Results

Selection of Antigen for vaccine candidate

Initially, we screened 9000 genes that are known to be conserved in *Leishmania* species by using microarray. We narrowed down to 99 genes. Further screening of 99 genes on the basis of significant changes in both avirulent and virulent strains, we came down to 32 genes. By doing real-time PCR of the chosen 32 genes, we picked only12 genes that showed maximal differential expression. Now, we screen those 12 genes by qPCR along with CD40 stimulation. 3% thioglycollate intraperitoneally injected into BALB/C mice, 5 days later, macrophages were harvested from the peritoneal fluid. The cells were seeded on the Petri plate, rested the cells for 48 hours; soon after, challenged with either *L. major* or *L. donovani* virulent and avirulent strain with 1:10 ratio. At 64th hours post-infection, cells were stimulated with anti-mouse CD40 (NA/LE) 3ug/ml antibody. 72 hours post-infection, lysed the cells with trizol for further quantification through real-time PCR. We observed that there was differential regulation in infected macrophages by CD40 stimulation, some genes were upregulated in LP and some were down-regulated in HP. Based on the fold change, we selected PAP (Phosphatidic acid Phosphatase) for cloning procedure **(Figure 7,8)**.

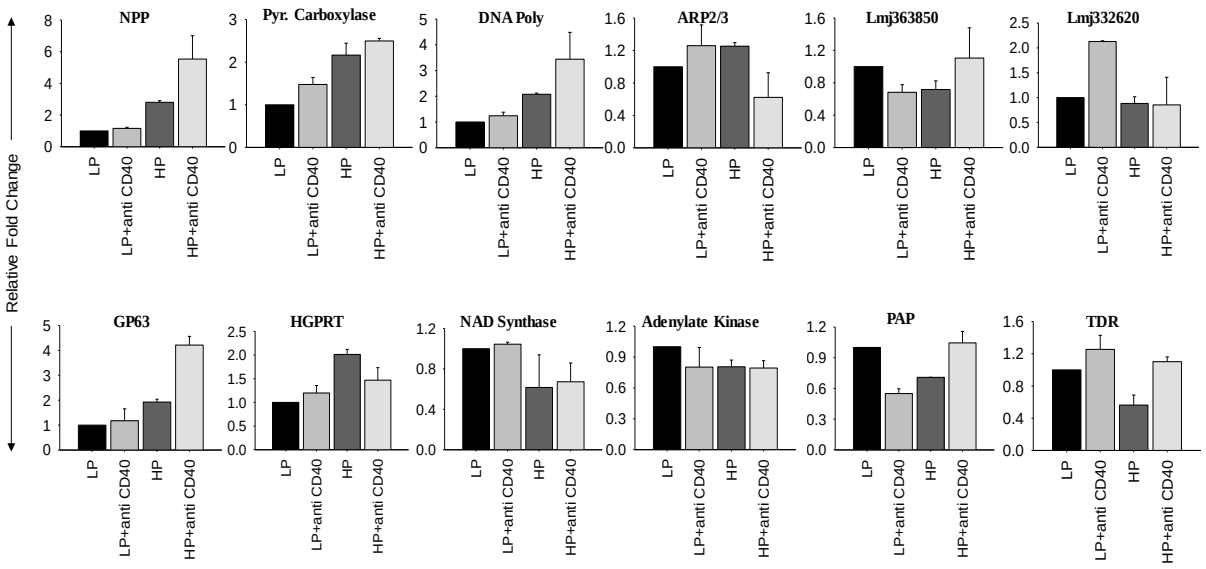

Figure: 7 Balb/c derived thioglycollate elicited peritoneal macrophage were challenge with *L. major* followed byCD40 stimulation for to check the expression of leishmanial gene.

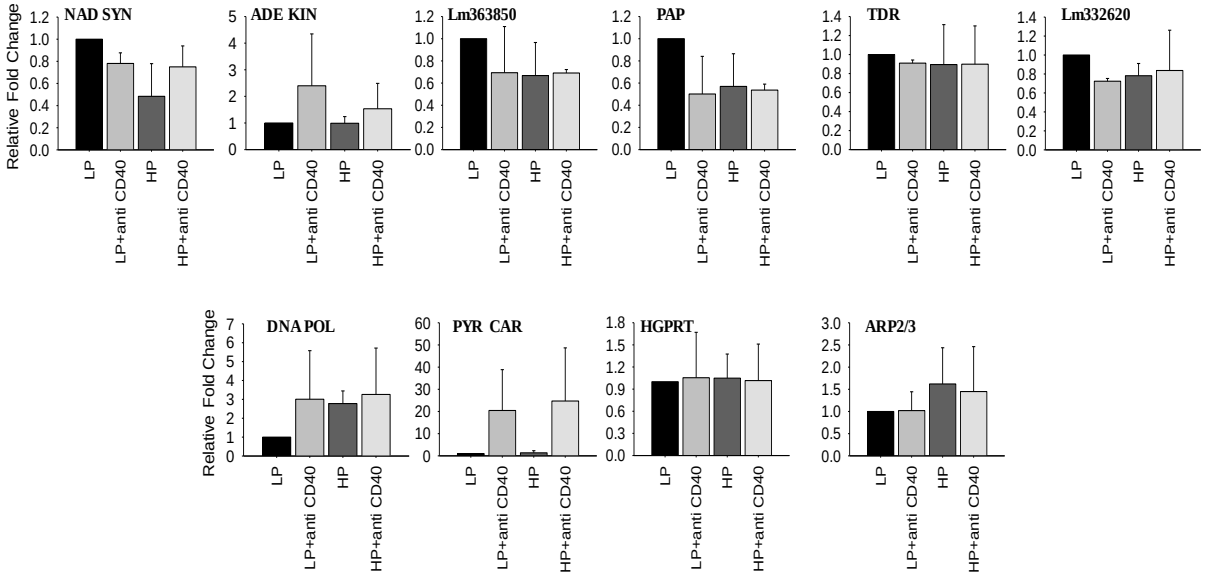

Figure: 8 Balb/c derived thioglycollate elicited peritoneal macrophage were challenge with *L. donovani* followed by CD40 stimulation for to check the expression of leishmanial gene.

Cloning of *LmjPAP* (Leishmania major Phosphatidic Acid Phosphatase) gene

The First *leishmania major* PAP gene was amplified by PAP-specific primer. Amplified PAP product was digested with restriction enzyme later on PAP gene were inserted in a PcDNA6/His A empty vector. *LmjPAP* was cloned in PcDNA6 His/A empty vector reveals from the colony PCR by using a specific primer as well as from restriction double digestion of recombinant plasmid pcDNA6HisA-PAP by BamHI and XbaI respectively. Double digestion releases the 1.2 kb insert of recombinant plasmid showing the presence of right insert PAP **(Figure 9)**. PAP clone was discontinued. So we have chosen MAPK10 as an alternative. Herein, we have select MAPK10 cloned leishmanial DNA for vaccination procedure because 15 MAP kinases are known in *Leishmania major* from them we pick

MAPK10 due to its homology with host ERK-1/2 for parasite survival. As we have earlier reported that *Leishmania major* MAPK10 shown promise in Balb/c mice against cutaneous leishmaniasis (117). So, we intended to test the efficacy of the same gene in *L. donovani* infection.

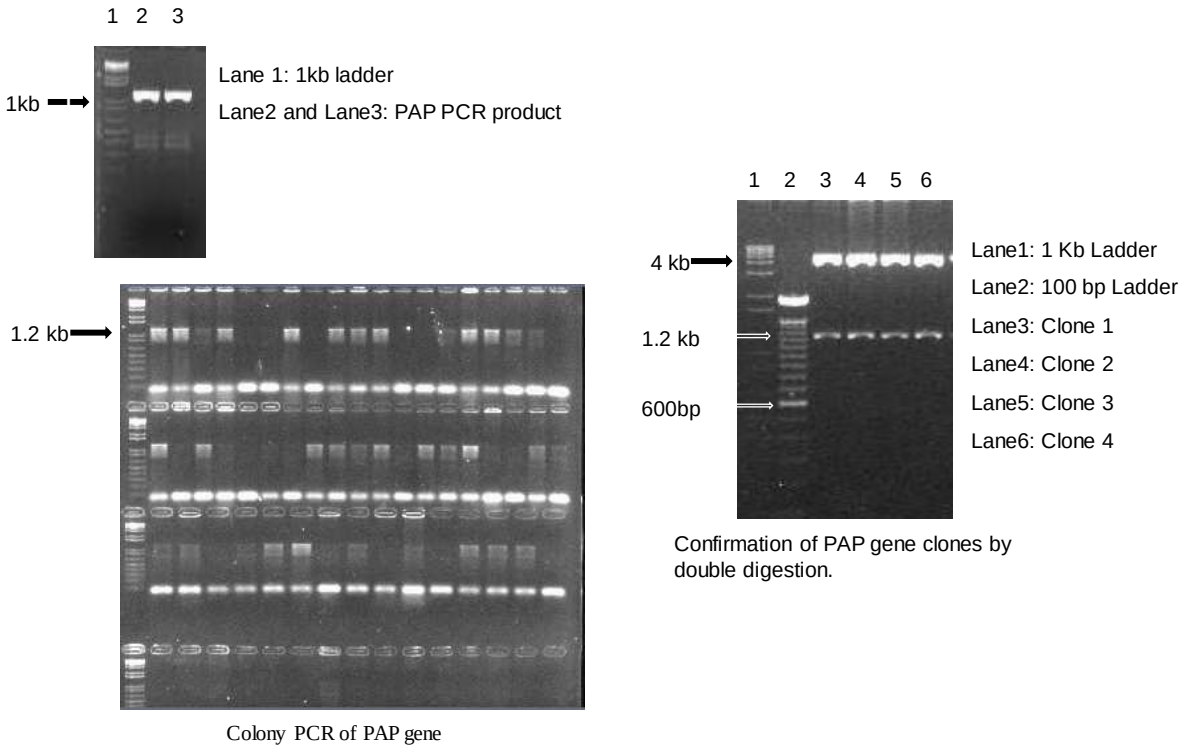

Figure: 9 Amplification product of PAP gene followed by cloning confirmation of PAP gene by colony PCR and restriction double digestion.

LmjMAPK10 DNA immunization significantly reduces the parasite burden against *L. donovani* infection

BALB/c mice were immunized on 0, 15, 30 days with 100ug each of Control DNA (i.e., Empty vector) or M10 DNA (Cloned leishmanial MAPK10 DNA). The mice were maintained with the intramuscularly (I.M) leishmanial DNA for 1month so that antigens were expressed in the myocyte muscle cell and presented at the cell surface allowing, as a result, memory T-cells development against M10 antigen. Soon after completion of incubation mice

were challenged with ($2x10^7$) stationary-phase *L. donovani* promastigotes on Day 14 and Day 28 respectively. Later spleen and liver were collected for determination of parasite load on indicated days. We found that M10 DNA immunized mice significantly decrease the parasite load in both visceral organs primarily spleen and liver. From that, we can conclude that splenic and Kupffer cell macrophages control the parasite replication in visceral organs following immunization M10 DNA. It indicates the cross-protective efficacy of *Leishmania major* MAPK10 DNA against *L. donovani* infection **(Figure 10)**.

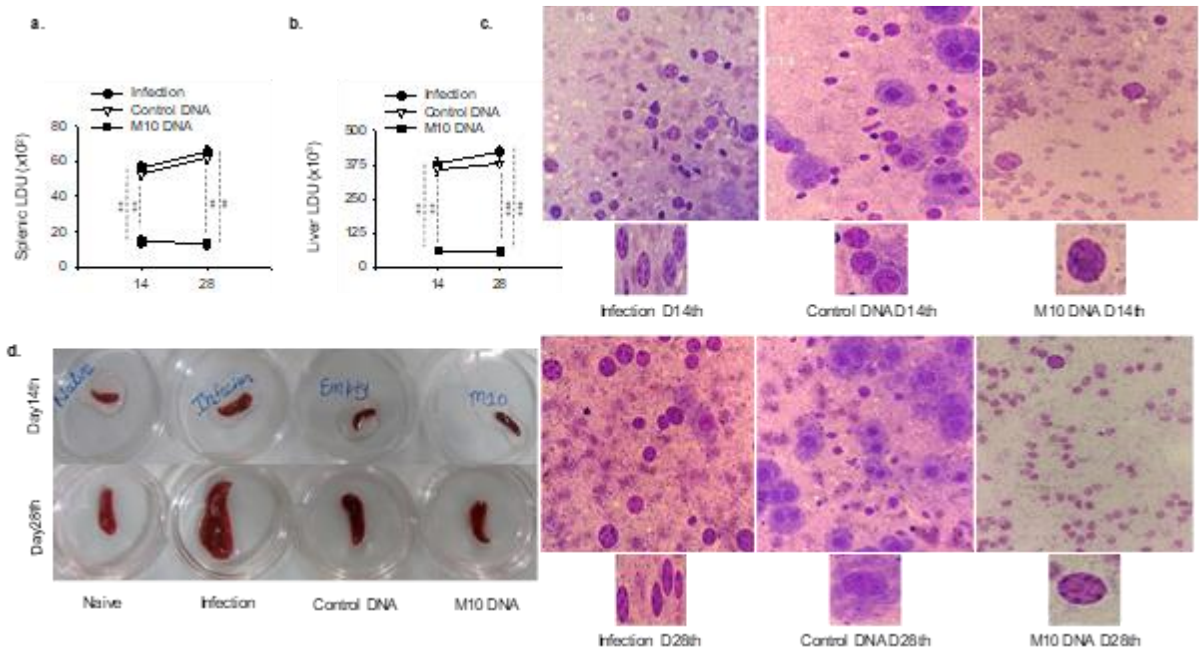

Figure: 10 (a) Splenic and (b) Hepatic parasite burden in BALB/c derived M10 primed *L. donovani* infected group (C) Giemsa stained Liver Tissue (D) Disease progression in spleen post-infection.

LmjMAPK10 immunization enhances the anti-leishmanial effect by reducing amastigote numbers

Thioglycollate elicited peritoneal derived macrophage were harvested for *L. donovani* infection in vitro. Let the infection establish, then we sort $CD4^+$T-cell (by FACS) from all the respective groups of mice. In vitro infected macrophage with sorted $CD4^+$ T-cell (1:3) ratio

was provided to all groups of mice and culture for another 56hr. Fix the slide in methanol and Giemsa stained. We found that the infected macrophage-T cell co-culture system, the T cells from the M10-immunized mice significantly reduced the amastigote count 14 and 28 days post-infection **(Figure 11)**.

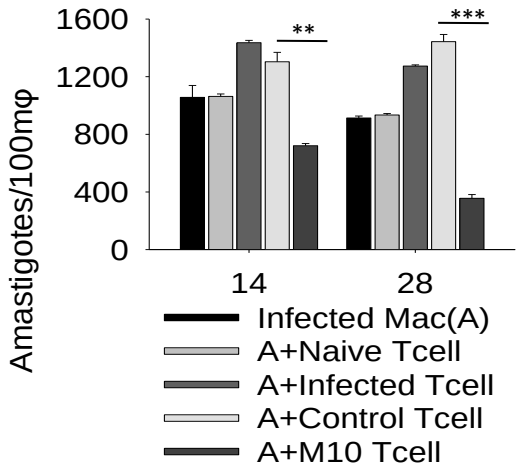

Figure: 11 *In vitro* macrophage T-cell co-culture *LmjMAPK10* primed mice leads to reduction in amastigote count.

LmjMAPK10 immunization enhances the anti-leishmanial effect with host-protective T cell functions

We collect supernatant from an infected macrophage T-cell co-culture system. The supernatants from the same co-culture were assessed to check pro and anti-inflammatory cytokine parameters. As it is well established that IL-12, IFN-γ & TNF-α are pro-inflammatory but anti-parasitic whereas IL-10 and IL-4 are associated with anti-inflammatory but pro-parasitic in nature. So, we intended to determine cytokine based Sandwich-ELISA from in vitro derived macrophage-T-cell co-culture group. We found that the M10 immunized group has higher production of IL-12, IFN-γ & TNF-α however, less IL-10 and IL-4. These observations suggest that the *L. major*-derived M10 immunization elicits host-protective T_H1-mediated response against *L. donovani* infected BALB/c mice **(Figure 12)**.

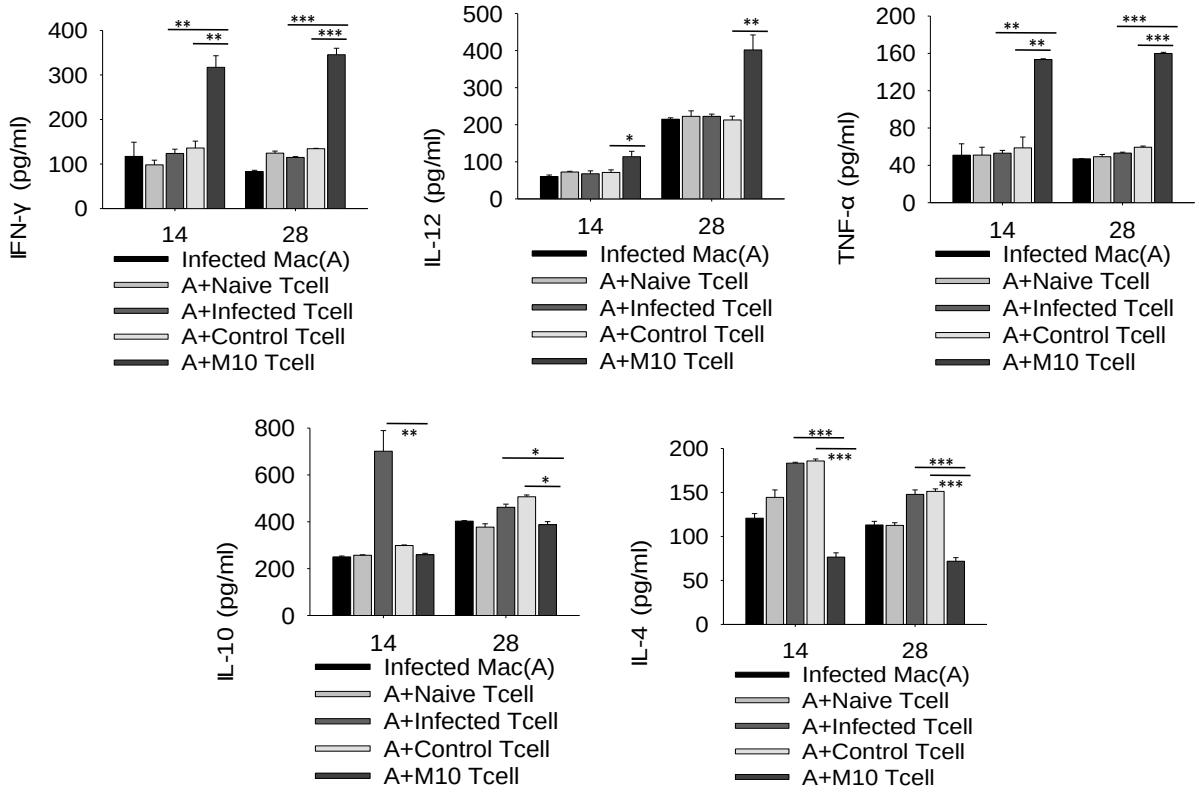

Figure: 12 Cytokine based Sandwich ELISA were done from *in vitro* macrophage T-cell co-culture supernatant of indicated group of mice.

LmjMAPK10 immunization enhances NO-mediated anti-leishmanial effect

In an infected macrophage T-cell co-culture system, the supernatant was collected to assess nitrite production. As it is well established that iNOS (inducible nitric oxide synthase) produce nitrite, which is responsible for controlling the replication of the *Leishmania* parasite. We observed that the M10 immunized group has a higher NO than the control (Empty DNA) group. These results indicate that M10 immunization has a potent anti-leishmanial function **(Figure 13)**.

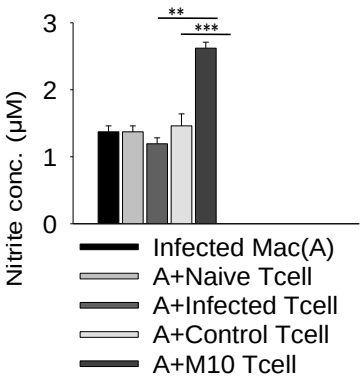

Figure: 13 NO production were measured from *In vitro* macrophage T-cell co-culture supernatant of indicated group of mice.

LmjMAPK10 immunization elicits humoral response against *L. donovani* infection

Immunized but *L. donovani*-infected mice group sera were collected before and after 14 and 28 days post-infection to assess the CSA-specific Ig isotypes. We observed significant changes in the leishmanial antigen-specific Ig isotype response. As it is well studied that IgG2a induce T_H1 and IgG1 induce T_H2 so we check Ig isotype during the course of pre and post-infection on Day 14 and Day 28. We found the heightened immune response in the M10 immunized group, by contrast, IgG1 and IgM levels were reduced during post-infection however, before infection, we did not observe any significant differences between respective Ig-isotype. As the cytokines, IL-4 and IFN-γ promote IgG1 and IgG2a response, the suppressed IgG1, but heightened IgG2a, responses suggest that M10 immunization induced T_H1-mediated immune response **(Figure 14)**.

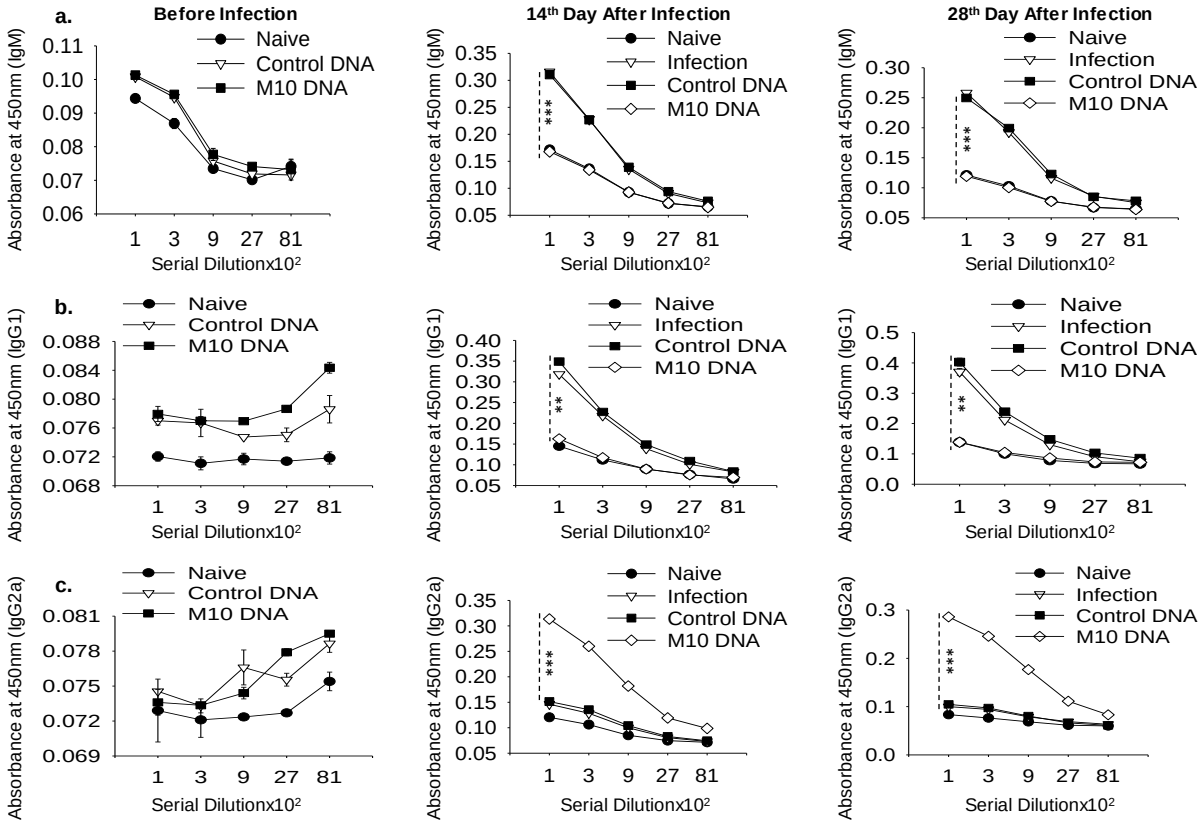

Figure: 14 Antibody mediated response of IgM, IgG1, IgG2a before and after infection of indicated group of mice.

LmjMAPK10 immunization induces cellular response against *L. donovani* infection

From M10-primed *L. donovani* infected mice, spleen were harvested and prepare single-cell suspension from splenocytes by stimulating with CSA (*L. donovani*) and culture it for 60hr. CSA-stimulated cultures of the splenocytes from M10-primed BALB/c mice were used for cytokine enumeration through ELISA. We found enhanced IL-12 and IFN-γ production in M10-immunized CSA stimulated group than medium alone 14 and 28 days post-infection. However, IL-10 and IL-4 levels were reduced during infection. It suggests that M10 immunization elicits cellular response against *L. donovani* infection **(Figure 15)**.

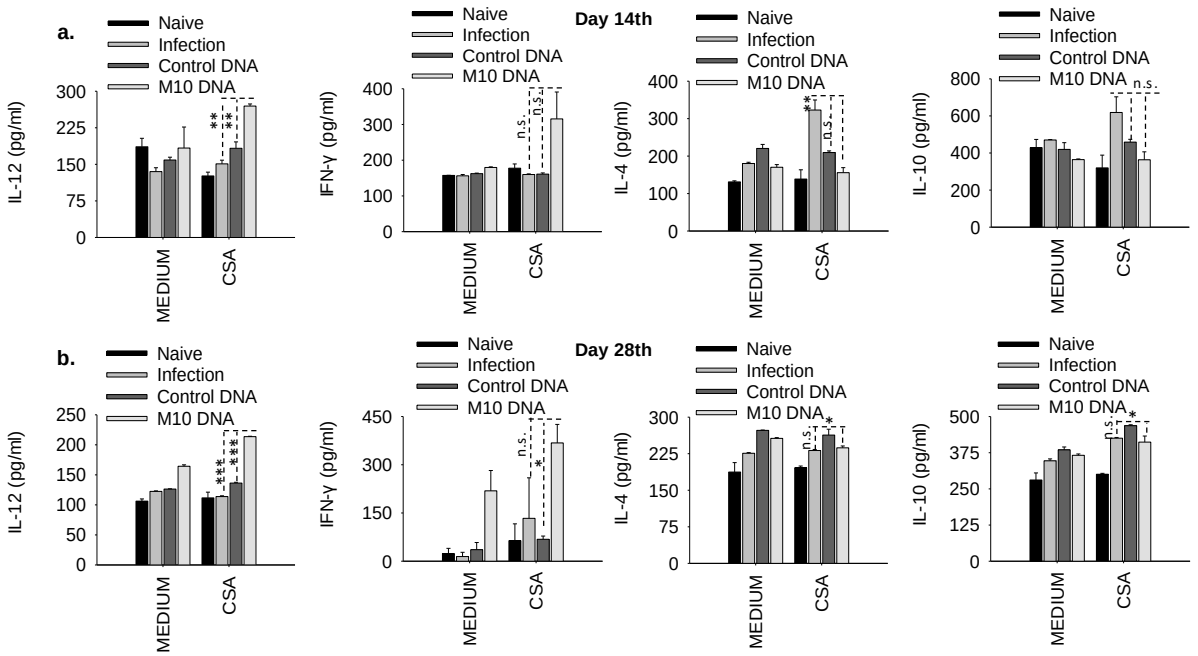

Figure: 15 Cytokine ELISA were performed from CSA stimulated splenocytes suspension from respective group of mice 14 and 28 days post-infection.

Cytokine profile indicates LmjMAPK10-elicited T_H1 response

Balb/c primed *L. donovani* infected mice from all groups were assessed for direct real-time PCR using RNA extracted from the splenocytes without sub-culturing in vitro. There was a reduced expression of IL-4, IL-10; Foxp3 a transcription factor associated with IL-10 expression, but an enhanced expression of IL-12, IFN-γ, and T-bet, the transcription factor associated with T_H1 response. Moreover, ROR-γt the transcription factor associated with IL-17 expansion was also increased.The cytokine profile also reveals similar responses of both pro- and anti-inflammatory genes at the transcription level **(Figure 16).**

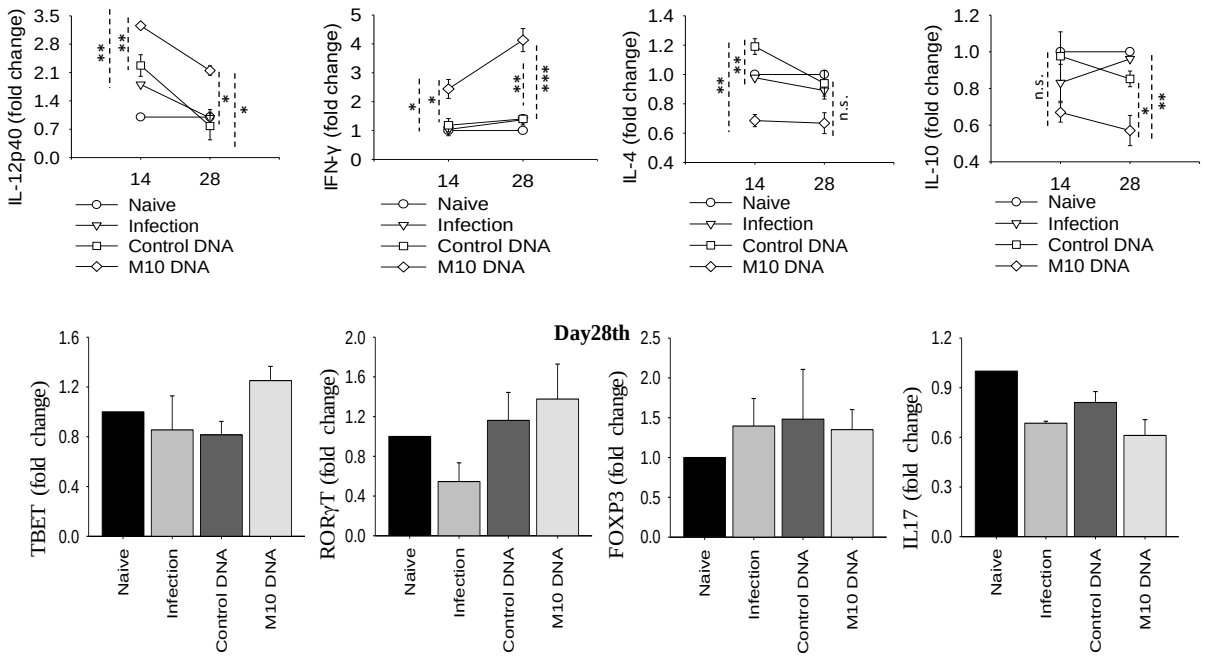

Figure: 16 Real-Time PCR were performed from splenocytes to check the pro and anti-inflammatory cytokine expression at RNA level.

LmjMAPK10 immunization reduces T_{reg} and T_H2 but enhances T_H1 cells in *L. donovani* infection

We further phenotyped the T cell subsets by multi-colour flow cytometry. The splenic T cells from the indicated immunized, infected or naïve BALB/c mice were analyzed for Treg cells- $FoxP3^+IL10^+$, $GITR^+IL10^+$, $GITR^+FoxP3^+$- by serial gating through $CD3^+CD4^+CD25^+CD127$low cells, for T_H1 cells- $T\text{-}bet^+IFN\text{-}\gamma^+$, T_H2 cells by $Gata3^+IL\text{-}4^+$ by serially gating through $CD3^+CD4^+$ cells or T_H17 cells by $Ror\gamma t^+IL17A^+$ by serially gating through $CD3^+CD4^+CD62L^+CD44^+$ cells. We observed increased T_H1, but reduced Treg, T_H2 and T_H17, cell numbers. These findings indicate LmjMAPK10 DNA-immunization elicited host-protective immunity by modulating $CD4^+$ T cell subsets **(Figure 17)**.

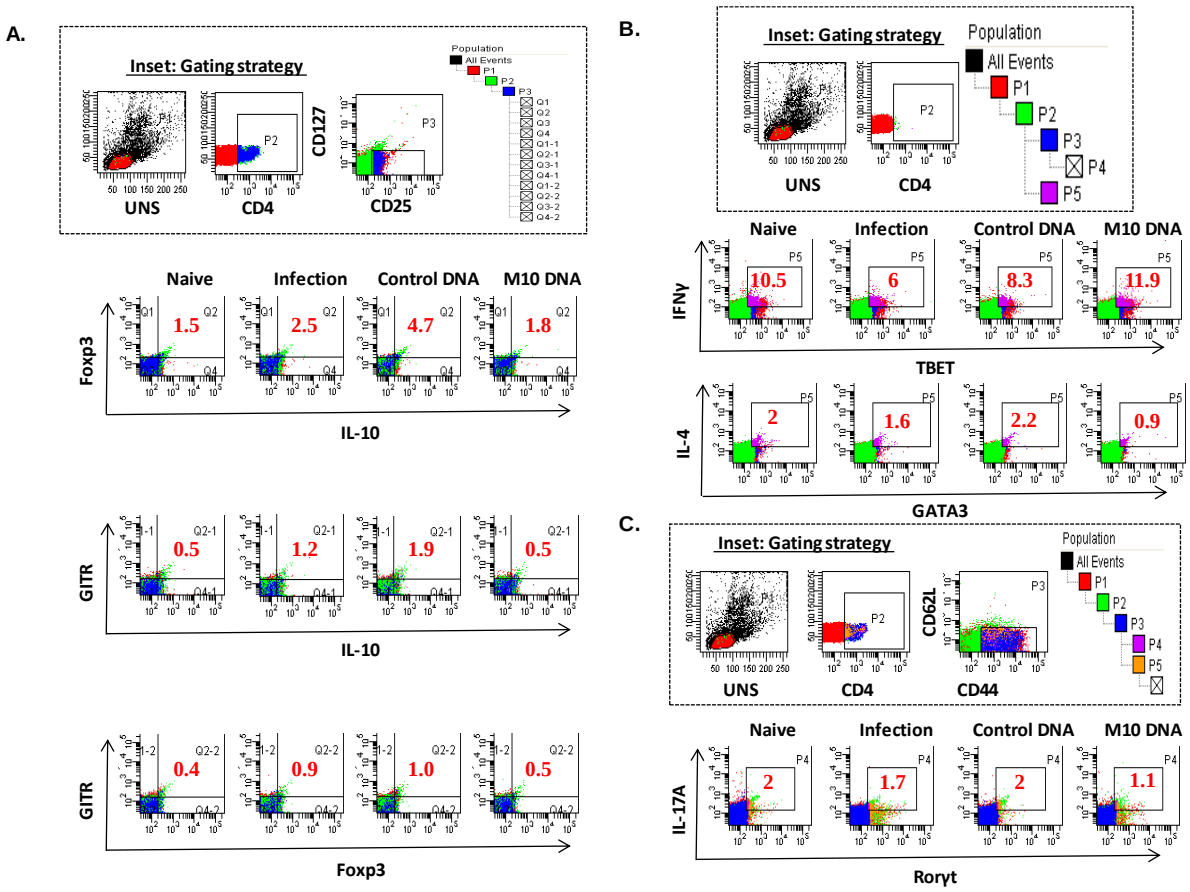

Figure: 17 Gating strategy for Treg, T_H1, T_H2 and $T_H17$population 14 and 28 days post-infection. Splenocytes T-cells were gated on $CD3^+$ $CD4^+$ cells as described in materials and methods.

M10+IL-7 immunization regulates anti-leishmanial function in visceral organ against *L. donovani* infection

Priming of BALB/c mice with 100ug each of Control DNA (i.e., Empty vector) or M10 DNA (cloned leishmanial MAPK10 DNA) on 0,15,30 days along with administration of recombinant murine IL-7 (rmIL-7 25ng each) on 2^{nd}, 17^{th}, 32^{nd} day respectively. Incubate the intramuscularly (I.M) leishmanial DNA and rmIL-7 through I.P (Intraperitoneal) route for 30 days so that antigenic protein from leishmanial DNA were expressed and represented at the cell surface with the help of MHC molecule as a result memory cell developed against M10 leishmanial antigen. Following incubation, mice were challenged with ($2x10^7$) stationary-

phase *L. donovani* promastigotes on Day 14 and Day 28 respectively. Later spleen and liver were collected to assess parasite load into a visceral organ on indicated days. We found that M10+IL-7 immunized mice significantly reduce the parasite load in both visceral organ spleen and liver. From that, we can assume that splenic and Kupffer cell macrophages restrict the parasite growth following immunization with IL-7+M10 DNA. Thus, we conclude that prime mice along with rmIL-7 regulate anti-leishmanial function by reducing the parasite load against the *L. donovani* challenge **(Figure 18)**.

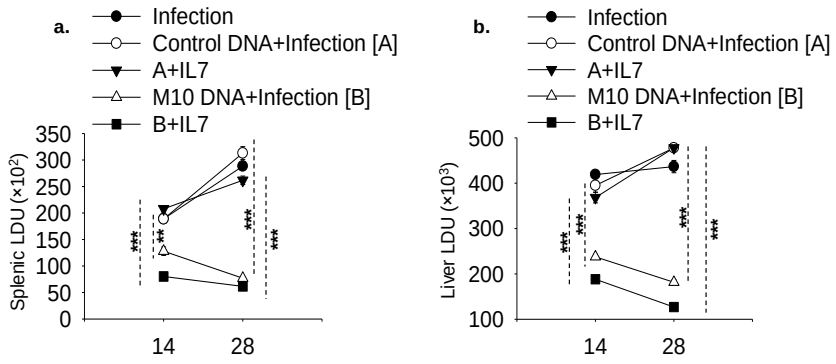

Figure: 18 Giemsa stained (a) Splenic and (b) Liver LDU in indicated group of mice post-infection.

M10+IL-7 immunization enhances the anti-leishmanial effect by reducing amastigote count in infected macrophages

Thioglycollate elicited peritoneal macrophage was harvested followed by infection with *L. donovani* in vitro. After the establishment of infection, $CD4^+$T-cell was sorted (by FACS) from splenocytes of all the immunized but infected groups of mice. In vitro infected macrophage along with sorted $CD4^+$ T-cell (1:3) ratio were provided to all the indicated group of mice and culture it for another 56hr. Fix the slide in methanol, dried and Giemsa stained and enumerated under a bright field microscope. We observed that the infected macrophage-T cell co-culture system, the T cells from the M10+IL-7 immunized mice had

significantly reduced amastigote numbers than M10 alone DNA. It suggests that M10+IL-7 has a better anti-leishmanial effect in the macrophage T-cell co-culture system **(Figure 19)**.

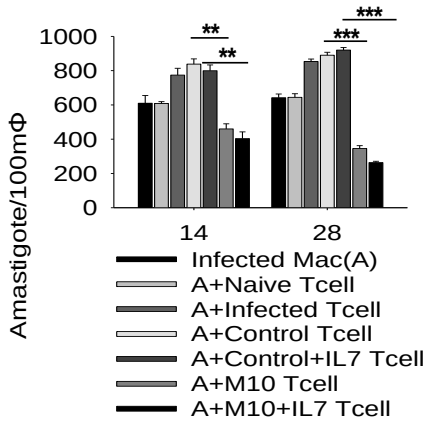

Figure: 19 Giemsa stained were used for to check parasite load in in vitro macrophage T-cell co-culture system.

M10+IL-7 immunization enhances IL-12 but reduces IL-10 in in vitro macrophage T-cell coculture system

Invitro macrophage T-cell co-culture system, we check both pro and anti-inflammatory cytokine parameters. Because it is well studied in terms of Leishmania infection, T_H1 based cytokine are pro-inflammatory but anti-parasitic however, T_H2 based cytokine are anti-inflammatory but pro-parasitic in nature. So, we want to assess both parameters IL-12 and IL-10 following M10+IL-7 prime *L. donovani* infected mice. We observed that the M10 immunized group showed higher production of IL-12 however, less IL-10 were produced 14 and 28 days post-infection. These observations suggest that *L. major*-derived M10+IL-7 immunization elicits host-protective T_H1 response against *L. donovani* infection **(Figure 20)**.

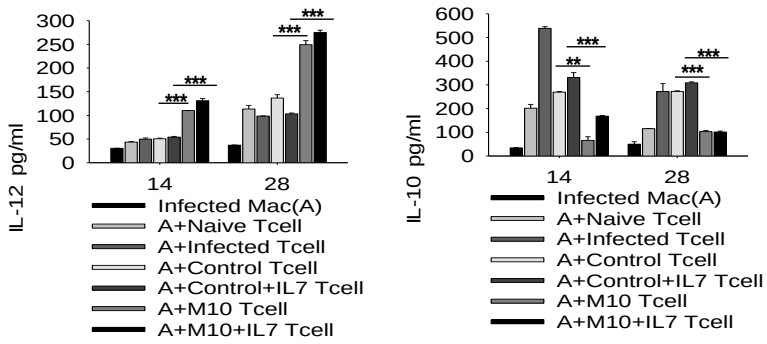

Figure: 20 Cytokine based ELISA were performed from in vitro macrophage T-cell co-culture supernatant.

M10+IL-7 immunization enhances NO not early but at a later point of infection

In an infected macrophage T-cell co-culture system, the supernatant was collected for a nitrite-based assay with the help of Griess reagent. As it is known that iNOS (inducible nitric oxide synthase) is responsible for the production of nitrite-ion, during *Leishmania* infection nitrite kills the leishmania parasite. We did not observe any significant NO production at 14 days post-infection but higher NO was produced at 28 days post-infection in the M10+IL-7 group. That means M10+IL-7 immunization restricts the amastigote growth at a later time point of infection **(Figure 21).**

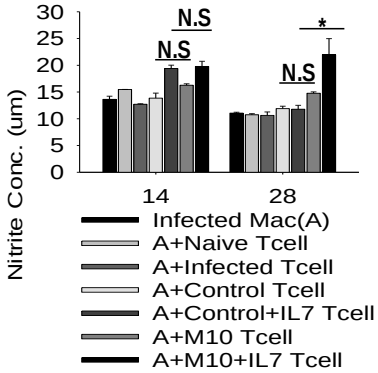

Figure: 21 NO production was measured from Griess reagent in an in vitro macrophage T-cell co-culture system.

M10+IL-7 immunization elicits an antigen-specific isotypic response

BALB/c prime *L. donovani*-infected mice group sera were collected before and after a challenge to assess the CSA-specific Ig isotypes. We did not observe comparable amounts of leishmanial protein in immunized mice before infection but we observed significant changes in the leishmanial antigen-specific Ig isotype response post-infection. We observe significantly reduced IgG1 and IgM post-infection however, IgG2a were comparable at 14 days but significantly enhanced at 28 days post-infection. Suppressed IgG1, IgM but heightened IgG2a responses suggest M10 +IL-7 immunization induced T_H1 type protective immune response **(Figure 22)**.

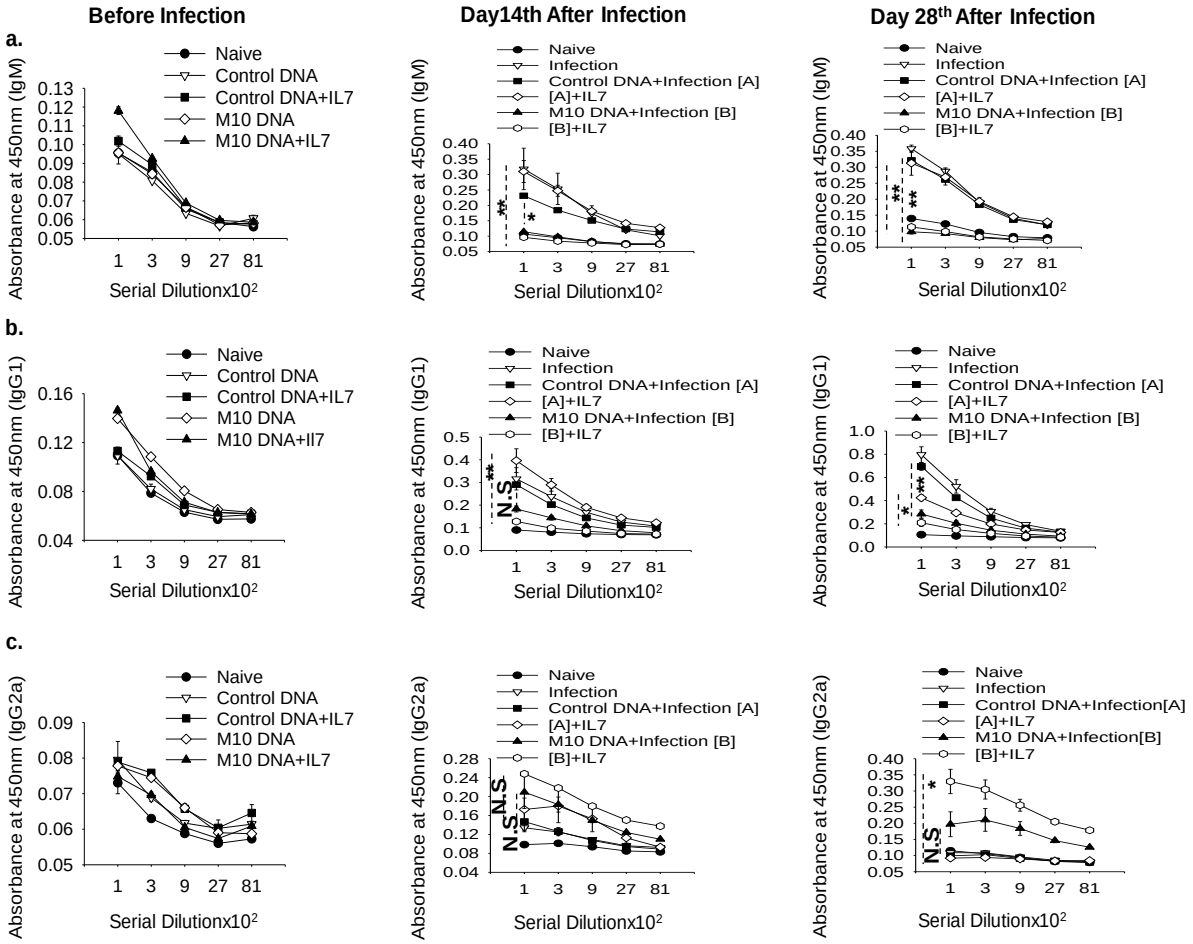

Figure: 22 Serially diluted serum samples were used to check antibody mediated Ig-isotype response of IgM, IgG1, IgG2a before and after infection.

M10+IL-7 immunization induces T_H1 but down-regulating T_H2 type cellular response

BALB/c primed *L. donovani* infected mice from all respective group spleen were harvested and prepare single-cell suspension by stimulating with CSA (Crude Soluble Antigen of *L. donovani*) and culture it for 60hr. CSA-stimulated cultures of the splenocytes from the indicated group of immunized mice were used for cytokine-based sandwich ELISA assay. We found significantly enhanced production of IL-12 and IFN-γ in CSA stimulated group 14 days post-infection, however, IL-10 was not significant but comparable IL-4. In addition, significantly enhanced IL-12 and IFN-γ however, reduced IL-10 and IL-4 28 days post-infection. Thus, M10+IL-7 immunization suggests cell-mediated response both early and late time points **(Figure 23)**.

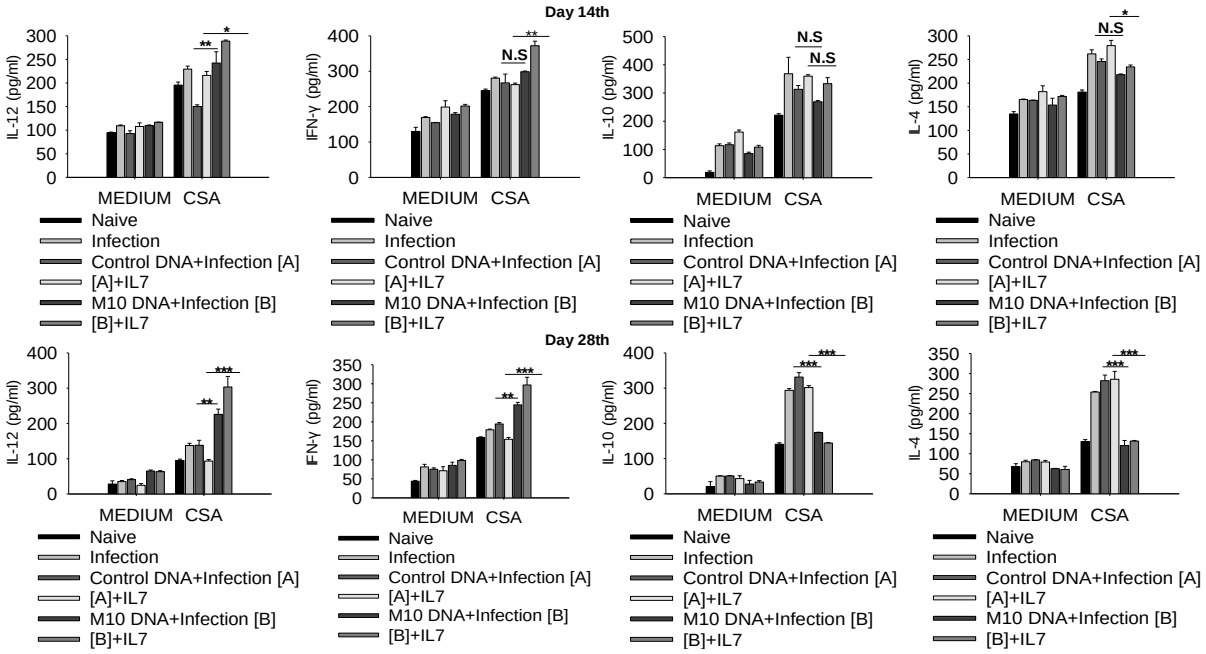

Figure: 23 Cytokine ELISA of splenocyte derived CSA stimulated indicated group from 14 and 28 days post-infection.

M10+IL-7 immunization induces T_H1, T_H17 and T_M but reduces T_{REG} and T_H2 cell population

We further phenotyped the T cell subsets 14 and 28 days post-infection by multi-colour flow cytometry. The splenic T cells from the indicated immunized, infected or naïve BALB/c mice were analyzed for Treg cells- $FoxP3^+IL10^+$, $GITR^+IL10^+$, $GITR^+FoxP3^+$- by serial gating through $CD3^+CD4^+CD25^+CD127$low cells, for T_H1 cells- T-bet^+ IFN-γ^+, T_H2 cells by $Gata3^+IL\text{-}4^+$ by serially gating through $CD3^+$ $CD4^+$ cells for T_H17 cells by $Ror\gamma t^+IL17A^+$ by serially gating through $CD3^+CD4^+CD62L^+CD44^+$ cells or for T_M Effector cells by $CD44^+$CD62L(low) $CCR7^-$ for central memory $CD44^+$CD62L(high) $CCR7^+$ by serially gating through $CD3^+CD4^+$ cells. We observed reduce the population of Treg either through GITR-FOXP3, FOXP3-IL10 or GITR-IL10 but comparable T_H17, T_H1, TH_2 and T_M14 days post-infection **(Figure 24A-E)**. In addition, at Day 28 enhance cell population of T_H1, T_H17 and T_m however, reduced T_H2 and Treg. These findings suggest M10+IL-7 immunization elicits host-protective immunity by modulating $CD4^+$ T cell subsets **(Figure 25A-D)**.

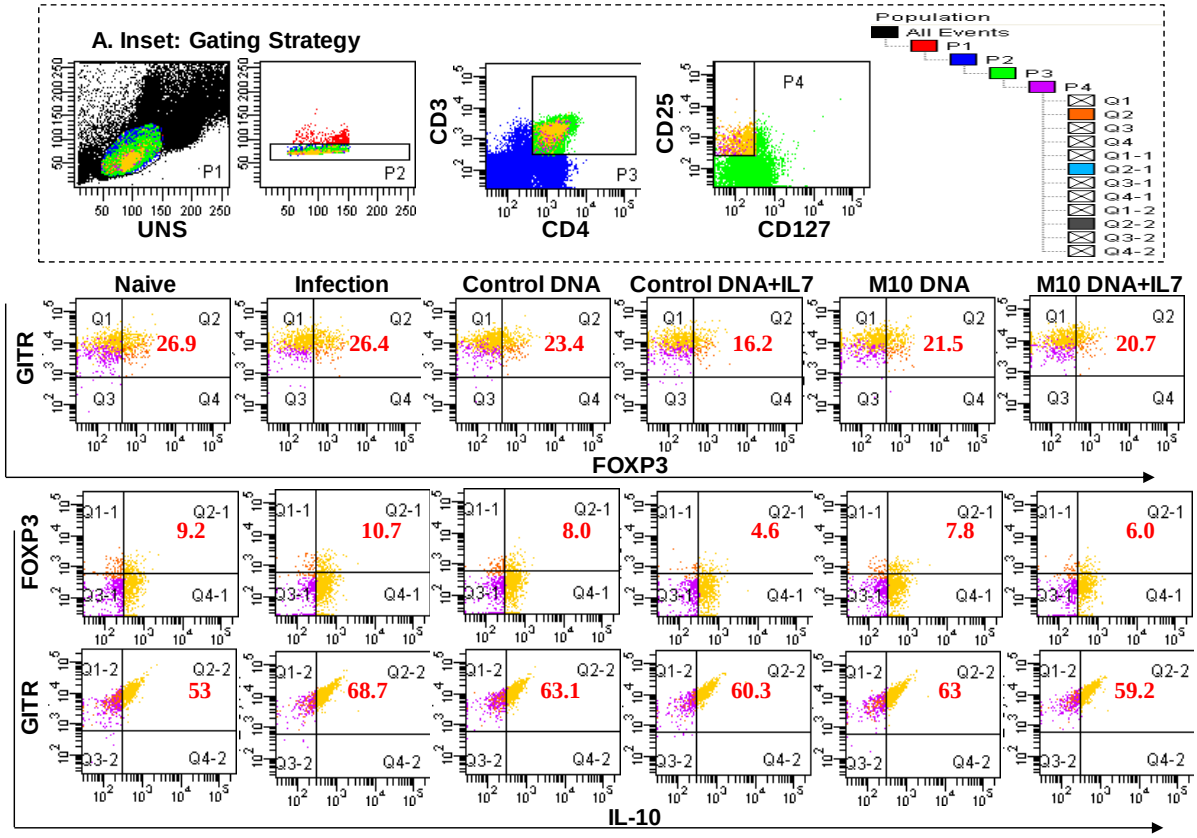

Figure: 24A Gating strategy for Treg cell population by serially gating on $CD3^{+}CD4^{+}CD25^{+}CD127^{+}$ T-cells 14th day post-infection.

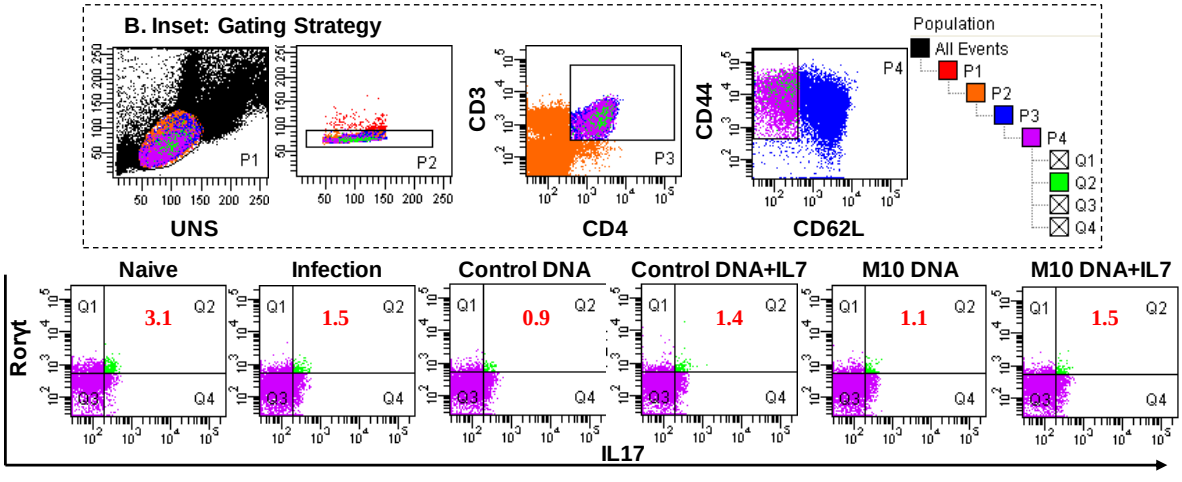

Figure: 24B Gating strategy for T_H17 cell population by serially gating on $CD3^{+}CD4^{+}$ $CD44^{+}CD62L^{+}$ T-cells 14th day post-infection.

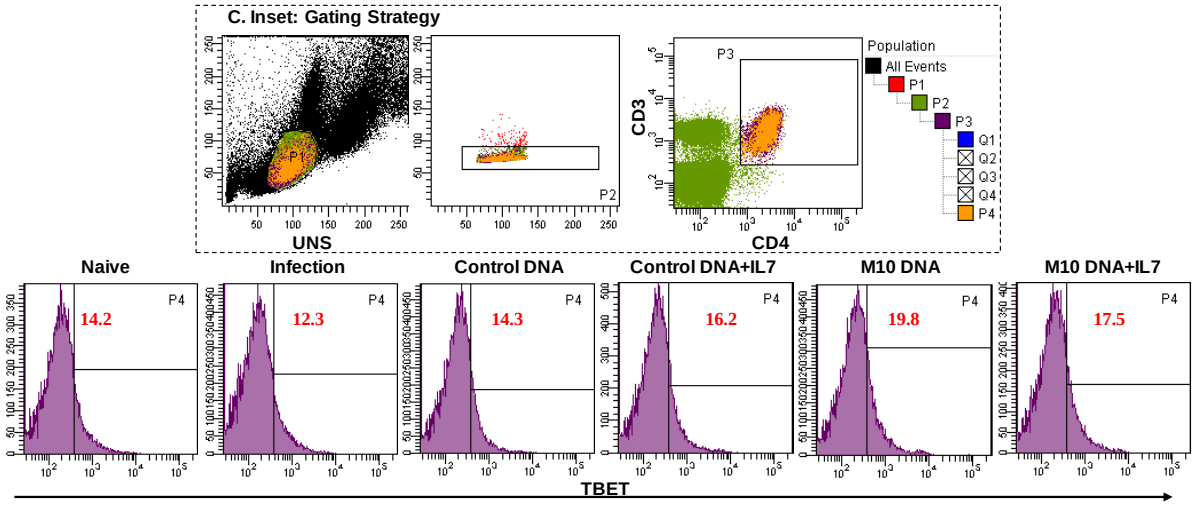

Figure: 24C Gating strategy for T_H1 cell population by serially gating on $CD3^+CD4^+$ T-cells 14th day post-infection.

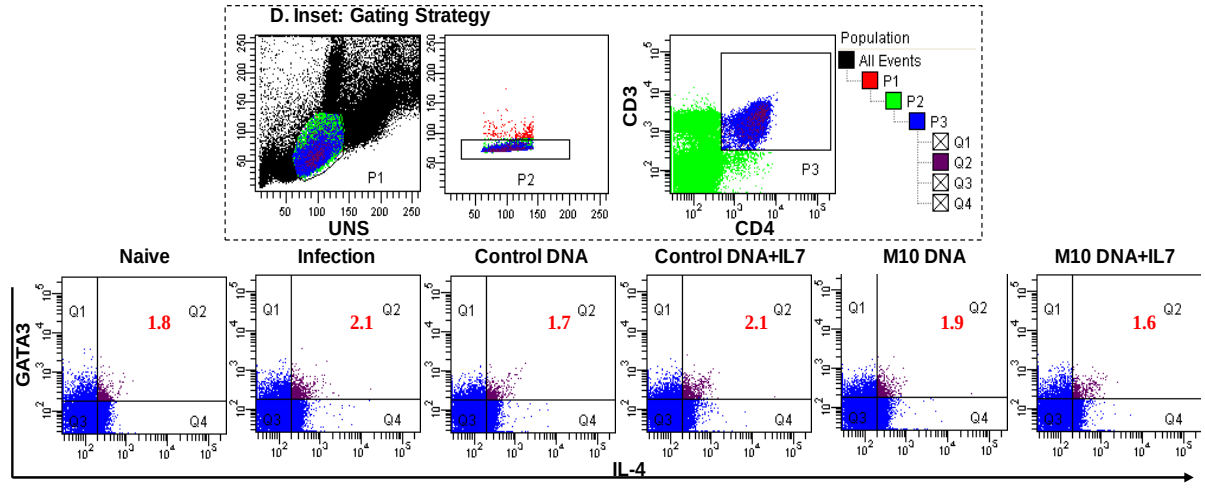

Figure: 24D Gating strategy for T_H2 cell population by serially gating on $CD3^+CD4^+$ T-cells 14th day post-infection.

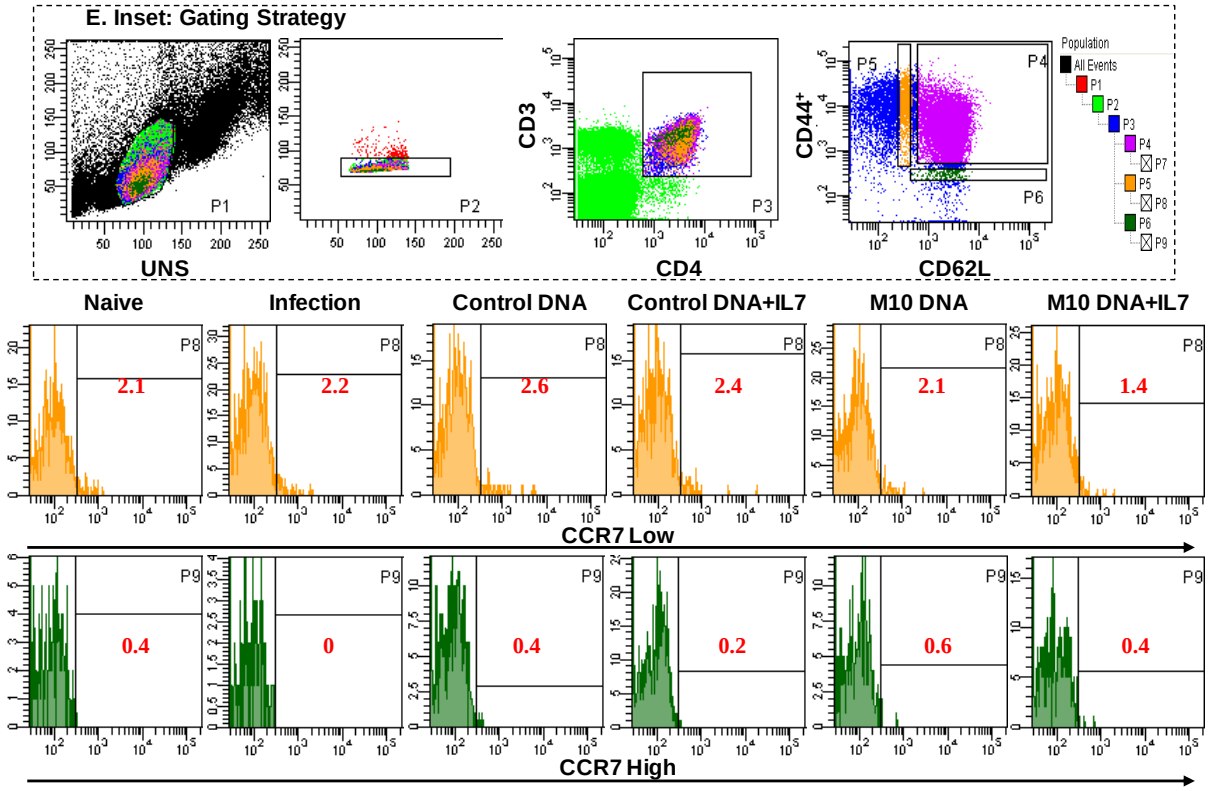

Figure: 24E Gating strategy for T_M cell population by serially gating on $CD3^+CD4^+$ $CD44^+CD62L^+$ T-cells 14th day post-infection.

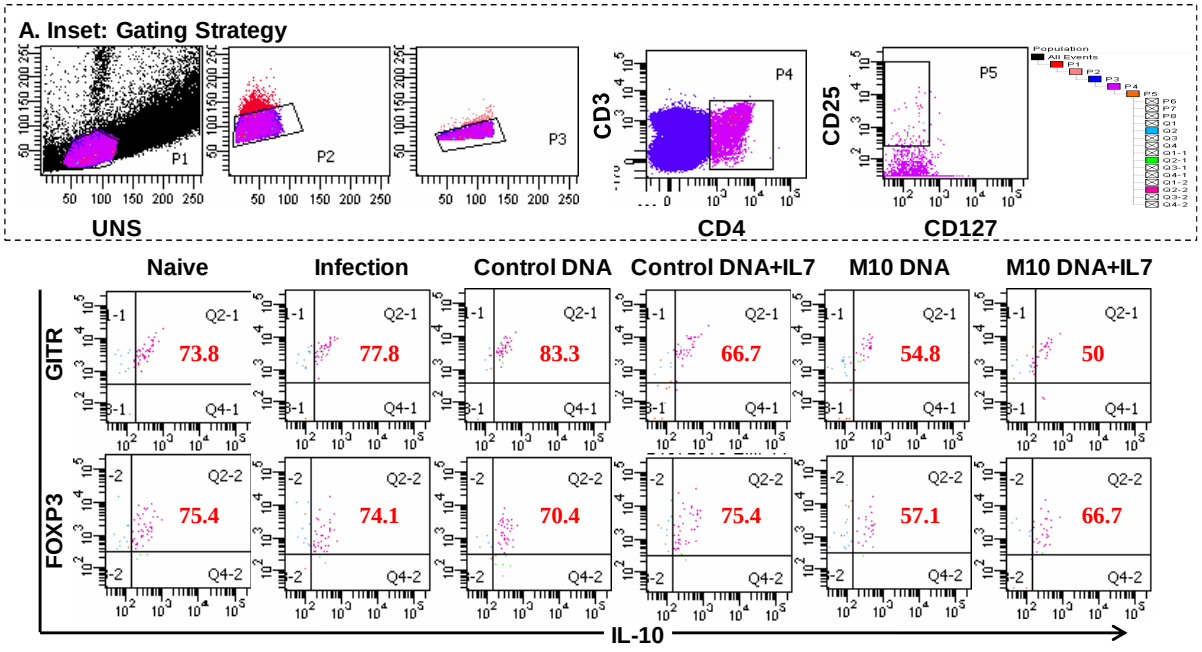

Figure: 25A Gating strategy for Treg cell population by serially gating on$CD3^+CD4^+$ $CD25^+CD127^+$ T-cells 28th day post-infection.

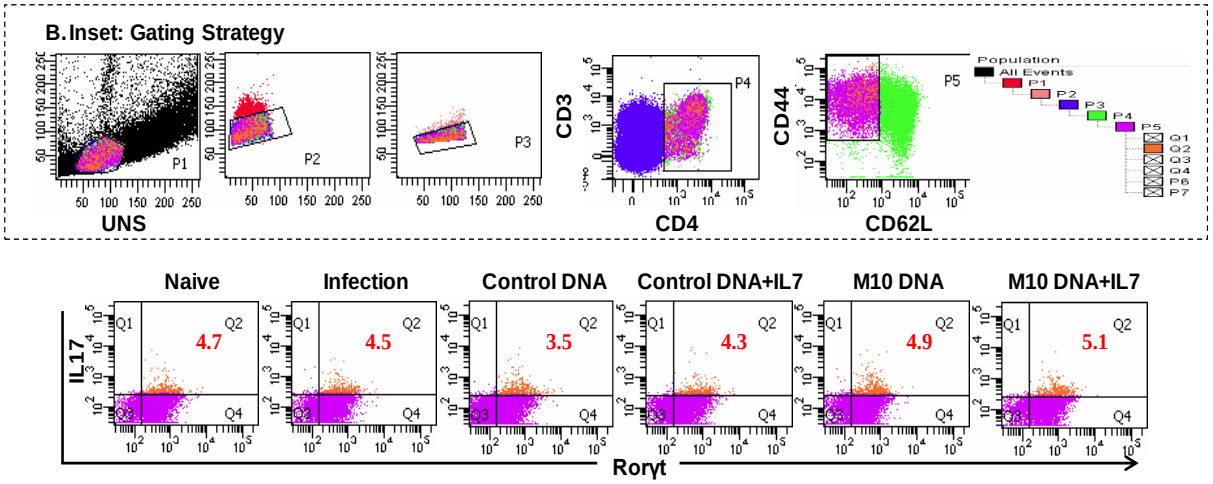

Figure: 25B Gating strategy for T_H17 cell population by serially gating on $CD3^+CD4^+CD44^+CD62L^+$ T-cells 28th day post-infection.

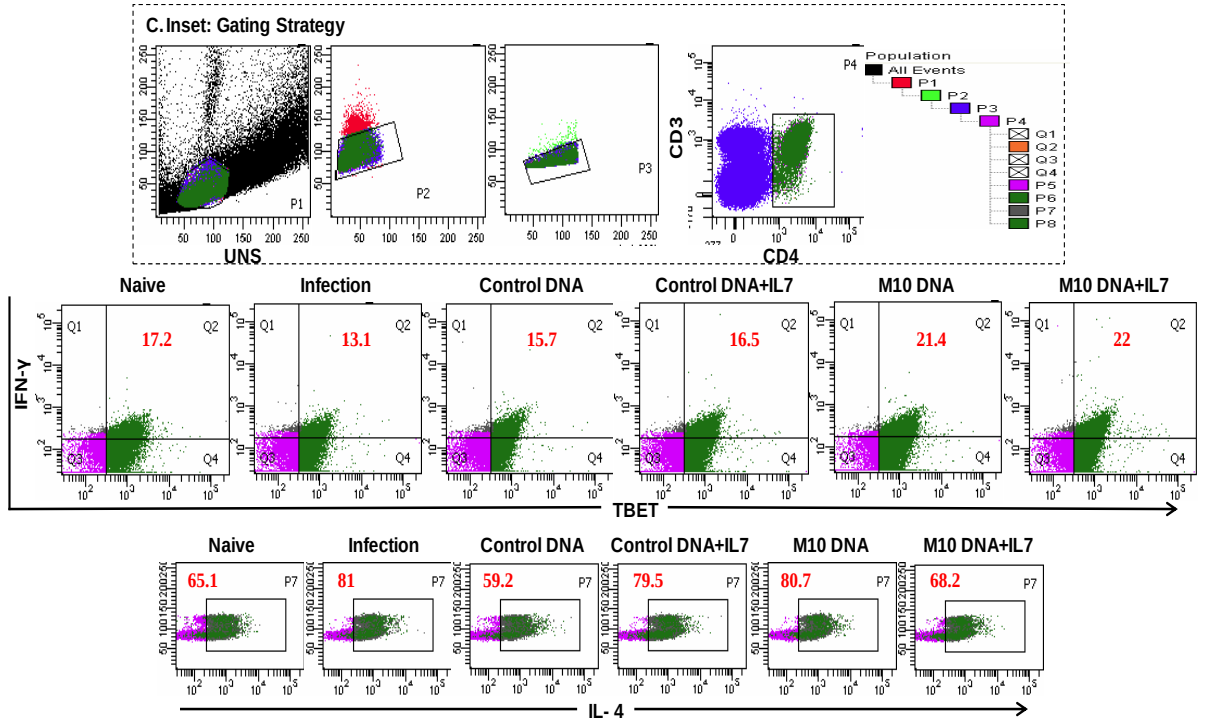

Figure: 25C Gating strategy for T_H1 and T_H2 cell population by serially gating on $CD3^+CD4^+$ T-cells 28th day post-infection.

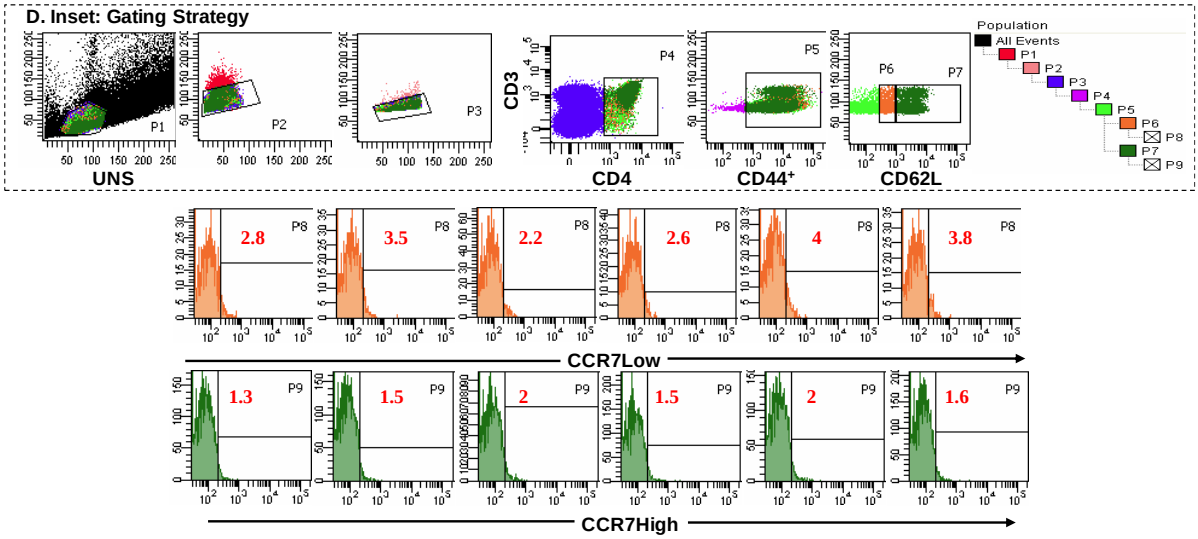

Figure: 25D Gating strategy for T_M cell population by serially gating on $CD3^+CD4^+CD44^+CD62L^+$ T-cells 28th day post-infection.