Summary and conclusions
In this study, we have tried to analyse the factors responsible for driving the response to distinct T helper subsets, in response to immunisation with live or heat-killed preparation of Stm in mice.

It has been shown before in this laboratory that, immunisation with live versus heat-killed Stm in BALB/c J strain, produces a distinct T helper response, as seen by increased DTH reaction, IFN-γ dominant cytokine profiles of the in vitro activated splenocytes as well as increased levels of IgG2a over IgG1 in mice that are immunized with live Stm, whereas mice that are immunized with heat-killed Stm, show the skewed Th2-like response.

What are the factors that may have a bearing on this skewing of T helper responses to the same recall antigen (Stm sonicate), when immunized with two different forms of Stm (live versus heat-killed) needed to be analyzed. We have carried out this study in three parts. First, we have looked at the effect of the host factor involved in Stm infection viz. Ity allele on the T helper development in the established Stm immunisation model. Secondly, we have looked at various bacterial factors involved, which include, entry into phagocytes like macrophages, and replication in vivo in the host cells. Finally, we have looked at various factors that are already shown to be involved in T helper development, such as the effect of some pharmacological agents, and MHC-peptide ligand density generated on the surface of APCs.

We have analyzed the major restriction elements involved in the response towards Stm using the MHC class II and TAP1 gene targeted mouse strains. It has been shown that the major element is MHC class II molecules as practically all the responses are diminished in mice deficient in MHC class II
expression, as seen by cytokine response after splenocytes have been activated \textit{in vitro} with Stm sonicate.

\textit{Ity} allele has been shown to be involved in clearing up the initial load of Stm after the infection. It has also been shown that there is initial surge of NK cell as well as \gamma\delta T cell mediated IFN-\gamma in this initial phase. Whether the reduced number of bacteria in the \textit{Itys} mice at the time of priming of T cell lead to any change in T helper development was chased. It can be seen from our experiments that both \textit{Itys} as well as \textit{Itys} mice show higher DTH when immunized with live Stm as compared to mice immunized with heat-killed Stm. It can also be seen that like \textit{Itys} mice, splenocytes from \textit{Itys} mice upon \textit{in vitro} stimulation with Stm sonicate show preponderance of IFN-\gamma dominated response, whereas, mice immunized with heat-killed bacteria show very low to undetectable IFN-\gamma. It can be seen that, the cytokine responses though dominated by IFN-\gamma in mice immunized with live Stm, are lower in magnitude in \textit{Itys} mice. This may be because of the faster clearing of the bacteria, hence less priming. This less priming however does not lead to qualitatively different response. When \textit{in vitro} proliferations are done over a long period (~90days), \textit{Itys} mice show small amounts of IFN-\gamma.

In the immune sera obtained from these immunized mice, it has been shown that both \textit{Itys} and \textit{Itys} mice show IgG2a dominance after immunisation with live Stm, as compared to IgG1 dominance after immunisation with heat-killed Stm, as seen by IgG1/IgG2a ratios.

In this study, we have chosen pairs of \textit{Itys} and \textit{Itys} mice of different MHC haplotype, to generalize the argument viz. BALB/c J & C.D2 (both H-2d) and C57BL6 J and C3H.SW (both H-2b). It has been shown here that, irrespective
of their MHC haplotype, \( \text{Ity}^S \) and \( \text{Ity}^I \) mice produce qualitatively same T helper development.

As can be seen from data shown, \( \text{Ity}^I \) mice do not clear the Stm \textit{in toto}, and hence to assess the role of replication of bacteria, somewhat cleaner method was to be adopted. Metabolic mutants of Stm, viz. \( \text{PurA} \) and \( \text{AroA} \) mutants are blocked in the synthesis of essential components of biochemical pathways. It has been reported that these attenuated strains do not grow beyond one or two cycles of replication \textit{in vivo}. But they are retained and not cleared as seen by our studies of \textit{in vivo} survival.

As can be seen from the data shown, the qualitative responses generated by these bacteria are Th1-like in mice immunized with either Stm754 or Stm \( \text{AroA} \) and \( \text{PurA} \) in live forms. Due to the decreased growth \textit{in vivo}, however, the responses quantitatively low. All the parameters tested, viz. DTH reaction, IgG1/IgG2a ratios as well as cytokine production by \textit{in vitro} stimulated splenocytes are suggestive of Th1-like response.

\( \text{E coli} \), another member of \textit{Enterobacteriaceae} however, when injected in live or heat-killed forms does not show the characteristic T helper cell development as seen after immunisation with live Stm. Mice immunized with live \( \text{E coli} \) do not show heightened DTH reaction in any of the mouse strains tested including \( \text{Ity}^I \) strains. Furthermore, the splenocytes from these mice show barely detectable IFN-\( \gamma \) production after \textit{in vitro} stimulation with either \( \text{E coli} \) or Stm sonicate. When sera from mice immunized with live \( \text{E coli} \) are analyzed, the antibody levels are very low as compared to the sera from live Stm immunized mice; hence isotype analysis could not be done from these sera. It is known that like Stm, which invade macrophages actively by inducing macrophage
membrane ruffling, followed by endocytosis, *E coli* HB101 can not do so. This probably leads to the responses towards Th2-like helper T cell development.

It was a logical possibility that live Stm was mounting Th1-like response because of certain proteins present in their native conformation, with possible contamination with macromolecules like LPS. Hence, to rule out the possibility that priming of T cells towards Th1-like cells is altered by heat killed bacteria, mice were immunized with soluble protein extract from heat-killed and live Stm. It is found out that both these immunizations lead to similar cytokine pattern indicating that the observed differences in T helper development are not possibly due to different structural forms of the proteins in live and heat-killed Stm immunizations. Moreover, it is also shown that, the Th2-like response in live *E coli* immunized mice are not due to some intrinsic defect in the recall ability of *E coli* sonicate, as *E coli* sonicate when used as a recall antigen in mice immunized with live Stm, shows the expected IFN-γ dominance. On the similar lines, Stm sonicate used as a recall antigen in mice immunized with live Stm, shows the expected IFN-γ dominance. On the similar lines, Stm sonicate used as a recall antigen in an *in vitro* splenocyte stimulation assay, does not show the IFN-γ dominance from the splenocytes of mice immunized with either heat-killed or live *E coli*. Mice immunized with irradiated Stm, which can not grow *in vivo*, do not mount IFN-γ dominant T cell response against Stm, unlike those immunized with live Stm. All these data, as a whole indicate that, intracellular localization as well as few rounds of intracellular replication, in response to distinct intracellular environment, leads to IFN-γ dominant responses.

*In vivo* MHC-peptide ligand densities generated on the surface APCs after injection with live or heat-killed preparation of Stm754, or Stm PurA or *E coli* were tested using a T cell hybridoma, which recognizes a shared epitope from Stm and *E coli*. It can be seen that densities generated after live Stm754
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injection is significantly better as compared to that from live Stm PurA or E coli injections. Injections with heat-killed forms of all these bacteria, produced lower ligand densities. Though, ligand densities generated from live Stm PurA injections are low, the response is IFN-γ dominant, and similar ligand densities are generated from live E coli injections; however, the in vivo T helper responses are Th2-like. Hence in the mouse model of Stm immunisation, factors other than only the ability to generate appropriate peptide-MHC ligand density could be relevant.

Pharmacological agents tested for their effects on T cell priming do not show any difference when a single dose is given before priming, possibly because of their faster clearance from the body. When POF as well as dbcAMP were given for a longer time, during priming, it led to quantitatively higher amounts of IFN-γ secretion in an in vitro stimulation assay. In view of the upcoming data from this lab, as has been quoted in discussion, this increase may be due to the increase in memory responses with these drugs and more work needs to be done to elucidate further mechanism.

Finally, in the efforts of looking at epitope specific T cell responses using recombinant Stm, expressing a known T cell epitope, we could not see the in vivo Ea specific priming in mice immunized with these bacteria. However these bacteria expressed the fusion protein, as seen by Western blot analysis using the anti-c-myc antibody. It is also known, based on the results obtained using a T cell hybridoma IA9.F10 that, the expressed fusion protein can be processed and presented on the surface of I-A\(^b\) APCs, as seen by its stimulation. This lack of in vivo priming may be because of epitopic competition between Stm peptides and Ea peptide. To resolve this issue further to see responses to a single epitope expressed in Stm, we are in the
process of priming mice with entire conalbumin as well as a T cell epitope of conalbumin expressed in Stm.