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Effects of TCH-013 on Acute Graft versus Host Disease

Internship report

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Learning Objectives/Internship Objectives

1. Understanding the effects of TCH-013 on graft versus host disease (GVHD) prevention by examining the effects on:
    - Dendritic cell maturation
    - Dendritic cell cytokine production
    - T cell proliferation
    - T cell cytokine production
    - T cell differentiation
    - In vivo weight/GVHD scores
    - In vivo survival
    - In vivo T cell proliferation and migration via bioluminescence imaging

2. Techniques / laboratory experience
    - Flow cytometry
    - Cell culturing
    - Bioluminescent imaging
    - Total body irradiation
    - Mice handling (ear punching, health checks, euthanization, dissection and harvesting of organs and cells)

Introduction

The transplantation of multipotent hematopoietic stem cells from bone marrow, umbilical cord and peripheral blood is called hematopoietic stem cell transplantation (HSCT). The three types of HSCT are – autologous (stem cells come from the patient), allogeneic (stem cells come from appropriate donor) and syngeneic (stem cells come from identical twins). Allogeneic HSCT is a very effective treatment of hematological diseases and immune disorders like multiple myeloma and leukemia [out.2pdf].

A post-transplantation host environment, where the donor T cells develops tolerance to the alloantigens and simultaneously maintains its ability to selectively detect and respond to a broader spectrum of foreign antigens indicates a successful transplantation [4]. Graft versus host disease (GVHD) is a medical complication arising from the receipt of transplant from a genetically different donor. We are concerned with the type of GVHD, which develops within the first 100 days of the transplant. This type of GVHD is called acute graft versus host disease (aGVHD)

Acute GVHD is the major cause of post-HSCT morbidity and mortality [out2][3]. The National Institute of Health characterizes aGVHD by its effects on skin, gastro-intestinal tract and liver [4]. Inflammatory cytokines primarily mediate aGVHD pathology. The tissue damage in aGVHD is the result of deregulated cytokines. The aGVHD in mice can be comprehensively described as a five step process –

a) **Immune priming** – The conditioning of the recipient mice with radiation, chemotherapy or monoclonal antibody therapy is done to create ample space in their bone marrow to hold the transplanted cells. The conditioning kills the gut bacteria and releases the bacterial endotoxins into the blood stream from the lumen of the small intestine. This phenomena triggers the release of the inflammatory cytokines (IL-1ß, TNF-α, IL-6 and IFN-ϒ), which in turn induces the upregulation of the major histocompatibility complex (MHC) and the surface adhesion molecules on the host antigen presenting cells (dendritic cells, macrophages, Langerhans cells and B cells). In our study we will consider dendritic cells in our in vitro experiments as the antigen presenting cell.

b) **T cell activation and co-stimulation** – The upregulation of the cell surface marker and the co-stimulatory markers on the antigen presenting cells causes the T-cells to recognize the surface and as a result causes T cell activation and co-stimulation.

c) **Alloreactive T cell expansion** – The recognition of the surface antigens and adhesion molecules on the host and donor APCs by the T-cell receptor (TCR) triggers the activation of the donor-derived T-cells. The activated donor T-cells produces IL-2 and IFN-ϒ (Th1 response), which activates more T-cells, natural killer cells and the macrophages. And in this way the T-cell populations are expanded as a response to various cytokine release. The donor-derived T cells responds to the alloantigens and proliferates in the lymph nodes in response to the antigens.

d) **Trafficking** – The T cell migration takes place with the help of the connective tissues like blood and lymph.

e) **Tissue injury and destruction** - The activated donor T-cells mediate cytotoxic damage to host cells through Fas-Fas ligand interaction, perforin-granzyme and TNF-α.

Steps to aGvHD in mice

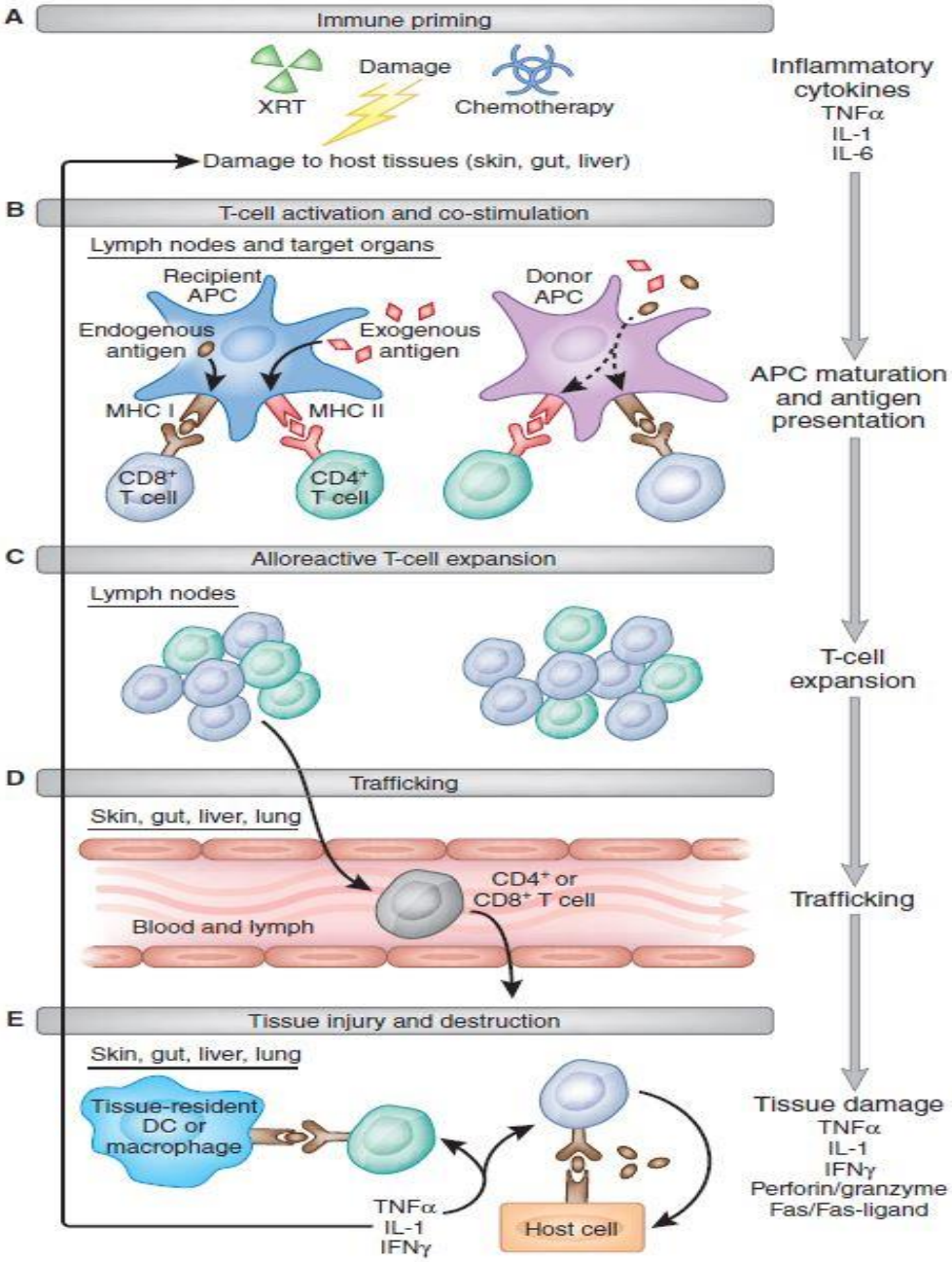


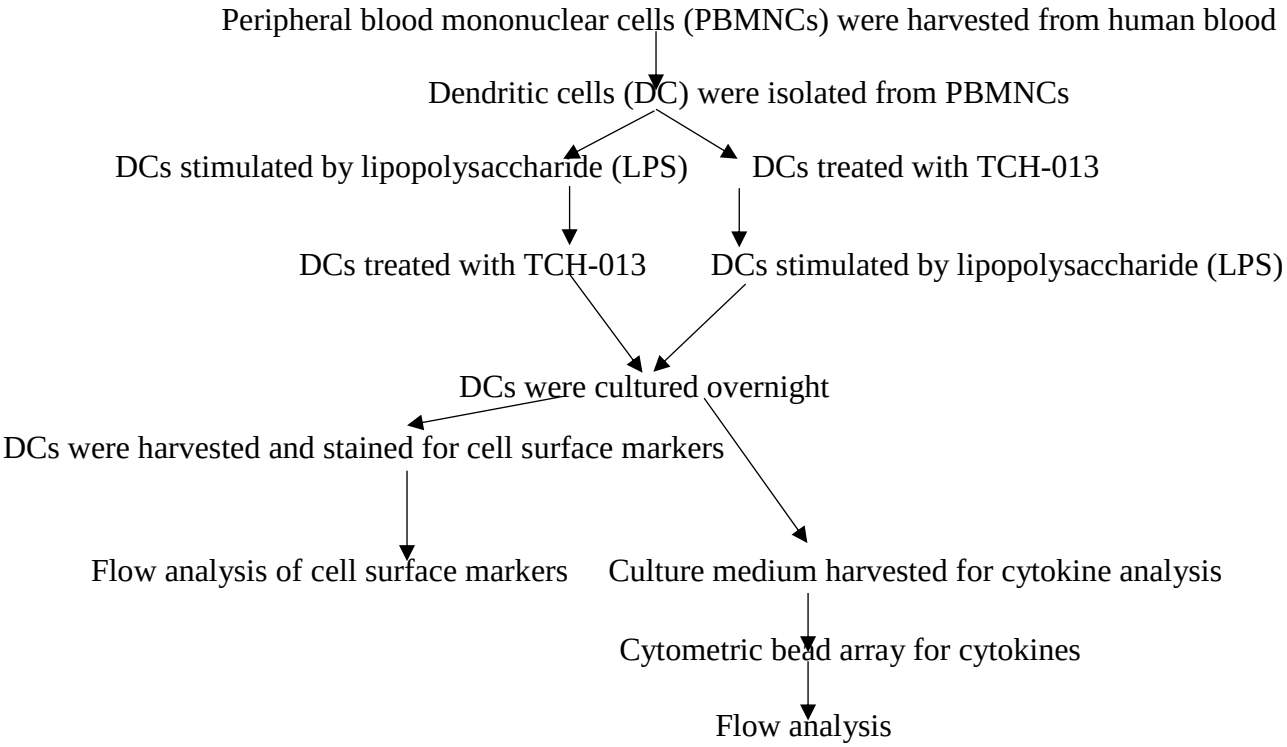
Figure 2. Steps to aGvHD in mice. The progression of events occurring in the development of aGVHD in the mouse is illustrated. The five crucial steps of immune priming (A), activation (B), T-cell expansion (C), T-cell trafficking (D) and host tissue injury (E) are outlined. DC, dendritic cell; XRT, radiation conditioning. See main text for full details. Adapted from - Mark A. Schroeder1 and John F. DiPersio1, Mouse models of graft-versus-host disease: advances and limitations

Nuclear factor-κβ (NF-κβ) is a transcription factor that causes a change in normal cell functioning. Activated nuclear factor-κβ (NF-κβ) induces the inflammatory immune response. TCH-013 is a proteasome modulator which inhibits the 26S proteasome to proteolyse the inhibitor of NF-κβ and thus helps in continued regulation of the NF-κβ transcription factor.

TCH-013 have not been studied in a transplant setting earlier. The focus of this research is to study the effects of TCH-013 on aGVHD. We performed in vitro and in vivo experiments to understand the effects of TCH-013 on aGVHD. The in vitro studies included – a) dendritic cell maturation; b) antigen cross presentation assay; c) T-cell proliferation assay. Our in vivo study included – a) the study of the effects of TCH-013 on aGVHD in a major mismatched murine model; b) Bioluminescence imaging of GvHD inducing donor T-cell proliferation and migration over time.

Description of Work

A. Dendritic cell maturation and Dendritic cell cytokine production

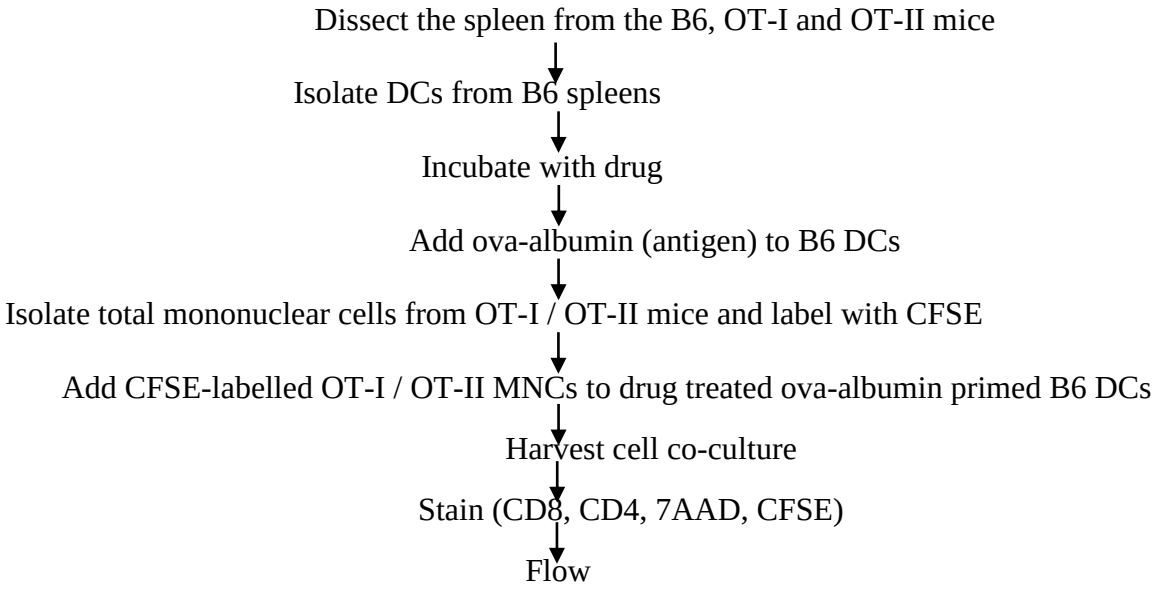


Leukocyte reduction filters from healthy human donors were obtained. Peripheral blood cells were eluted from the filters then added to the top of the lymphoprep solution (1:1 volume). The PBMNC were separated out from the mixture using a density gradient, after a density spin. After the density spin, the PBMNC layer was separated from the polymorphnuclear cell, erythrocyte and the plasma layers. Dendritic cells were isolated from the PBMNC using a magnetic based negative selection system (STEMCELL Technologies). The isolated dendritic cells from each filter were then treated as followed –

1. untreated
2. stimulated with LPS
3. stimulated with LPS then treated with TCH-013 12 µM
4. stimulated with LPS then treated with TCH-013 24 µM
5. stimulated with LPS then treated with Bortezomib 10 nM
6. treated with TCH-013 12 µM then stimulated with LPS
7. treated with TCH-013 24 µM then stimulated with LPS
8. treated with Bortezomib 10 nM then stimulated with LPS

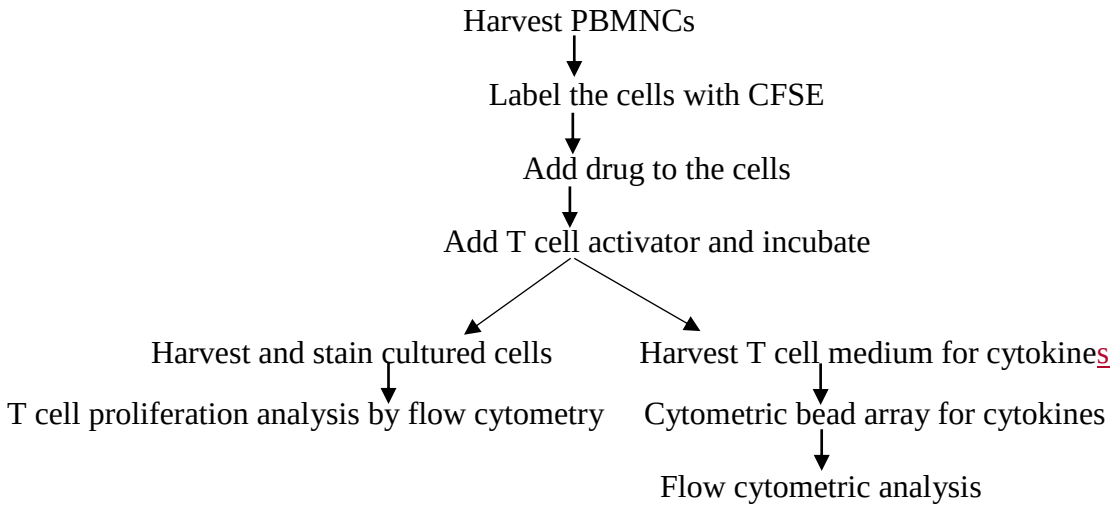
DCs from each treatment groups were stained for analysis of cell surface markers (CD86, CD54, CD83, CD40 and CD80). The culture medium was extracted from the well for human inflammatory cytokine (IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70) analysis using flow cytometry (BD Cytometric Bead Array Human Inflammatory Cytokines Kit).

B. Antigen cross presentation assay

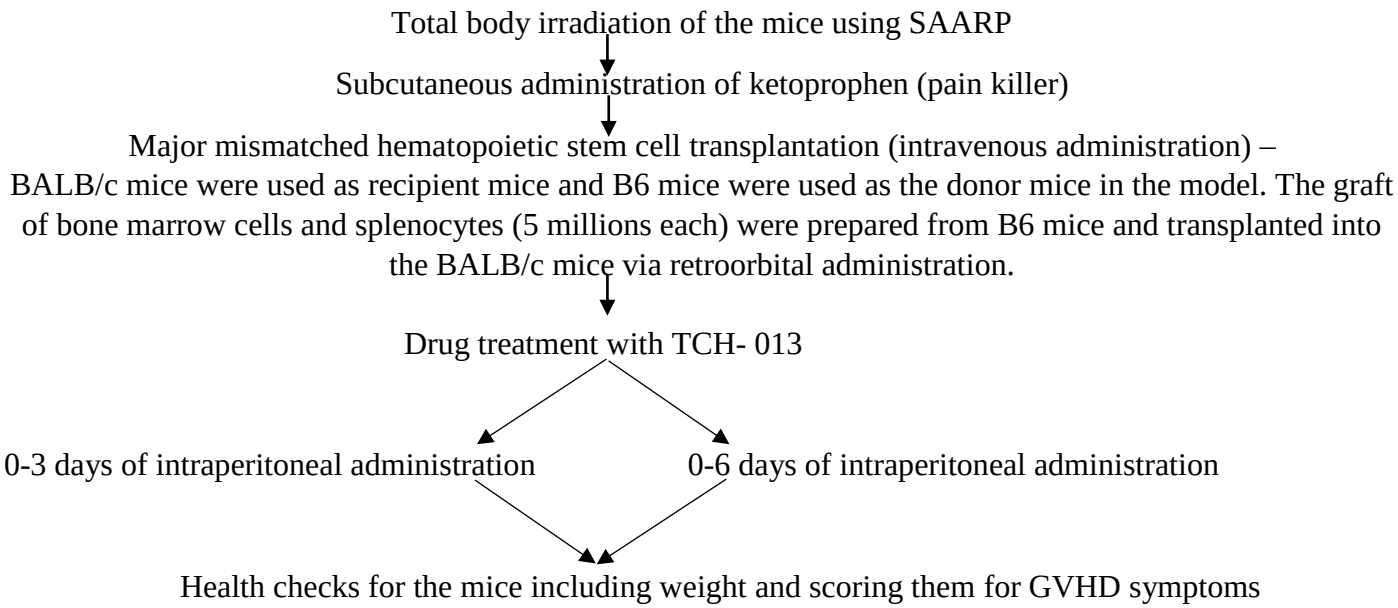


The spleens were dissected out of the B6 (C57B6), OT-I (transgenic mice designed to express CD8+ T cells, which primarily recognize $OVA_{257-264}$ when presented by MHC I molecule) and OT-II mice (transgenic mice designed to express CD4+ T cells, which primarily recognize $OVA_{323-339}$ when presented by MHC II molecule). The spleen of the B6 mouse was dissociated in spleen dissociation buffer to ensure the dissociation of DCs from the white pulp of the spleen. The DCs were isolated by negative selection (Manufacturer protocol – STEMCELL Technologies) from the harvested splenocytes of the B6 mouse. The dendritic cells were incubated with drug in a 96 well culture plate. Ova-albumin ($OVA_{257-264}$ and $OVA_{323-339}$ respectively) were added to the B6 dendritic cells. The splenocytes of the OT-I and OT-II mice were gently dissociated in DPBS (Dulbecco's phosphate buffer saline) and the mononuclear cells were labelled with Carboxyfluorescein succinimidyl ester (CFSE). The CFSE-labelled OT-I and OT-II mononuclear cells were added to the respective drug-treated ova-albumin primed B6 DCs. The co-culture was harvested after appropriate incubation, stained for CD8, CD4, CFSE and 7AAD, and analyzed on a flow cytometer

C. T cell proliferation assay



The *in vivo* study on BALB/c mice –



** The GVHD scoring of mice is based on – bodyweight, activity, posture, gut, fur, and skin

We used a major-mismatched murine model of the BALB/c mice as the recipient and the B6 mice as the donor mice. We conditioned the BALB/c mice by giving them 10 Gray total body irradiation in SAARP irradiator. After the conditioning of the recipient mice, we extracted the bone marrow cells and the splenocytes from the B6 mice. The two population of the cells were counted and diluted accordingly to get a graft of 5* 10^6 cells for each cell population. Anaesthesia was performed on the recipient BALB/c mice and they were administered with ketoprophen (pain killer), before the transplant. The BALB/c mice were then administered with the graft via retro-orbital (intravenous) administration. Post-transplantational administration of the drug TCH 013 was performed via intraperitoneal administration.

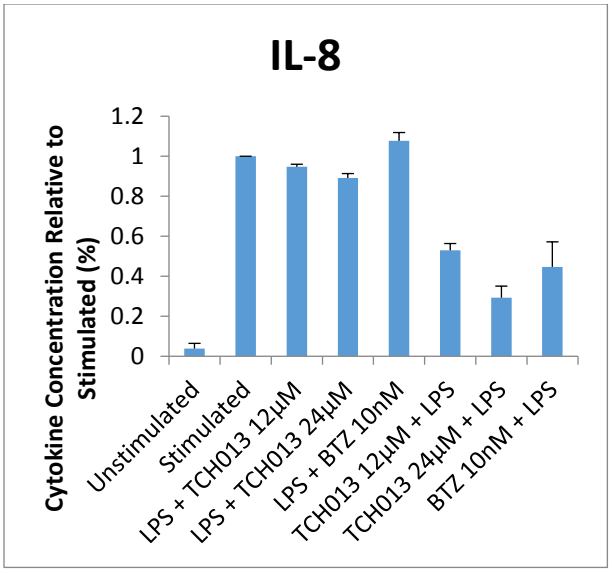
Observation and Analysis –

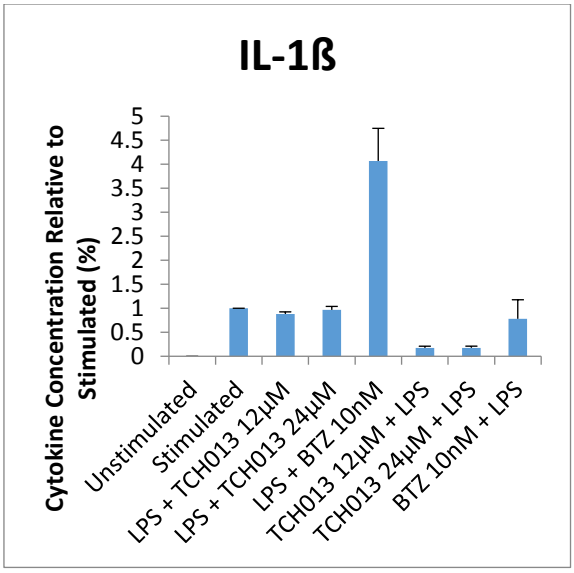
Dendritic cell maturation –

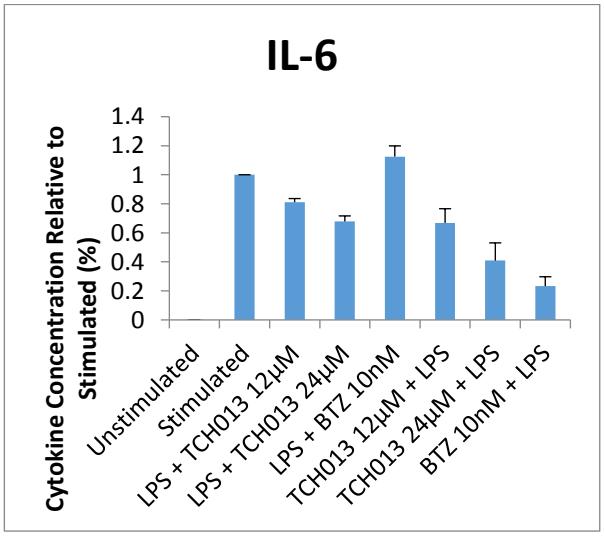
The dendritic cell treated with TCH-013 exhibited less amount of surface markers, when compared to the stimulated, untreated dendritic cells. The analysis of marker expression was done using the flow cytometric analysis. The TCH-013 have shown same pattern of results as our positive drug control, bortezomib. The analysis is supported by the microscopic observation of the dendritic cell under the treatments, where the dendritic cells treated with the TCH-013 and bortezomib. The results from the dendritic cell maturation experiment supports the fact that the TCH-013 starts acting in the earlier phases of the aGVHD onset.

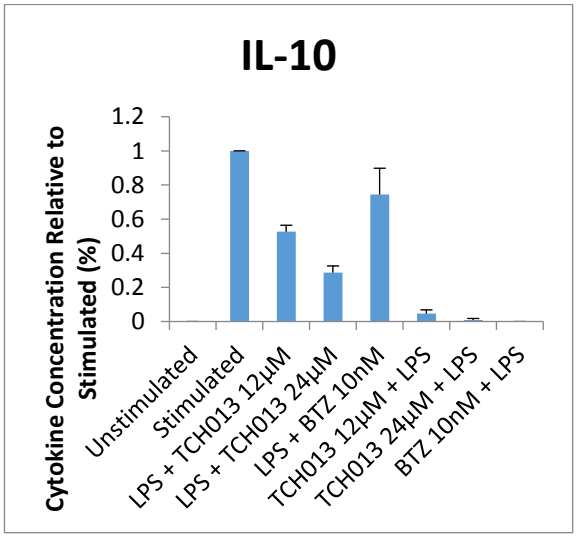
Dendritic cell cytokine production –

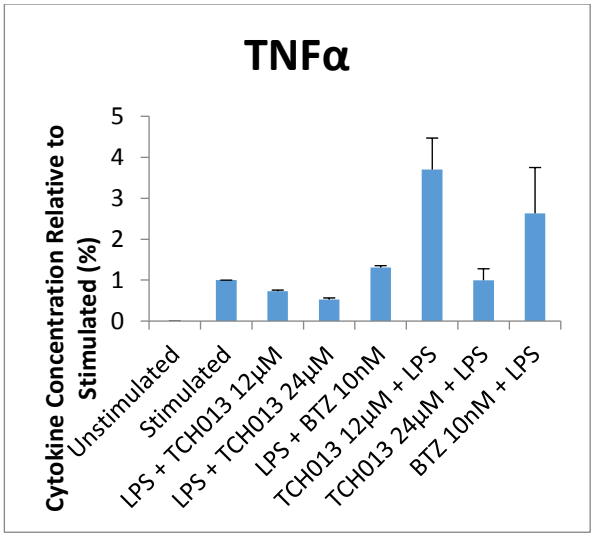
The dendritic cell cytokine production decreased after being treated with TCH-013 and this could be the result of the effect of TCH-013 on the DC maturation. The decrement of the DC cytokine level in TCH-013 treated cell media supports the observation that the drug is working on the dendritic cells. The results of the DC maturation and the DC cytokine production supports each other.

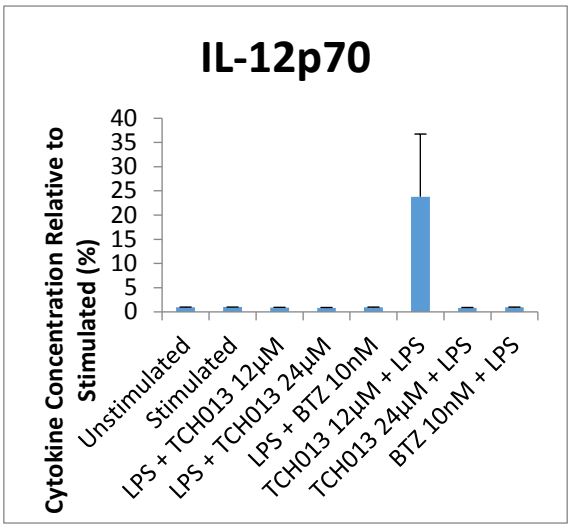






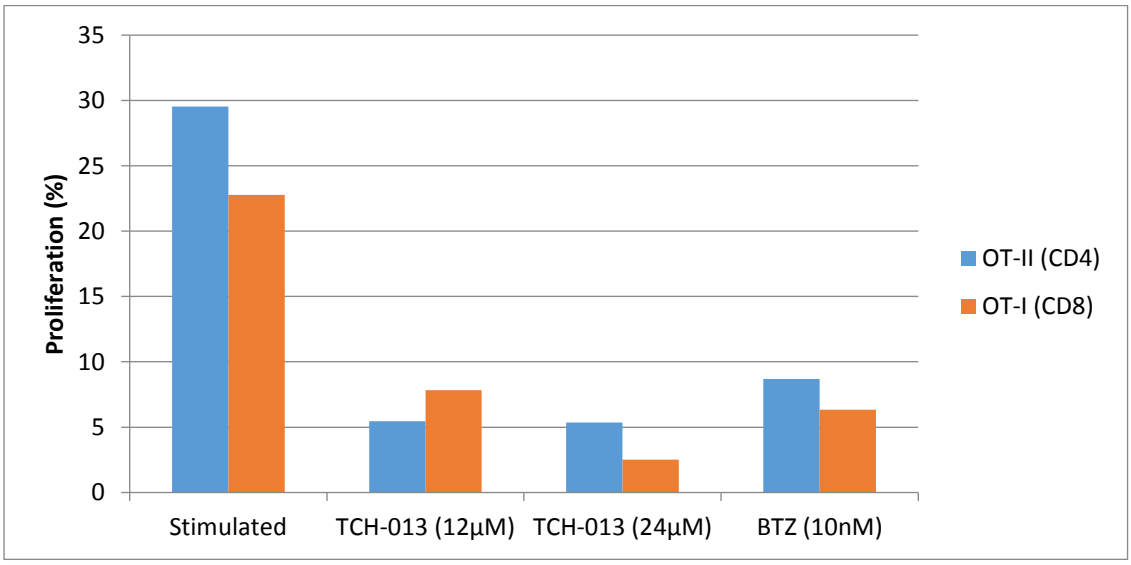






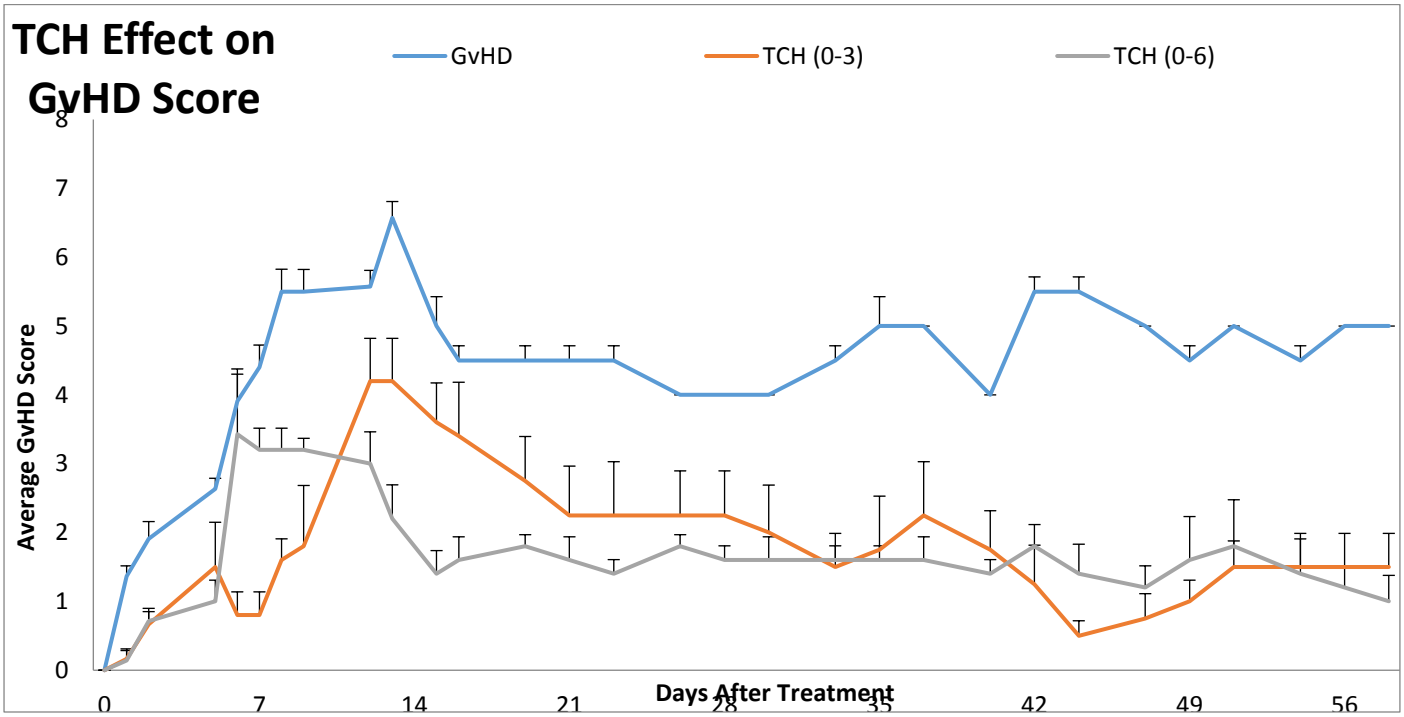
Antigen cross presentation assay –

The graph indicates that the expression of the CD4 and CD8 markers of the T-cell have gone down on treatment with the TCH-013 drug. This indicates that TCH-013 plays a role in the antigen cross presentation between the recipient and the donor antigen presenting cells. This result is consistent with the DC maturation and the DC cytokine production results.

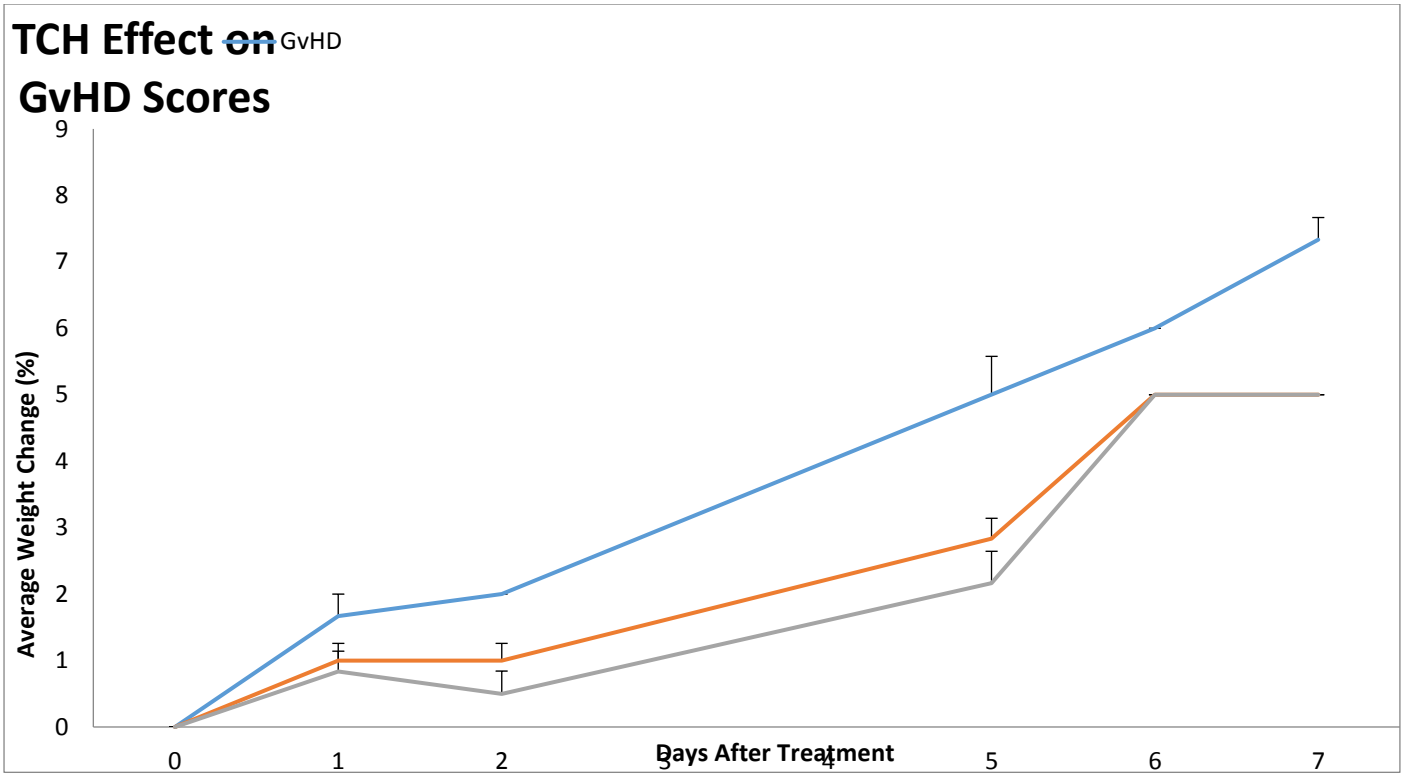


Effects of TCH-013 on GvHD score –

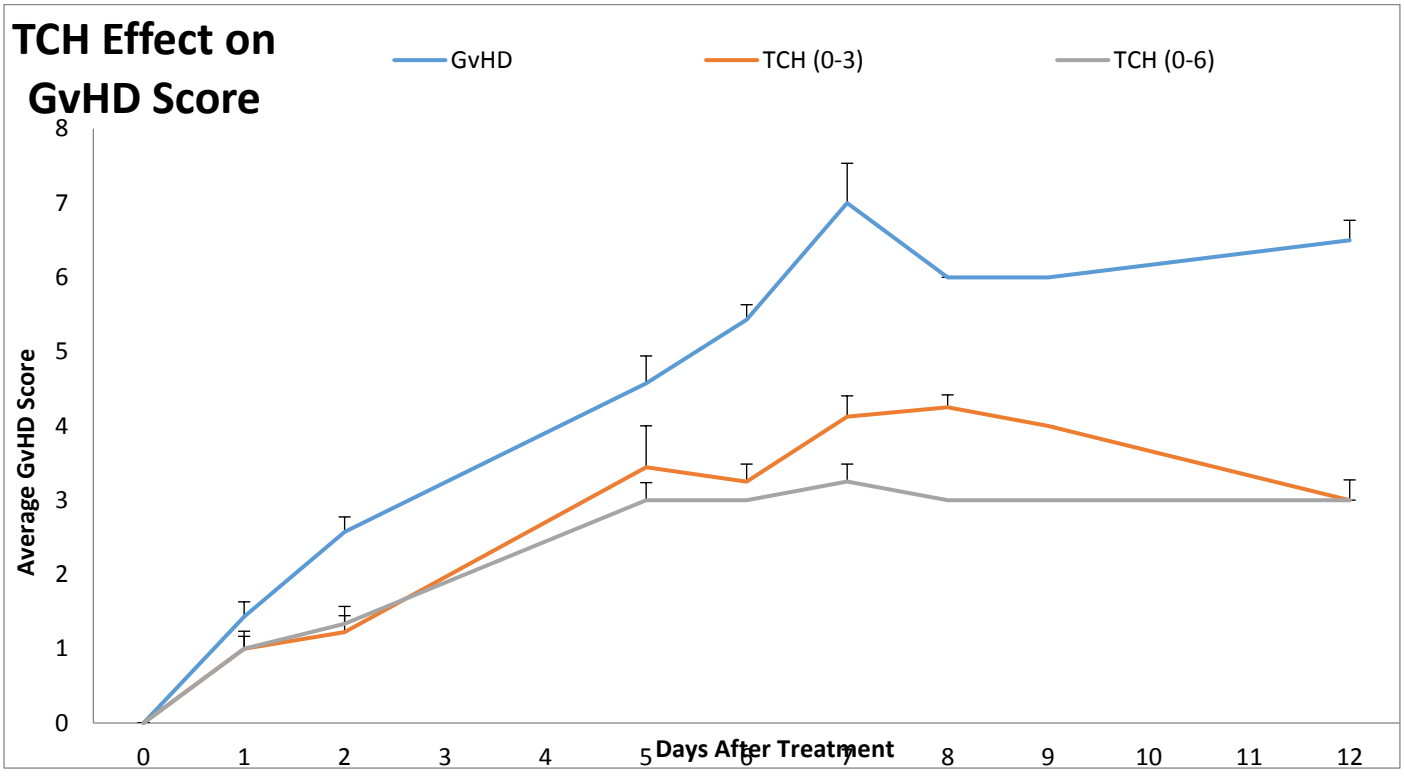
The experiments BMT 19, BMT 20 and BMT 21 were designed to induce different severity models of aGvHD in the BALB/c mice. When the average GvHD scores (y-axis) were plotted against the days after treatment (x-axis), a similar trend was noticed in all the three cases. It is evident from the trend that the post transplantational administration of TCH-013 is showing a positive effect on the aGVHD. The GVHD scores were given to the mice on the basis of the bodyweight, activity, posture, gut, fur, and skin. The graphical trend shows that there is a decrease in the GVHD scores when the mice were treated with TCH-013. The decrement of the GVHD score signifies a shift of the health of the mice towards recovery from aGVHD.



BMT 19


TCH Effect on GvHD
GvHD Scores
Average Weight Change (%)
Days After Treatment
0
1
2
3
4
5
6
7
8
9

BMT 20


TCH Effect on
GvHD Score
GvHD
TCH (0-3)
TCH (0-6)
Average GvHD Score
Days After Treatment
0
1
2
3
4
5
6
7
8
9
10
11
12

Discussion –

TCH-013 have shown to decrease the expression of the cell surface markers on the dendritic cells as well as the level of the dendritic cell cytokines went down when the dendritic cells were treated with the TCH-013. Moreover, TCH-013 also affected the antigen cross presentation as a result of which the CD4+ and CD8+ T cell population went down when treated with TCH-013. These three observations showed the similar patterns and supports each other. In other words, the TCH-013 downregulates the cell surface marker expression on the dendritic cells and also downregulates the dendritic cell cytokines as a result of which the antigen presentation in the dendritic cell as a whole decreases. And as the antigen presentation capacity of the dendritic cells are decreased, less CD4+ and CD8+ T cells were activated, when compared to the stimulated untreated control. TCH-013 shows a positive trend in decreasing the antigen presenting capacity of the dendritic cells but the mechanism behind this pattern is yet to be studied.

References –

[4] Socie G, Blazar BR, Acute graft-versus-host disease from the bench to the bedside, Blood, 2009;1144327–36

Internship Discussion

This section contains a discussion of the internship and should address the following points:

- Were the objectives achieved?
  The objectives of the project were met. The learning objectives of the internship was also met.

- What skills (scientific and professional) were learned during the internship?
  The professional skills I have gained from this internship are-

  □ Isolation of peripheral blood mononuclear cells (PBMNCs) from leukocyte reduction filters
  □ Dendritic cell negative selection using magnetic separation
  □ Dendritic cell stimulation, drug treatment, and culturing

  □ Analysis of flow cytometric data including statistical outputs using Flowing Software
  □ Cytometric Bead Based Array for Human inflammatory cytokines
  □ T-cell proliferation assay
  □ Operation of BD FACSCanto and BD FACSCalibur
  □ Analyze flow cytometric data
  □ Handling, ear punching, weighing, GvHD health scoring, isoflurane anesthesia, subQ drug injection, euthanization, dissection/ organ harvest, and irradiation of mice

- Did the PSM coursework properly prepare the student for the scientific content of the internship?

  The PSM coursework helped me to understand the scientific content of the internship. This internship heavily relates to immunology and the PSM CMB coursework prepared me well to grasp the concepts of the research very quickly.

- Did the PSM coursework properly prepare the student for the professional content of the internship?

  PSM CMB course have given me the opportunity to equip myself with good lab technique skills, which helped me a lot to cope up with the *in vitro* part of the research.

- What challenges did you experience during the internship?

  Mice handling was a challenge to me, especially injecting the mice and the ear punching were a challenge to me at first, but I gained experience and improved on those weaknesses, during the internship.

- What is your overall evaluation of the internship experience?

  I am very satisfied with my internship experience, as I have gained a lot of new skills. I was specifically looking for the flow cytometry experience, which I got from this internship.