REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad

1.	Name and designation of ICMR- IF	: Geetanjali Sachdeva, Scientist F
2.	Address	: Primate Biology Laboratory, Indian Council of Medical Research-National Institute for Research in Reproductive Health (ICMR-NIRRH), Parel, Mumbai-400012, India
3.	Frontline area of research in which training/research was carried out	: Reproductive Immunology
4.	Name & address of Professor and host institute	: Prof Sarah Robertson, Director, Robinson Research Institute, The University of Adelaide, North Adelaide, South Australia, 5006
5.	Duration of fellowship with exact date	: 13th January 2020 to 19th May 2020 (Four months, 1 week)
6.	Highlights of work conducted	: Please see Annexure 1
	i) Technique/expertise acquired	:
	ii) Research results, including any papers, prepared/submitted for publication	
	iii) Proposed utilization of the experience in India	:

Signature of ICMR-IF

Geetanjali Sachdeva

ICMR Sanction No.
INDO/FRC/452/S-13/2019-20-IHD

Annexure 1

Title: Exploring the Potential of all-Trans Retinoic Acid (atRA) as a Treg Booster for Improved Pregnancy Outcome

Principal Investigators:

Dr. Geetanjali Sachdeva, Indian Council of Medical Research-National Institute for Research in Reproductive Health (ICMR-NIRRH), Parel, Mumbai, India

Professor Sarah Robertson, Director, Robinson Research Institute, Adelaide Health and Medical Sciences, Australia

1) **Technique/Expertise acquired**: During my stay in Prof Robertson's laboratory, I acquired technical expertise in immunophenotyping using multi-parametric flow cytometry, data analysis using Flow-Jo, Handling of LSR-Fortessa X20; intraperitoneal administration of drugs/proteins in mice, collection of biological samples – uterine draining lymph nodes, mesenteric lymph nodes, spleen, uterine horns; preparation of single-cell suspensions, immunostaining for regulatory T cells, assessment of pregnancy outcomes.

2) **Research results, including any papers, prepared/submitted for publication**

Regulatory T cells (Treg cells) play a cardinal role in maternal tolerance to an allogenic fetus during pregnancy. Successful embryo implantation and placentation critically rely on Tregs. Reduced frequency and /or function of Tregs have been associated with various pregnancy disorders such as recurrent miscarriages, preterm birth, intrauterine growth retardation, pre-eclampsia. Thus there exists tremendous scope for modulating Treg frequency or function for effective management of various pregnancy complications, especially those ensued by poor implantation or placentation. Towards this, Dr. Robertson's group is currently testing the therapeutic utility of various protein biologicals (recombinant proteins/antibodies), as Treg boosters in pregnancy disorders. The present

study was undertaken to explore whether small molecules such as all-trans Retinoic acid (atRA- a vitamin A metabolite) are also effective as Treg boosters in-vivo in an animal model. known to have Treg defects and poor reproductive outcomes.

atRA, also known as Tretinoin, is reported to induce the generation of CD+CD25+ FOXP3+ regulatory T cells by increasing FOXP3 demethylation in CD4+ T cells (Sun et al, 2018). Under physiological conditions, atRA is synthesized by dendritic cells and in association with TGFβ and IL-2, atRA leads to differentiation of naïve T cells into Treg cells. atRA also inhibits the polarization of naïve T cells into Th17 cells, even in the presence of an inflammatory microenvironment. Thus atRA treatment aids in maintaining the suppressive phenotype of Tregs. This prompted us to probe whether atRA administration modulates Treg proportions in the uterine draining lymph nodes and thereby improves pregnancy outcome in an abortion- prone mouse model.

Methodologies:

Animal experimentations: The study was approved by the University of Adelaide Animal Ethics Committee (M-2017-101). Female CBA/J and male DBA/J mice housed in the laboratory animal services facility (LAS) at the University of Adelaide were used for the study. 1-2 adult (8-10 week old) female CBA/J mice were cohabitated with male DBA/J mice of proven fertility. The day of a vaginal plug detection in mated female mouse was referred 0.5-day post-coitum (dpc). Mated female mice were removed and treated with either atRA or vehicle. atRA (Sigma) stock solution (40mg/ml) was prepared in dimethylsulfoxide (DMSO). The stock was diluted to 1mg/ml by adding cottonseed oil. Mated CBA/J mice (n=12) were intraperitoneally injected with 300ug atRA for three consecutive days from day 0.5 dpc to day 2.5 dpc. Control mice (n=12) were similarly administered with 300ul of DMSO: Cottonseed oil (1:40) from day 0.5 dpc to day 2.5 dpc. On day 3.5, atRA/vehicle-treated animals were anesthetized by intraperitoneally administrating avertin. Blood was collected by cardiac puncture. Animals were cervically dislocated and paraaortic lymph nodes (uterine draining lymph nodes), mesenteric lymph nodes, uterine horns, and spleen were collected for immunophenotyping. All the tissues were suspended in sterile PBS at 4C till further processing.

In a parallel set of experiments, atRA or vehicle-treated mated female CBA/J mice were sacrificed on day 17.5 to assess the number of implantation sites, resorption sites, fetal weights, placental weights, and the number of fused placentas as the surrogates for abnormal implantation sites.

Immunophenotyping for regulatory T cells (Tregs):

Single-cell suspensions from spleen, paraaortic lymph nodes, and mesenteric lymph nodes were prepared by mechanical disruption using frosted glass slides. Cell suspensions from the slides were collected in PBS supplemented with 2% fetal calf serum (FCS). Lymph node suspensions were filtered through 70um strainer, centrifuged at 1500 rpm, and suspended in 300ul PBS. To get rid of RBC contamination from splenocytes, splenocyte suspension (300ul) was overlaid onto 500 ul of mammal Lymphoprep and spun for 30 min at 1800rpm at room temperature (RT). The lymphocyte layer was collected, washed with PBS at 1500 rpm for 2 mt, and suspended in 300ul PBS. Blood sample was diluted (1:1) with PBS and layered onto mammal Lymphoprep, centrifuged at RT for 30 mt at 1800 rpm. The buffy coat was collected and washed with PBS. The pellet was suspended in 100ul PBS.

To prepare uterine cell suspension, uterine horns were removed of mesenteries and fat tissue. Uterine tissues were minced into small pieces and incubated in 1 ml RPMI1640 containing 10% heat-inactivated fetal calf serum, 1 mg collagenase 1A, and 2.5 mg DNase at 370C for 1 hour with gentle shaking. The suspension was filtered through a 70um strainer by thorough rinsing with PBS. The filtrate (4.5 ml) was underlaid with 1/10th volume of FCS and this suspension was spun at 2200 rpm for 10 min at RT. The pellet was resuspended in 300ul PBS. Splenocytes, uterine cell suspensions, paraaortic lymph node, and mesenteric lymph node cell suspensions, and peripheral blood mononuclear cell (PBMC) suspensions were analyzed for Treg immunophenotyping. Single-stained splenocytes (1 million per fluorochrome) were used as compensation controls to distinguish a negative and a positive population for each fluorochrome used for staining experimental samples.

Experimental samples/ compensation control/ count wells were incubated for 10 min at RT in dark with viability stain (LD-APC-Cy7) diluted 1:1000 in PBS with murine Fc Block (1:100). This was followed by surface staining of the experimental samples with CD4-BUV395 (1:50), CD25-PE-Cy7 (1:20), NRP1-BV421 (1:12.5) antibodies in Brilliant Stain buffer for 20 mins at RT in dark. Cells were then washed with PBS by centrifugation at 1500 rpm for 2 min. Cells were fixed and permeabilized using permeabilizing concentrate diluted to 4 times with the diluent (provided in the Intracellular Fixation and Permeabilization Buffer Set- ebiosciences) and stored in dark at 4 0C for 12-14 hrs. Cells were washed with Permwash (permeabilization buffer diluted with MilliQ water-1:10) and stained with FOXP3-APC (1:100), CTLA-PE (1:100), Ki67-FITC (1:125) antibodies diluted in Permwash solution for 1 hour at 4 0C in dark.

Cells were washed with Permwash for 1 hr at 40C, suspended in PBS. In some experiments, the absolute number of Tregs was counted by labeling cells with LD-APC-Cy7 (diluted to 1:1000) and CD4-BUV395 (1:50). Labeled cells were fixed, permeabilized, and washed. This was followed by the addition of Count Beads (1.5 x 104 Count bright). Samples (experimental, compensation controls and Count)

were assessed for their immunofluorescence using LSR-Fortessa X-20 (BD) with FACSDIVA (BD-Biosceinces). Flow-JO software was used to set up gates to include the lymphocyte population and singlets using forward and side-scatter profiles. CD4 positive lymphocytes were gated within viable lymphocytes (LD-APC-Cy7 negative lymphocytes). CD4 positive population was gated for the proportion of FOXP3+ CD25+ cells. CD4+ FOXP3+ CD25+ cells were defined as Regulatory T cells (Tregs). Gates were established on the CD4+ FOXP3+ CD25+ population to determine the proportion of thymic Treg (NRP1+) and peripheral Treg (NRP1-) cells. Further Ki67+, CTLA4+, and double-positive Ki67+CTLA4+ cell proportions within thymic and peripheral Treg populations were determined. Median Fluorescence Intensity (MFI) was used to compare FOXP3, CTLA4 and Ki67 expression in Tregs from control and atRA treated samples.

Results: Intraperitoneal administration of atRA from day 0.5 to day 2.5 dpc (preimplantation phase) in mated CBA/J female mice (n=12) led to an increase in the proportion of regulatory T cells (CD4+FOXP3+CD25+) in the para-aortic lymph nodes (uterine draining lymph nodes) (Fig 1).

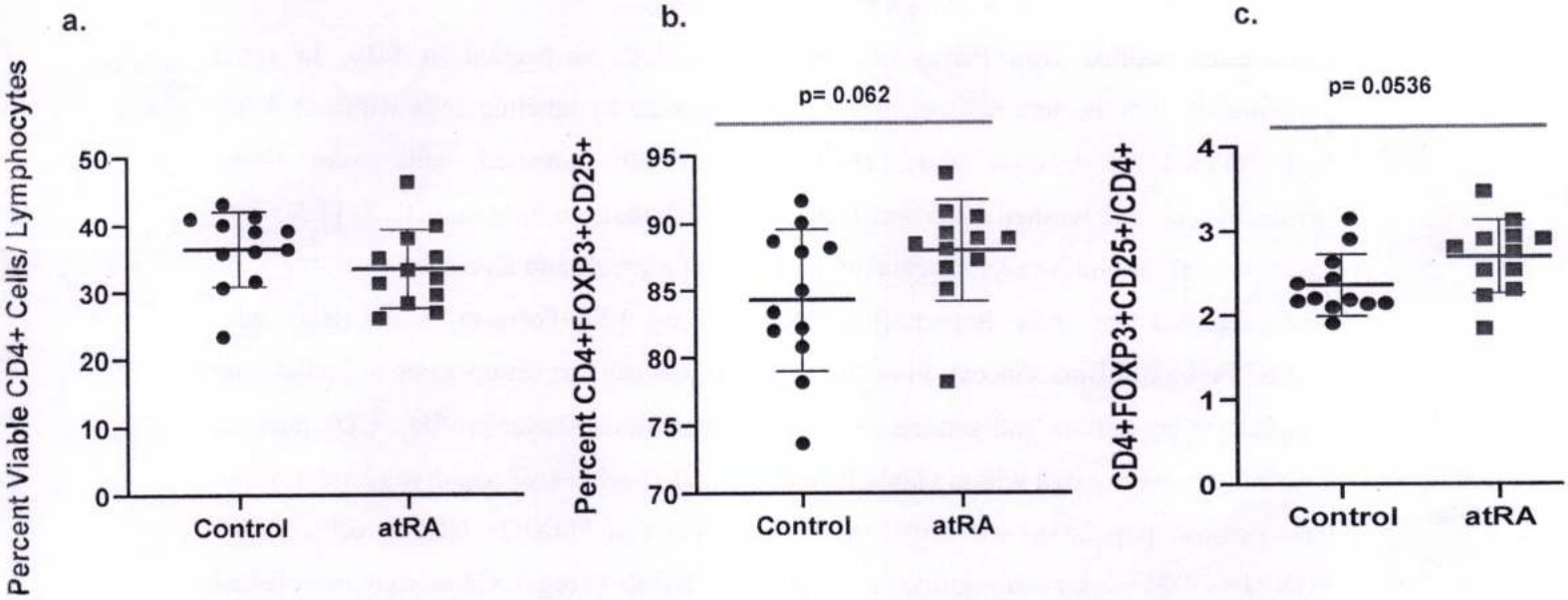

Fig 1: Effect of atRA administration (i. p.) on the proportions of viable CD4 cells in lymphocytes (a), CD25+ cells in CD4+ FOXP3+ population (b); proportion of Tregs (CD4+FOXP3+CD25+ cells) (c) in the uterine draining lymph nodes of DBA/J mated CBA/J female mice. Unpaired student's t test was used to compare these proportions in atRA and vehicle treated groups.

Further, a significant proportion of Tregs in the uterine draining lymph nodes from atRA treated mice revealed a proliferative phenotype, as indicated by Ki67 positivity (Fig 2). This suggests that atRA leads to the expansion of Tregs in the uterine draining lymph nodes of mated CBA mice.

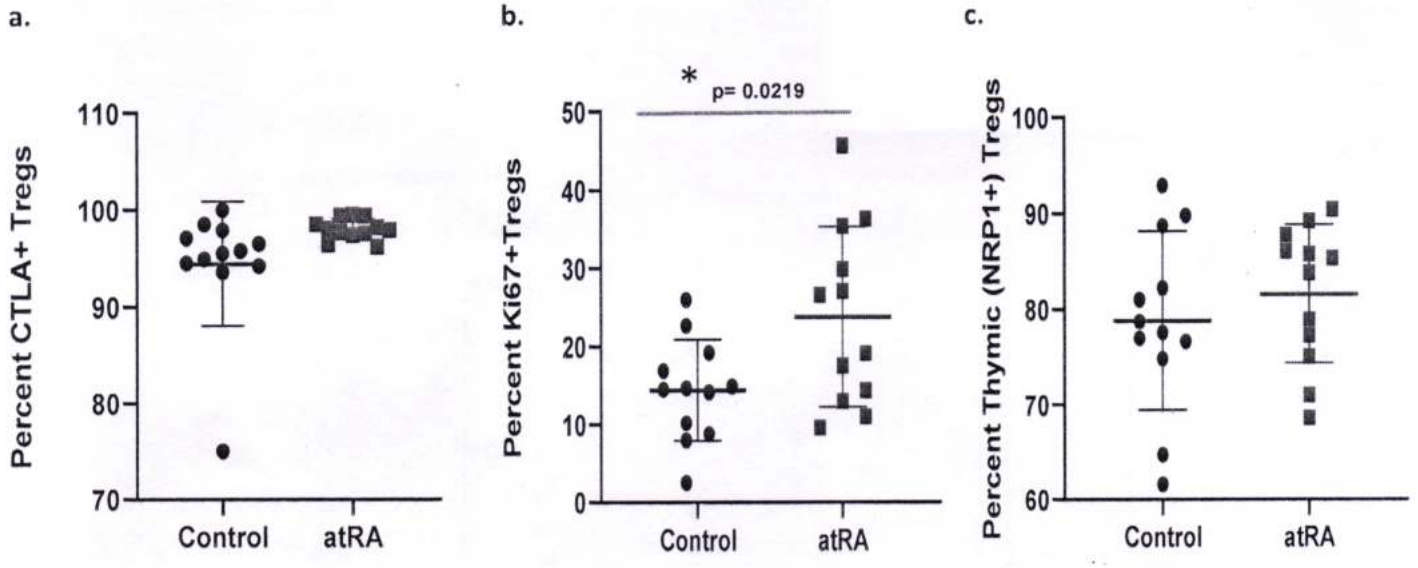

Fig 2: Effect of atRA administration (i.p.) on the proportions of Tregs expressing CTLA4 (a), Ki67 (b) and NRP1 (c) in the uterine draining lymph nodes of DBA/J mated CBA/J female mice. Unpaired student's t test was used to compare the proportions of CTLA4 or Ki67 or NRP1 expressing Tregs in atRA and vehicle treated groups. *p value < 0.05

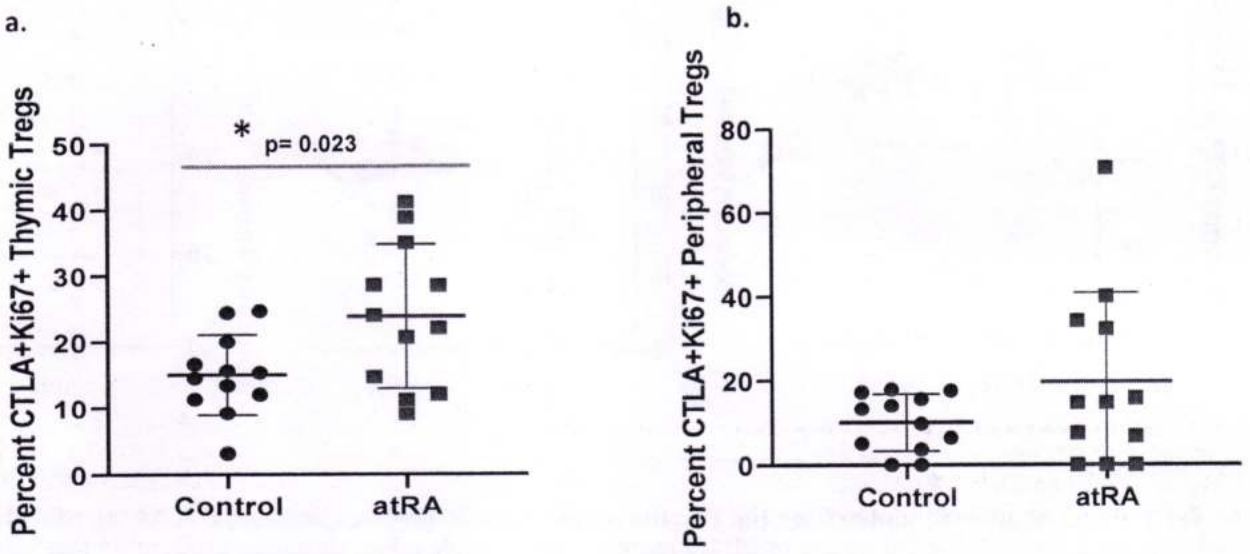

Fig 3: Effect of atRA administration (i.p.) on the proportions of thymic (a) and peripheral (b) Tregs expressing CTLA4 and Ki67 in the uterine draining lymph nodes of DBA/J mated CBA/J female mice. Unpaired student's t test was used to compare the percentage of CTLA4+ Ki67+ positive cells in thymic (NRP1+) or peripheral (NRP1-) Tregs in atRA and vehicle- treated groups. *p value < 0.05

This significant increase in the proportion of Ki67 positive cells was particularly evident in thymic Tregs, identified by neuropilin-1 positivity (Fig 3). Further, atRA treatment also caused a significant increase in the expression of FOXP3 and CTLA4 (a marker of suppressive phenotype) in the Tregs residing in the para-aortic lymph nodes (Fig 4).

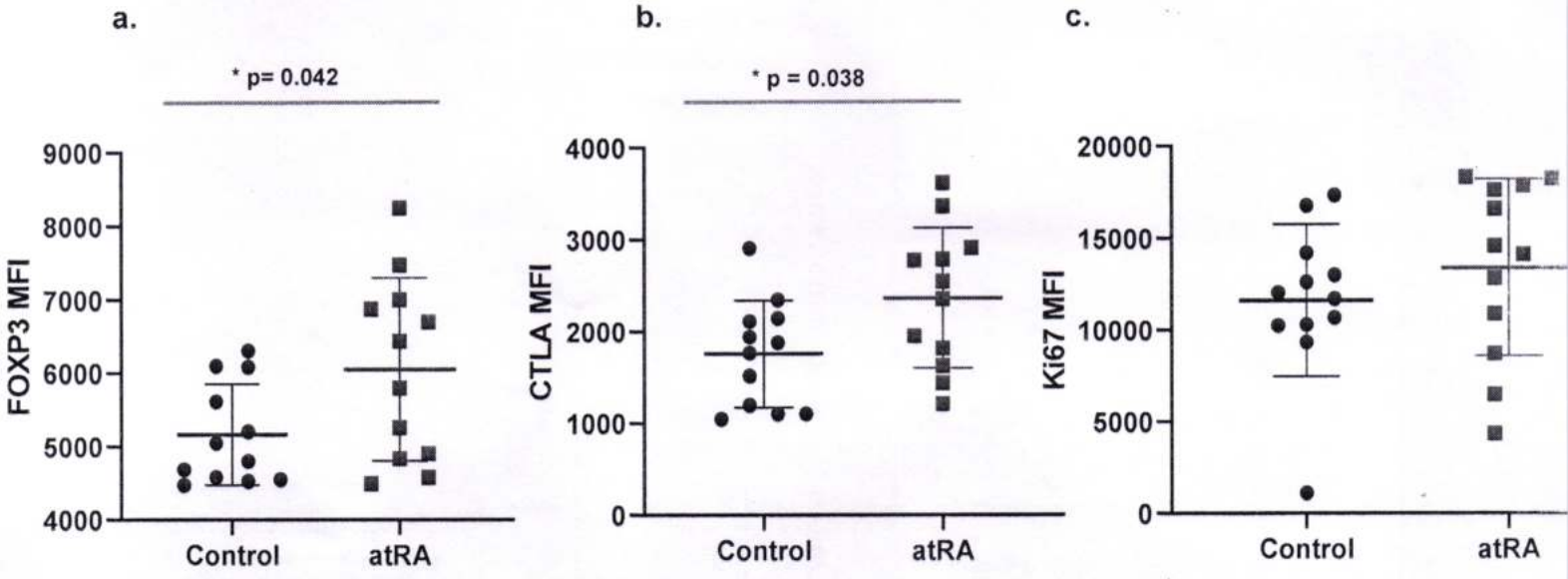

Fig 4: Effect of atRA administration (i. p.) on FOXP3, CTLA4 and Ki67 expression in Regulatory T cells of the uterine draining lymph nodes of DBA/J mated CBA/J female mice. Panels a, b and c show median fluorescence intensities (MFIs) of FOXP3, CTLA4 and Ki67, respectively. Data represent mean ± SD. Unpaired student's t test was used to compare the MFIs for each marker in atRA and vehicle treated groups. *p value < 0.05

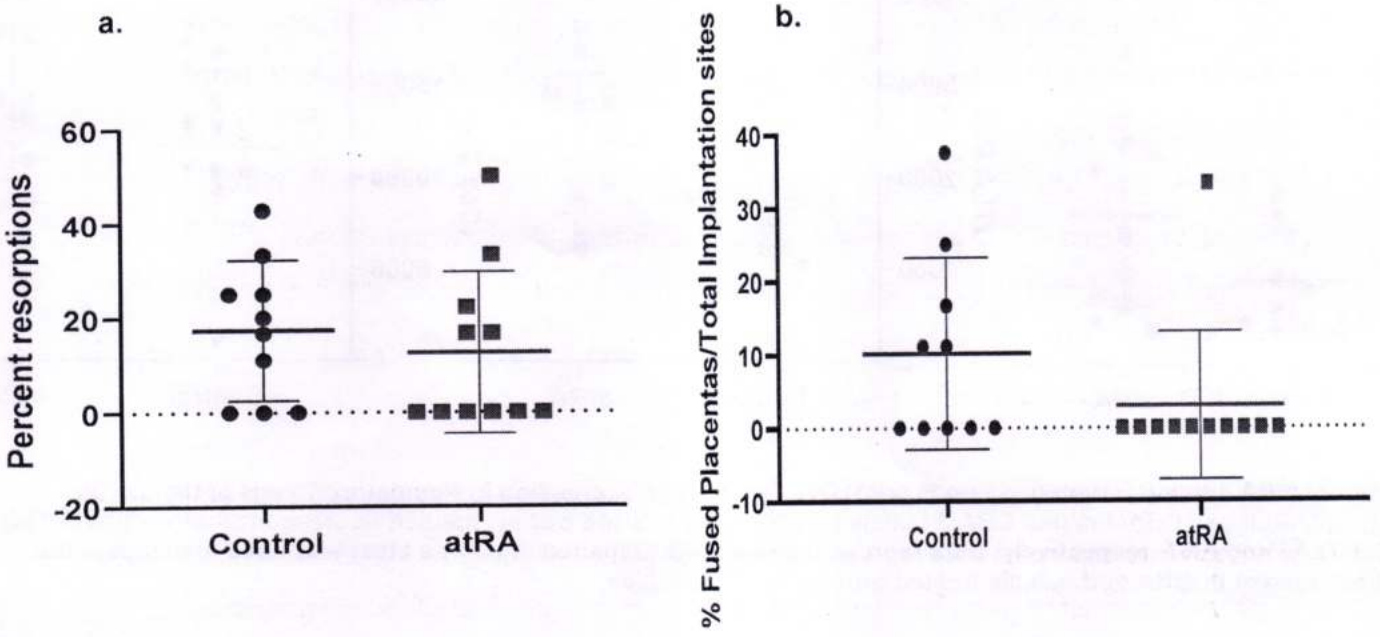

Fig 5: Effect of atRA administration (i. p.) on embryo resorptions (a) and percentage of fused placentas /total implantation sites (b) in DBA/J mated CBA/J female mice.

Collectively, these observations suggest that treatment with atRA (300 μg) during the preimplantation phase modulates the phenotype of Tregs in the uterine draining lymph nodes of CBA/J female mice. However, this modulation in the Treg phenotype was not accompanied by an improvement in the pregnancy outcome in CBA/J mice mated with DBA/J, known for higher resorptions. Fetal weight, placental weight, and fetus: placental weight ratios, number of implantation sites, did not differ significantly in atRA-treated and vehicle-treated groups, although the number of mice with no embryo resorptions appeared to be higher in the atRA treated group (6/11), compared to the vehicle-treated group (3/10) (Fig 5). Further investigations in larger sample size are warranted to corroborate this inference. Also, the percentage of the fused placenta (a surrogate for abnormal implantation sites) was lower in atRA treated group, compared to vehicle-treated groups.

Overall, it may be surmised that the increase observed in Treg proportions in the uterine-draining lymph nodes was not adequate to prevent the embryo resorptions to a significant

level in mated CBA females, administered with 300 μg atRA in the pre-implantation phase. It is also possible that the phenotypic changes observed in Tregs in these mice were not sustained for sufficient duration (till the completion of implantation or placentation). While the dose used (300μg dose each day for three days) appears to be very high, it is well established that atRA has a very short half-life. Thus it is possible that the bioavailable dose in the present study was lower than that required for eliciting an optimal response in terms of Treg number or function in the uterine draining lymph nodes. It will also be of interest to investigate whether atRA treatment if given at a pre-conception stage, generates sufficient number of functional Tregs and rescues abortions in DBA mated abortion-prone CBA/J mice. atRA treatment before conception/ mating may allow a longer window for the induction of Treg differentiation and thus ensure the availability of sufficient and functional Tregs during the peri-implantation period.

Overall, these "proof of principle" experiments suggest that in-vivo administration of atRA in abortion-prone mice modulates the phenotype of regulatory T cells (Tregs) in the uterine draining lymph nodes. Although no significant improvement was observed in the pregnancy outcome of abortion- prone CBA/J female mice, further refinement of treatment modalities (atRA administration before conception, route of administration, co-administration with other known Treg boosters) may reveal whether atRA has a potential to improve pregnancy outcomes.

iii) Proposed Utilization of the Experience in India: The experience gained by training in Prof Robertson's laboratory will be of great help in our ongoing studies on the relevance of alarmins in implantation, wherein our focus is to determine the mechanism by which an excess of alarmins in the uterine cavity leads to implantation failure. In addition, we are also formulating studies to determine whether immune homeostasis in the uterus is governed by alarmins present in the uterine fluid. The expertise gained by working with Prof Robertson will help us execute these studies with a better perspective on the immunological basis of implantation. Further, the study carried out in Prof Robertson's laboratory has provided some interesting leads that can be pursued further in collaboration with Prof Robertson's group.