if there is no increase in estrogen levels, GPER-1 stimulation could still be occurring. The fact that lower concentrations of ATR have proven to be more potent than higher concentrations of ATR in animal models (36) suggests that ATR may only need to cause weak interactions in order to mediate adverse health effects.

Since antagonizing GPER-1 has proven to be difficult, more definitive conclusions could be drawn if we were able to use GPER-1 knockout mice. If GPER-1 mice are not available, it would be possible to use siRNA to knock down GPER-1 expression. If treatment of these cells (cells derived from GPER-1 knockout mice or cells whose GPER-1 expression is inhibited) with 30μM ATR does not affect proliferation or activation as severely as wild type mice, then it would suggest that GPER-1 stimulation is involved.

If it turns out that ATR is increasing the level of estrogen in our *in vitro* cultures, it is possible that there is synergy between the classical estrogen receptors and GPER-1. ERα/ERβ stimulation along with GPER-1 stimulation have been shown to stimulate proliferation of mouse Sertoli (testicular) cells (84) and decrease testosterone production (85). The increased levels of estrogen could be binding ERα/ERβ and GPER-1 causing the increased inhibition on proliferation and activation. In order to test if ERα and GPER-1 stimulation synergize, we would need to culture cells in the presence of PPT and G-1. The effects of ERβ alone and in conjunction with GPER-1 still need to be assessed. It would be beneficial to culture cells in the presence of ATR and PPT or ATR and G-1 in order to see if we can mimic the increased severity seen in ATR treated cultures supplemented with estrogen. It would also be possible to test these effects in ERα and ERβ knockout mice. The expression levels of ERα/ERβ and GPER-1 on ATR treated CD4+ T cells still need to be assessed.

Since ATR has been shown to disrupt multiple tissues, the *in vivo* effects of ATR should also be assessed. ATR is found in many tissues throughout the body and can

elicit different effects within the body than it can *in vitro* (13). One of the main differences is that ATR can increase hormone release from the hypothalamic-pituitary-adrenal axis which may significantly alter hormone concentrations compared to *in vitro* cultures (86). We may also get additional differences in sex hormone levels since ATR can affect reproductive tissues/organs (34). The effects of ATR during an active immune response (challenge with antigen *in vivo*) would also be interesting to examine since our data indicates differences in $CD4^+$ T cell activation and proliferation and changes in the frequency of $Foxp3^+$ T cells. After we are able to fully characterize the effects of ATR *in vivo* and *in vitro*, the next step would be to examine the effects of major metabolites, like DACT (13), on $CD4^+$ T cells.

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